Original Article

PRMT5 attenuates regorafenib-induced DNA damage in hepatocellular carcinoma cells through symmetric dimethylation of RPL14

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Background: Regorafenib has been approved for second-line treatment of hepatocellular carcinoma (HCC) following sorafenib failure, but resistance to targeted therapy remains a major challenge. Enhancing the therapeutic sensitivity of HCC cells to regorafenib is crucial for improving treatment outcomes. This study aims to elucidate the role of PRMT5 in HCC and its impact on regorafenib sensitivity. Specifically, it focuses on the regulatory relationship between PRMT5 and RPL14, investigating their influence on DNA damage repair and drug resistance mechanisms in HCC.

Methods: A stable PRMT5-overexpressing HCC cell line was constructed via lentiviral infection. Immunoprecipitation was employed to examine whether PRMT5 catalyzes the symmetric dimethylation of RPL14 at arginine residues. Western blot (WB) was used to assess changes in DNA damage markers (γ-H2AX) and DNA repair markers (RAD51) after RPL14 knockdown. Huh7 cells with PRMT5 overexpression, RPL14 knockdown, and combined PRMT5 overexpression and RPL14 knockdown were treated with regorafenib. DNA damage repair-related factors were analyzed using WB and immunofluorescence.

Results: Mass spectrometry and immunoprecipitation confirmed the interaction between PRMT5 and RPL14, with PRMT5 catalyzing symmetric dimethylation of RPL14. RPL14 knockdown inhibited HCC cell proliferation, increased sensitivity to regorafenib, and disrupted DNA damage repair, while overexpression had the opposite effect. Regorafenib-treated PRMT5-overexpressing cells showed reduced γ-H2AX expression and improved survival, whereas RPL14 knockdown enhanced γ-H2AX levels and decreased survival. Notably, simultaneous PRMT5 overexpression and RPL14 knockdown significantly elevated γ-H2AX expression compared to PRMT5 overexpression alone, leading to reduced cell viability. These results suggest that PRMT5 modulates DNA damage repair through RPL14, influencing the sensitivity of HCC cells to regorafenib.

Conclusions: PRMT5-mediated symmetric dimethylation of RPL14 stabilizes the protein, promoting DNA damage repair and contributing to regorafenib resistance in HCC. RPL14 plays a key role in PRMT5-driven enhancement of DNA damage repair and reduced drug sensitivity, identifying RPL14 as a potential

therapeutic target to overcome regorafenib resistance in HCC.

Keywords: Hepatocellular carcinoma (HCC); regorafenib; DNA damage repair; PRMT5; RPL14

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Introduction

Hepatocellular carcinoma (HCC) is associated with a poor prognosis and represents the third leading cause of mortality among malignant tumors worldwide. In 2020, the global mortality rate associated with HCC reached 830,000, with an age-standardized mortality rate of 8.7 per 100,000, underscoring the significant impact of this disease on public health (1). The primary pharmacological agents for systemic treatment of intermediate and advanced HCC are targeted therapies, such as sorafenib (2), lenvatinib (3), cabozantinib (4), pirarubicin (5), among others. In recent years, advancements in research on tumor combination therapy have led to gradual enhancements in both tumor control rates and patient survival rates (6).

PRMT5, a type II protein arginine methyltransferase with a molecular weight of 72 kDa, catalyzes the symmetric dimethylation of arginine residues within a range of substrate proteins, encompassing chromatin-associated proteins and transcription factors (7-9). PRMT5 has been the subject of extensive study concerning its involvement in tumor-related regulatory processes, including cell cycle progression, apoptosis, transcription, DNA damage response, and RNA metabolism. Elevated or dysregulated expression of PRMT5 has been observed in many cancers, including lung cancer and breast cancers, where PRMT5 is highly expressed. Furthermore, it regulates the activity or stability of transcription factors, thereby affecting their transcriptional regulation of vascular endothelial growth factor. This suggests that it is closely related to tumorigenesis and development. It has been demonstrated that the knockdown or inhibition of PRMT5 expression impedes tumor cell growth, induces apoptosis, and reduces cell migration ability. Consequently, PRMT5 is regarded as a prospective therapeutic target for cancer therapy. A substantial amount of evidence suggests that PRMT5 is intimately linked to the progression of various cancer types and holds notable clinical significance (10). Nevertheless, the present study aims to elucidate the role of PRMT5 in HCC development and to determine its impact on regorafenib sensitivity in HCC cells, as well as the underlying mechanisms.

The present screening has revealed RPL14 to be a pivotal downstream target gene of PRMT5. Prior research has demonstrated that loss of heterozygosity for RPL14 has been reported in lung cancer and head and neck squamous cell carcinomas (11). However, there is a paucity of research investigating the role of RPL14 in HCC. This study addresses this gap in the literature by examining the role of RPL14 in HCC and its underlying mechanism.

RPL family proteins play a significant role in the process of DNA damage repair. The DNA damage response is closely associated with cancer susceptibility and drug sensitivity, influencing individual responses to DNA

Highlight box

Key findings

- PRMT5 promotes DNA damage repair through RPL14 and leads to regorafenib resistance in hepatocellular carcinoma (HCC). RPL14 plays a critical role in enhancing PRMT5-driven DNA damage repair and reducing drug sensitivity, and has been identified as a potential therapeutic target for overcoming regorafenib resistance in HCC.

What is known and what is new?

- PRMT5 has been extensively studied in tumor-related regulatory processes, including cell cycle progression, apoptosis, transcription, DNA damage response, and RNA metabolism. As a result, PRMT5 is considered a potential therapeutic target for cancer treatment. However, the mechanism of action of PRMT5 in the development of liver cancer is not yet clear, and there is no evidence that it is associated with regorafenib sensitivity.
- In this study, we investigated the role of PRMT5 in HCC and found that it can catalyse the symmetric dimethylation of arginine residues in RPL14, prolong its half-life, and maintain RPL14 protein stability. This, in turn, promotes the occurrence of DNA damage repair in HCC cells and reduces the cells' sensitivity to regorafenib.

What is the implication, and what should change now?

- Co-targeting PRMT5 or RPL14 is expected to be a new strategy to improve the sensitivity of regorafenib.

damage-targeted antitumor therapies (12). It has been demonstrated that several pathogenic factors linked to the development of HCC can induce DNA damage responses, DNA adduct formation, and chromosomal abnormalities. Post-translational modifications of DNA repair genes, including methylation, are known to affect repair capacity and increase cancer risk (13). The aberrant activation of the DNA repair system is frequently linked to tumor progression and therapeutic response in HCC (14). Consequently, an investigation into the mechanisms underlying DNA damage repair in HCC will facilitate a deeper comprehension of its pathogenesis and prognosis, thereby providing novel insights for the development of more efficacious therapeutic strategies for HCC.

This study aims to investigate the impact of PRMT5-mediated arginine methylation of proteins on the sensitivity of HCC to regorafenib, as well as the underlying mechanism. It seeks to determine whether PRMT5 can regulate the enhanced DNA damage repair ability of HCC cells through RPL14, thereby reducing the sensitivity of cells to regorafenib. Furthermore, this study investigates the impact of PRMT5 and RPL14 expression in HCC on the prognosis of HCC patients. The objective is to elucidate the roles of PRMT5 and RPL14 in HCC development and therapeutic resistance, identify key molecules and markers, and propose novel strategies for precision clinical interventions. We present this article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-737/rc).

Methods

Cell culture

The human hepatoma cell lines Huh7, HCCLM3, HepG2, Hep3B, MHCC-97H, MHCC-97L, PLC/PRF/5/, and HEK-293T used in this project are from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. All stable cell lines of Huh7 cells used in this study were purchased from OBiO Technology.

Western blot (WB)

The appropriate concentration gel was selected according to the molecular weight of the target protein, and it was configured according to the instructions. An equal amount of protein (20–40 μg) was added to each well, and an equal amount of protein marker was added to both lanes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein electrophoresis was performed at 180 V for 45 min. The sponge, filter paper, gel, nitrocellulose (NC) membrane, filter paper, and sponge were placed sequentially from the bottom (negative pole) to the top (positive pole) in the membrane transfer tank, the prepared membrane transfer solution was poured in, placed in a prepared ice bath, the current was set to 230 mA, and the membrane was transferred for 60 min. The NC membrane was cut according to the location of the target protein bands and placed in the prepared 5% bovine serum albumin (BSA) closed membrane. The NC membrane was cut according to the position of the target protein bands and placed in the prepared 5% BSA blocking solution, then placed on a shaker for 1 hour at room temperature. After cutting, the membrane was placed in the appropriate proportion of diluted primary antibody at 4 ℃ overnight, including RAD51 (Abcam, Cambridge, UK; ab88572), γ-H2AX (CST, Boston, USA; 80312), PRMT5 (Abcam, ab109451), RPL14 (Abcam, ab181200). The membrane was then washed 3 times with 1× tris-buffered saline with tween-20 (TBST), each time for 5 min. The membrane was placed in the secondary antibody corresponding to the primary antibody and incubated at room temperature for 2 hours, protected from light, and then washed 3 times with 1× TBST, each time for 5 min.

Co-immunoprecipitation (Co-IP)

An appropriate amount of 5 μg of the primary antibody was added to 500 μL of cell lysate, while another tube of 500 μL of cell lysate was taken, to which 5 μg of an equal amount of isotype IgG was added as positive control, and all samples were spun on a carousel at 4 ℃ overnight. Prepare new EP tubes, 500 μL of 1× tris buffered saline (TBS) was added to each tube, then 30 μL of ProteinG was added to magnetic beads, wash twice, carefully aspirate the TBS, the antibody from the eppendorf (EP) tube was added on day 1 to the corresponding magnetic beads, and the tube was slowly rotated at 4 ℃ for 2 hours. The bead-antibody-antigen complexes was collected at 4 ℃ for 100 g for 30 s, and then it was washed three times by adding 500 μL of 1XTBS, each time for 5 min. 100 μL 1× SDS was added to each tube, denatured on a honeycomb oven at 100 ℃ for 5 min, immediately placed on ice for more than 2 min (to prevent protein denaturation), and stored at –20 ℃ for spare parts.

Liquid chromatography-tandem mass spectrometry experiments (LC-MS/MS)

After enzymatic digestion of the target protein bands, the extracted supernatant was subjected to peptide desalting. Peptide desalting was performed using a packed column prepared with C18 material. The volatilized peptide samples were redissolved using 0.1% formic acid solution [nano-high performance liquid chromatography (HPLC) buffer A] (CNW, Shanghai, China; CAS:64-18-6). 40 µL methanol was added to the column and centrifuged, the bottom effluent was discarded, and this procedure was repeated twice. 40 µL nano-HPLC buffer A was passed through the column and centrifuged, the bottom of the column was discarded, and the procedure was repeated twice. After passing the sample through the column, the bottom effluent was removed, and the sample was passed through the column again. The sample was passed through the column with 40 µL nano-HPLC buffer A and centrifuge, the column bottom was discarded, and repeated twice. The tube was replaced with a new EP tube, the column was passed through 40 µL of acetonitrile solution containing 0.1% formic acid (buffer B) (ThermoScientific, Massachusetts, USA; A955-4), and centrifuge, bottom effluent was collected, and repeated once. The peptide samples obtained were evaporated dry with 80 µL of eluent in buffer B. The peptide samples were then dried.

Following volatilization, the peptide samples were re-dissolved in nano-HPLC buffer A. Nano-HPLC separation was conducted using an EASY-nLC1200 liquid system. The samples were loaded onto a trap column via an autosampler and subsequently separated in a 75 µm × 150 mm analytical column at a flow rate of 300 nL/min. Each sample was then analyzed, followed by a 30-minute mobile-phase gradient wash using a blank solvent. Comprehensive chromatographic and mass spectrometric analyses were performed.

The Cancer Genome Atlas (TCGA) database analysis

The RNA-seq data were normalized by transcript fragment number per kilobase per million mapped reads (FPKM), and the survival data were obtained from the TCGA Prostate Cancer (TCGA-PRAD) dataset, which is available from UCSC XENA (https://xenabrowser.net/datapages/). Samples lacking corresponding clinical or survival data were excluded. The study comprised 371 tumor samples and 50 normal samples. The limma package was employed to analyze the differences in PRMT5 and RPL14 expression, with a significance threshold of $P<0.05$ and $|\log_2(\text{fold change})| \geq 1.5$. Box plots of gene expression were constructed using data from the TCGA dataset. Kaplan-Meier (KM) survival analysis was performed on PRMT5 and RPL14 using the Survival package (version 3.3-1), with a $P<0.05$ considered statistically significant. Co-expression patterns were explored using Pearson and Spearman correlation analyses with the Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) database, and correlation scores were calculated and presented using R software.

Statistical analysis

The statistical analysis was conducted using SPSS 22.0 and GraphPad 8.0.2 for the statistical analysis and graphing of the data. The results were presented in the form of histograms, scatter plots, line graphs, and so forth. The appropriate tests were selected according to the type of experimental data. In this study, the results of the relevant experiments were presented as mean ± standard error (SE). When the data were normally distributed and the variance was homogeneous, a *t*-test and an analysis of variance were employed. Conversely, when the data were not normally distributed or the variance was not homogeneous, a nonparametric rank sum test was used. The threshold for statistical significance was set at $P<0.05$.

Patient cohorts

Cancer tissue and paraneoplastic normal liver tissue samples were obtained with informed consent from HCC patients at Eastern Hepatobiliary Surgery Hospital (EHBH). The study was approved by the ethics committee of EHBH (No. EHBHKY2024-K011-P001), and conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Results

PRMT5 reduces the sensitivity of HCC cells to regorafenib by activating DNA damage repair

PRMT5 has been shown to play a critical role in tumorigenesis and cancer progression (15). The initial objective was to examine the influence of regorafenib on the proliferation of diverse HCC cell lines. Cell-counting-kit-8 (CCK8) assays showed that regorafenib inhibited

HCC cell proliferation with notable differences in 50% inhibiting concentration (IC50) values across cell lines (*Figure 1A*,*1B*). The IC50 of regorafenib in Huh7 cells was determined to be 12.28 μM. The IC50 in HepG2 cells was determined to be 3.64 μM. Based on the IC50 results, subsequent experiments used different concentration gradients of regorafenib in Huh7 cells (6 and 12 μM) for extended treatment periods. Huh7 cells were treated with 6 and 12 μM regorafenib for extended periods. The expression levels of the PRMT5 protein were observed in the cells. WB analysis showed a significant increase in PRMT5 expression in the regorafenib-treated group relative to the control group (*Figure 1C*). We also found that PRMT5 affects the sensitivity of HCC to regorafenib. Cloning formation assay showed that the combined treatment of regorafenib and PRMT5 inhibitor (EPZ015666) significantly attenuated the cell proliferation and colony formation ability of Huh7 cells compared with cells treated with regorafenib or EPZ015666 alone (*Figure 1D*). CCK8 results showed that the survival of Huh7 cells treated with regorafenib and EPZ was significantly lower than that treated with regorafenib alone (Figure S1A). To further address whether PRMT5 inhibition increases tumor responsiveness to regorafenib, we employed patient-derived organoids (PDO) to mimic the *in vivo* environment. PDO was treated with regorafenib and EPZ, which blocks CRIPTO/GRP78 binding and CRIPTO signaling or both in combination. In PDO experiments, HCC cell viability was significantly reduced when combined with regorafenib and EPZ, suggesting that HCC is more sensitive to regorafenib when PRMT5 is inhibited (*Figure 1E*).

To further explore the role of PRMT5 in HCC progression and its underlying mechanisms, we conducted the following experiments. A stable Huh7 cell line overexpressing PRMT5 was constructed using a full-length PRMT5 plasmid. After multiple passages and screening, we constructed Huh7-PRMT5, verified its PRMT5 expression, and determined the cell line construction (Figure S1B,S1C). We used transcriptome sequencing technology to sequence Huh7-PRMT5 and Huh7 Ctrl cells after regorafenib treatment for 48 h to determine the activation of various signaling pathways and the expression of transcripts in HCC cells in the two groups. For the transcriptome sequencing results, the discrimination between the two groups was high, while the within-group reproducibility was good (Figure S1D,S1E). The transcriptome sequencing results were analyzed. Deseq2 software was used to compare the differences between Huh7-PRMT5 and Huh7 Ctrl (screening condition P<0.05). regorafenib treatment resulted in the upregulation of 698 genes and downregulation of 685 genes in PRMT5-overexpressing Huh7 cells compared to the control. Enrichment analysis of 1,383 differentially expressed genes showed that the expression patterns of the differentially expressed genes within the group were the same. Among 1,383 differential genes, 18 differential genes were selected for Gene Ontology (GO) enrichment analysis. Among the top 30 significantly enriched GO terms (P<0.05), the pathway of events related to DNA damage repair was enhanced in PRMT5 overexpressing cells, and the function of DNA repair complexes was significantly enriched (*Figure 1F*, Figure S1F,S1G). We then treated Huh7-PRMT5 and control cells under the same conditions and harvested the cells 48 hours later to detect the expression of related genes. WB analysis confirmed that DNA damage markers in the Huh7-PRMT5 group cells after regorafenib treatment showed a decrease in γ-H2AX expression, while the expression of the DNA repair marker RAD51 increased (*Figure 1G*).

In summary, regorafenib induces DNA damage in HCC cells and inhibits their proliferation. However, PRMT5 overexpression facilitates DNA repair and reduces the extent of DNA damage caused by regorafenib, suggesting a correlation between PRMT5 and regorafenib resistance.

PRMT5 interacts with RPL14 to catalyze symmetric demethylation and maintain protein stability

To explore the mechanism of PRMT5 regulating DNA damage repair in HCC cells, we used regorafenib to treat Huh7 cells overexpressing PRMT5 and then used PRMT5 as bait protein to search for proteins that can interact with it through a silver staining experiment, Co-IP, and mass spectrometry analysis. Mass spectrometry and Co-IP identified several RPL family proteins, including RPL14, as interacting partners of PRMT5 (*Figure 2A*,*2B*). TCGA database analysis revealed higher RPL14 expression in HCC tumor tissues compared to adjacent normal tissues, suggesting potential biological significance (Figure S1H). According to the analysis of the human protein atlas database, RPL14 messenger RNA (mRNA) was low expressed in normal liver tissues, while the protein level showed moderate expression (Figure S2A,S2B). The above differences suggest that RPL14 protein expression may be regulated by post-translational modifications, such as methylation. To study the interaction between PRMT5 and RPL14, we used Huh7-PRMT5 overexpression to stably transform the

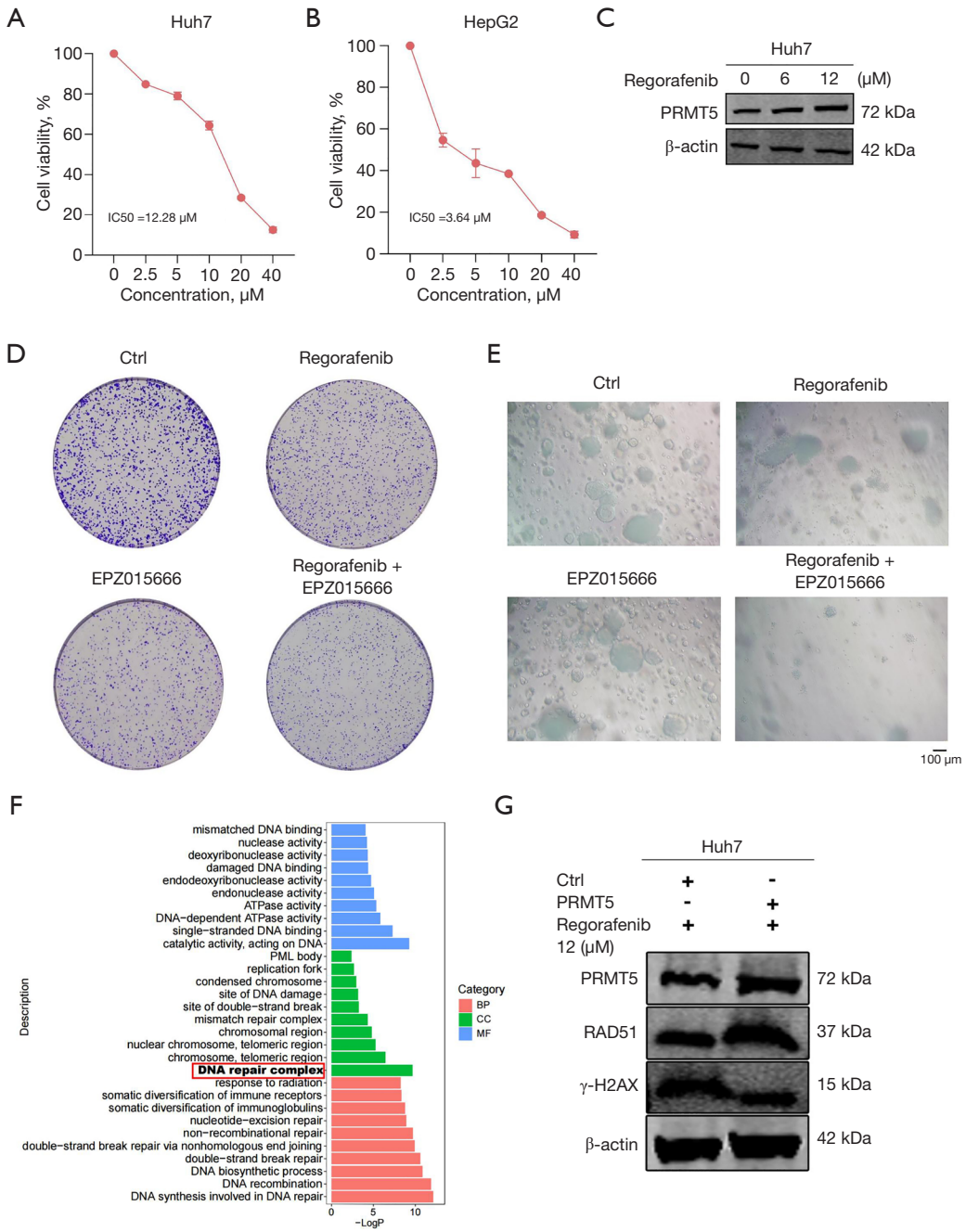

Figure 1 PRMT5 decreases the sensitivity of HCC cells to regorafenib by enhancing DNA damage repair mechanisms. (A) Survival and IC50 of Huh7 cells treated with different concentrations of regorafenib for 48 hours were determined. (B) Survival and IC50 of HepG2 cells treated with different concentrations of regorafenib for 48 hours were determined. (C) WB was used to detect the expression of PRMT5 in Huh7 cells after regorafenib treatment for 7 days. (D) Colony formation capabilities of Huh7 cells treated with regorafenib (5 μM), EPZ015666 (5 μM), regorafenib (5 μM) plus EPZ015666 (5 μM). Used crystal violet staining solution. (E) PDO treated with regorafenib (10 μM) and EPZ (10 μM), and bright field images showing the morphology of organoids derived from the HCC PDO after 96 h of culture (Ctrl, regorafenib, EPZ015666, regorafenib + EPZ015666 10 μM). (F) GO enrichment analysis. (G) The expression of γ-H2AX and RAD51 in the PRMT5 overexpression group and the control group after regorafenib treatment. Scale bar 100 μm. HCC, hepatocellular carcinoma; WB, western blot; IC50, 50% inhibiting concentration; PDO, patient-derived organoids; GO, Gene Ontology; PML, promyelocytic leukemia; BP, biological process; CC, cellular component; MF, molecular function.

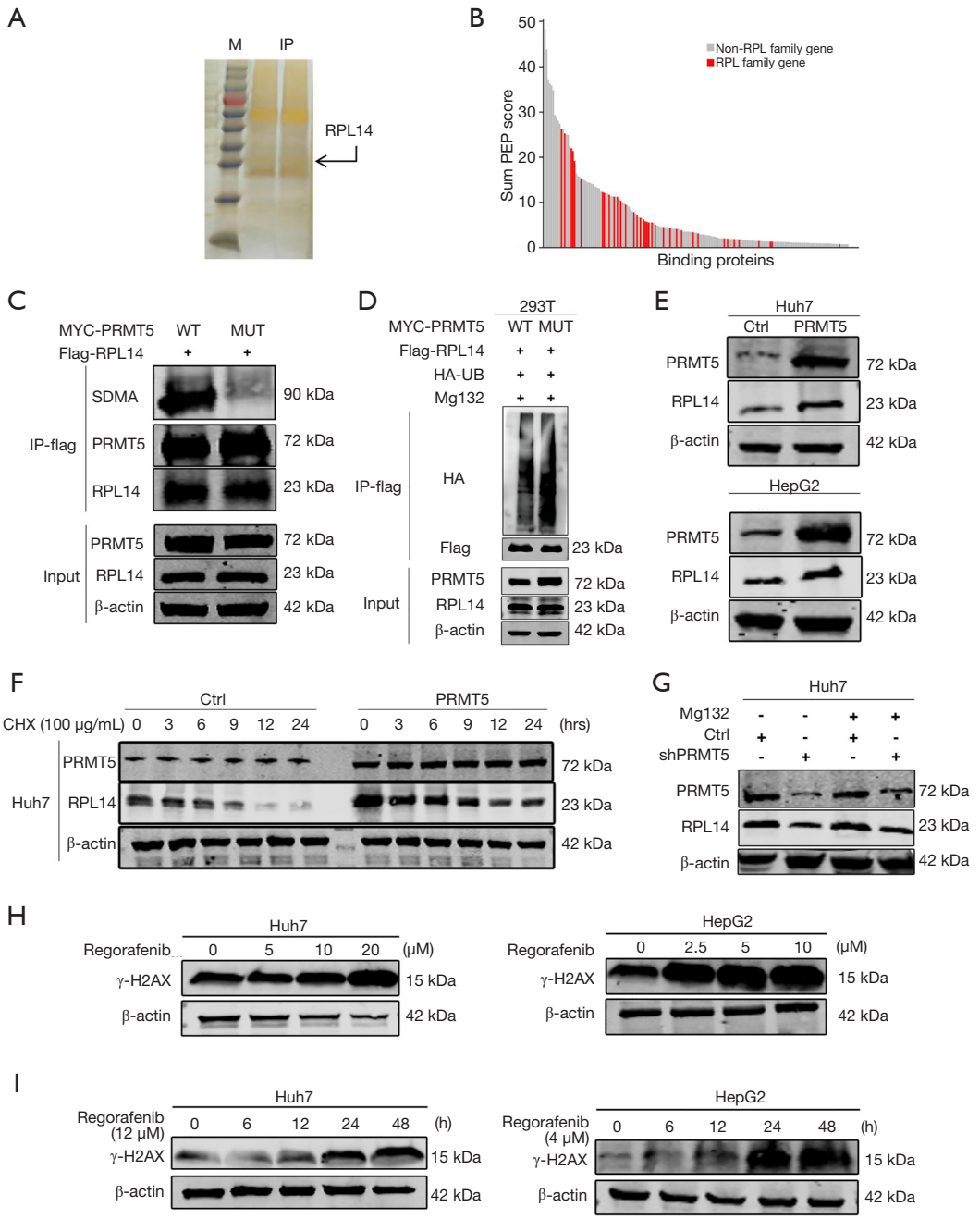

Figure 2 PRMT5 interacts with RPL14 to catalyze symmetric demethylation and maintain protein stability. (A) Results of Co-IP and silver staining experiments. (B) Mass spectrometry results analyzing the proteins interacting with PRMT5 in the RPL family. (C) Detection of RPL14 symmetric dimethylation and interaction with PRMT5 by CO immunoprecipitation combined with WB. (D) Ubiquitination level detection of RPL14. (E) RPL14 expression was detected by WB after overexpression of PRMT5. (F) After the addition of CHX, the protein expression levels of RPL14 in the control and PRMT5 overexpression groups were at different time points. (G) RPL14 protein expression in Huh7 cells after knockdown of PRMT5 and addition of MG132. (H) Effects of different concentrations of regorafenib on the expression level of γ-H2AX in HCC cells. (I) The effect of regorafenib treatment on the expression level of γ-H2AX in HCC cells as a function of treatment time. M, mark; IP, immunoprecipitation; RPL, ribosomal protein large; GO, gene ontology; WB, western blot; CHX, cycloheximide; HCC, hepatocellular carcinoma; PEP, posterior error probability; WT, wild type; MUT, mutant; HA-UB, hemagglutinin-tagged ubiquitin.

strain and detect it by LC-MS/MS. The results showed that the specific peptide of RPL14 was identified, which further confirmed that there was indeed an interaction between the two (Figure S2C).

According to the literature review and analysis, PRMT5, as an arginine methylase, may catalyze arginine symmetric dimethylation after interacting with RPL14 (16). A specific antibody was used to detect the symmetric dimethylarginine (SDMA) modification of exogenously expressed RPL14, and it was found that overexpression of wild-type PRMT5 could significantly modify RPL14. Based on the wild-type PRMT5 overexpression plasmid, the mutant of its catalytic site was constructed, that is, the arginine at position 368 in the PRMT5 amino acid sequence was mutated to alanine. Co-IP and WB confirmed that both wild-type and mutant PRMT5 can bind to RPL14, but only wild-type PRMT5 mediates SDMA modification. Therefore, the catalytic active site mutation of the PRMT5 enzyme does not affect the interaction of PRMT5 with RPL14 (*Figure 2C*). On the other hand, we co-transfected wild-type and mutant PRMT5 plasmids with RPL14 and hemagglutinin-tagged ubiquitin (HA-UB), respectively, into 293T cells. The Co-IP results suggested that RPL14 could undergo more ubiquitination modification after PRMT5 mutation (*Figure 2D*).

WB analysis revealed that the expression level of RPL14 was significantly upregulated in the Huh7 and HepG2 cells overexpressing PRMT5 compared to the control group (*Figure 2E*). We added cycloheximide (CHX) (100 μg/mL), a protein synthesis inhibitor, to the Huh7 control cells and PRMT5 overexpressing cells, respectively, and detected the protein level of RPL14 in the cells after collecting the cells at different time points (0, 3, 6, 9, 12, 24 hours). We found that overexpression of PRMT5 could maintain the protein stability of RPL14 in cells and promote the expression of RPL14 in cells (*Figure 2F*). The proteasome inhibitor MG132 (100 μg/mL, 12 hours) was added to the cells of the control group and the cells of the PRMT5 knockdown group to detect the expression of RPL14 in the cells. WB analysis indicated that knockdown of PRMT5 could reduce the expression of RPL14, and the addition of MG132 could significantly inhibit the degradation of RPL14 in the knockdown PRMT5 group (*Figure 2G*).

To summarize, RPL14 interacts with PRMT5 and serves as a target protein for PRMT5, undergoing SDMA modification through its catalytic action. PRMT5 further regulates RPL14 by inhibiting its ubiquitination and degradation, thereby enhancing its protein expression and stability. This effect is mediated by the suppression of the ubiquitin-proteasome degradation pathway specific to RPL14.

Regorafenib induces dose- and time-dependent DNA damage in HCC cells

Abnormal activation of the DNA damage repair system in tumor cells is one of the major mechanisms of anti-tumor drug resistance (17). To investigate the extent of the effect of regorafenib on DNA damage in HCC cells, we treated Huh7 cells with different concentrations of regorafenib (0, 5, 10, 20 μM) and HepG2 cells with different concentrations of regorafenib (0, 2.5, 5, 10 μM). After 48 hours, increasing concentrations of regorafenib led to a dose-dependent increase in γ-H2AX expression, indicating greater DNA damage (*Figure 2H*). Furthermore, Huh7 cells were treated with 12 μM regorafenib (0, 6, 12, 24, 48 hours), and HepG2 cells were treated with four μM regorafenib (0, 6, 12, 24, 48 hours), and the expression level of intracellular γ-H2AX was detected by WB. The results showed that with the prolongation of regorafenib treatment time, the expression of intracellular γ-H2AX gradually increased (*Figure 2I*). These results indicate that regorafenib induces DNA damage in HCC cells in a dose- and time-dependent manner.

To validate the impact of regorafenib on DNA damage in HCC cells, we employed indirect immunofluorescence to monitor changes in intracellular γ-H2AX under consistent experimental conditions. The results showed that with increasing regorafenib treatment time or concentration, the number of γ-H2AX foci in the nucleus gradually increased, i.e., the degree of DNA damage in HCC cells gradually worsened, and this effect was dose- and time-dependent (*Figure 3A*,*3B*).

In conclusion, we treated HCC cells with regorafenib at different times and concentrations and detected the level of intracellular γ-H2AX by WB and indirect immunofluorescence technology. The results showed that regorafenib could induce DNA damage in HCC cells, and with the increase of regorafenib treatment time or concentration, the degree of DNA damage in HCC cells gradually aggravated, suggesting that regorafenib induced DNA damage in HCC cells in a dose and time-dependent manner.

RPL14 promotes DNA damage repair and inhibits regorafenib-induced HCC cell death

Next, we continued to investigate the role of RPL14

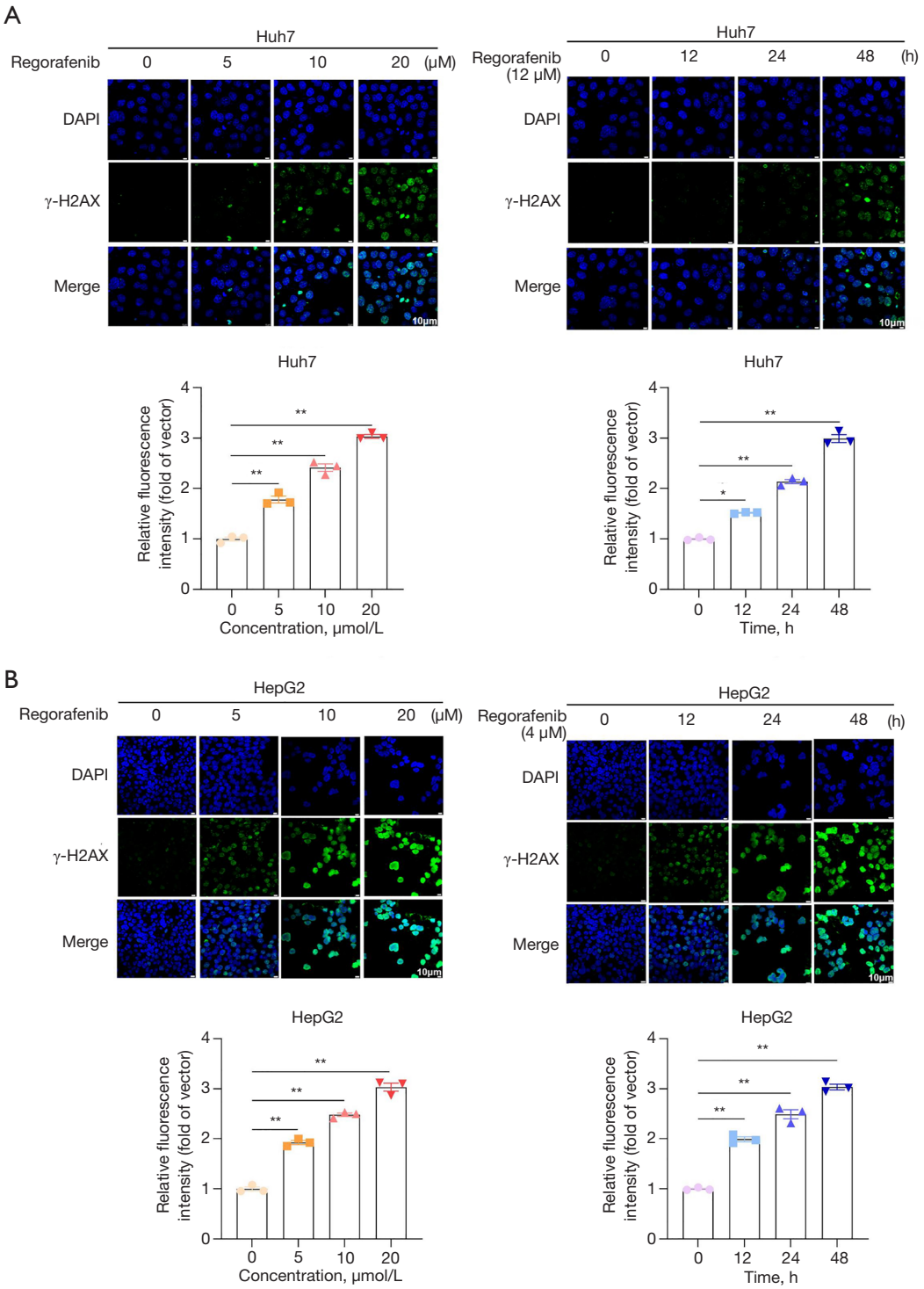

Figure 3 Regorafenib induces dose- and time-dependent DNA damage in HCC cells. (A) Indirect immunofluorescence detection indicated that the expression level of γ-H2AX in Huh7 cells treated with regorafenib was dose- and time-dependent. (B) Indirect immunofluorescence detection indicated that the expression level of γ-H2AX in HepG2 cells treated with regorafenib was dose- and time-dependent. Scale bar 10 μm. *, P<0.05; **, P<0.01. DAPI, 4',6-diamidino-2-phenylindole; HCC, hepatocellular carcinoma.

in HCC. First, we used WB to detect the expression of RPL14 in multiple HCC cells. The results indicated that there was little difference in the expression levels of RPL14 among different HCC cells (Figure S3A). Next, we treated Huh7 cells with 6 and 12 μM regorafenib, respectively, for extended periods and observed the expression levels of RPL14 protein in the cells. WB analysis confirmed that the expression of RPL14 was increased in the regorafenib-treated group compared to the control group (Figure S3B). We then knocked down RPL14 in Huh7 and HepG2 cells (Figure S3C,S3D) and used the CCK8 cytotoxicity assay to detect the effect of RPL14 knockdown on the proliferation of HCC cells. After 0/24/48 hours of incubation and culture, the proliferation rate of Huh7 and HepG2 cells in the RPL14 knockdown group was significantly slower compared to the control group (*Figure 4A*). Huh7 and HepG2 cells were then treated with different concentrations of regorafenib (0, 2.5, 5, 10, 20 μM) for 48 hours. The results showed that compared with the control group, the proliferation rate of cells in the RPL14 knockdown group was significantly slowed down, and the cell viability decreased more significantly. RPL14 could effectively inhibit HCC cell death caused by regorafenib treatment (*Figure 4B*). Knockdown of RPL14 may improve the sensitivity of HCC cells to regorafenib. The above results showed that RPL14 knockdown could inhibit the proliferation of HCC cells and improve the sensitivity of HCC cells to regorafenib.

To investigate whether RPL14 could repair DNA damage in HCC cells, we first performed WB. The results showed that compared with the control group, the expression of intracellular γ-H2AX was significantly upregulated in Huh7 cells with RPL14 knockdown, while the expression of intracellular DNA repair marker RAD51 was significantly downregulated (*Figure 4C*), suggesting that RPL14 has a repairing effect on regorafenib-mediated DNA damage in HCC cells. To further verify this effect, we overexpressed RPL14 in Huh7 cells (Figure S3E,S3F) and used indirect immunofluorescence experiments to detect the effect of RPL14 overexpression on γH2AX expression levels. The results showed that the expression of γ-H2AX was significantly reduced in HCC cells overexpressing RPL14 compared to the control group (*Figure 4D*). In conclusion, RPL14 can promote the repair of DNA damage in HCC cells caused by regorafenib.

PRMT5 regulates the DNA damage repair signaling pathway through RPL14 and reduces the sensitivity of HCC cells to regorafenib

PRMT5 overexpression, RPL14 knockdown, PRMT5 overexpression along with RPL14 knockdown, and control cell lines were constructed based on wild-type Huh7 cells and HepG2 cells. The results of the IC50 assay of regorafenib in Huh7 and HepG2 cells indicated that Huh7 cells and HepG2 cells were treated with 12 and 4 μM regorafenib at different concentration doses, and the corresponding proteins were collected after 48 hours. WB analysis was then used to detect the expression of the DNA damage marker γ-H2AX and the DNA repair marker RAD51 in the cells of each group. The results demonstrated that the expression of γ-H2AX was reduced in the PRMT5 overexpression group relative to the control group in Huh7 cells and HepG2 cells. Conversely, the expression of γ-H2AX was augmented in the RPL14 knockdown group. Furthermore, the expression of γ-H2AX in the cells of the PRMT5 overexpression and simultaneous RPL14 knockdown group was significantly increased in comparison to that of the PRMT5 overexpression alone group. Additionally, RAD51 expression was observed to be elevated in the PRMT5 overexpression group relative to the control group in both Huh7 and HepG2 cells. Conversely, RAD51 expression was found to be reduced in the RPL14 knockdown group. Furthermore, the expression of RAD51 in the cells of the PRMT5 overexpression and simultaneous RPL14 knockdown group was significantly decreased in comparison to that observed in the PRMT5 overexpression group alone (*Figure 5A,5B*).

The detection of RAD51 focal point formation in the nucleus may serve as an indicator of DNA repair activity within the cell. An indirect immunofluorescence assay was employed to detect RAD51 focus formation in PRMT5 overexpression, RPL14 knockdown, PRMT5 overexpression along with RPL14 knockdown, and control stable-transformed Huh7 cell lines following 12 μM regorafenib treatment for 48 hours in the four groups of cells. The results demonstrated that RAD51 expression was elevated in the PRMT5 overexpression group relative to the control group. Conversely, RAD51 expression was reduced in the RPL14 knockdown group. Notably, the expression of RAD51 in the cells of the PRMT5 overexpression concomitant with the RPL14 knockdown group exhibited

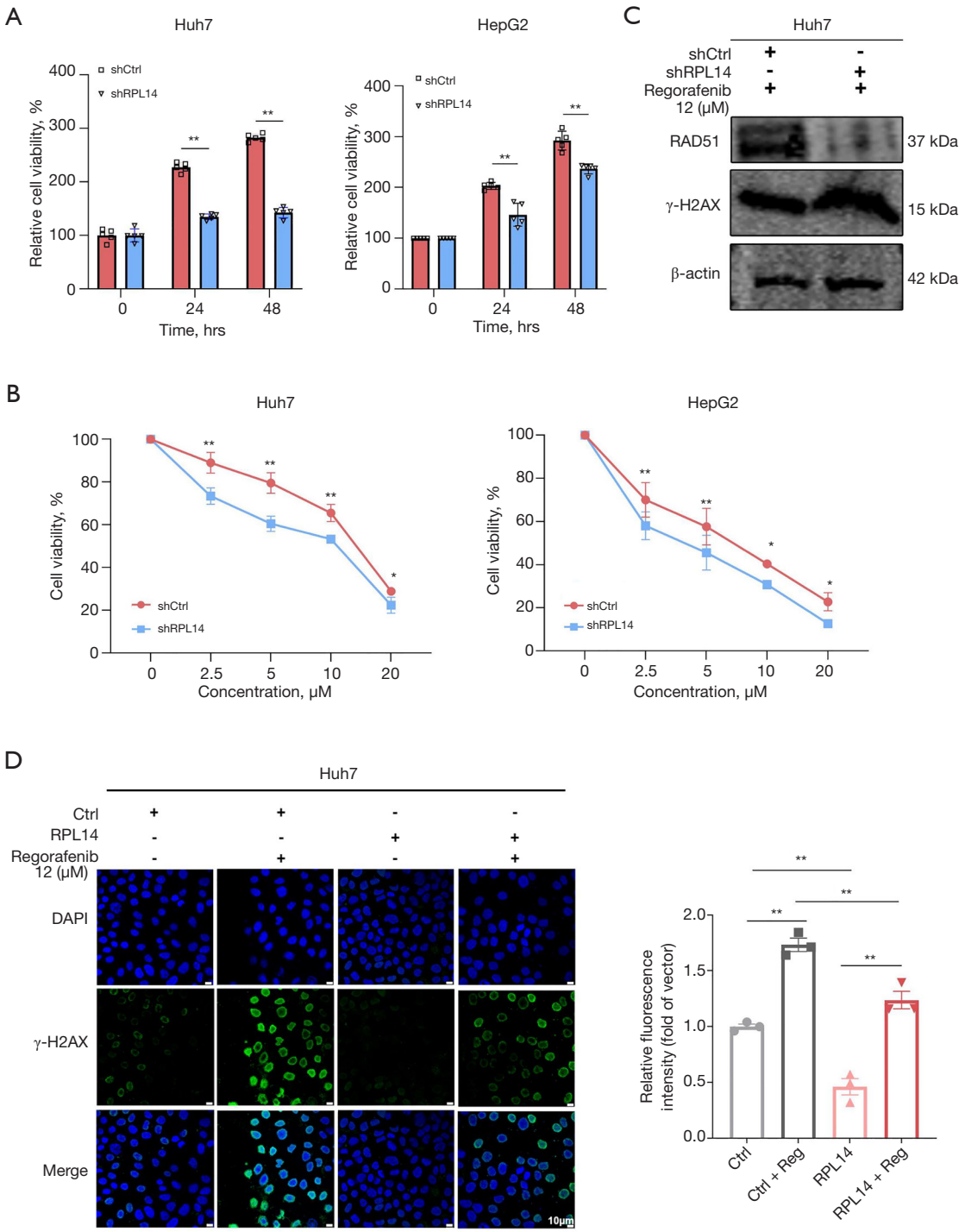

Figure 4 RPL14 promotes DNA damage repair and inhibits regorafenib-induced HCC cell death. (A) The proliferation of HCC cells with RPL14 knockdown. (B) Changes in cell viability of HCC cells with RPL14 knockdown after treatment with different concentrations of regorafenib. (C) The effect of RPL14 knockdown in HCC cell lines on the protein expression levels of γ-H2AX and RAD51. (D) Focal point formation and indirect immunofluorescence detection of the DNA damage marker γ-H2AX in nuclei of cells overexpressing RPL14 and subjected to different treatments. Scale bar: 10 μm. *, P<0.05; **, P<0.01. DAPI, 4',6-diamidino-2-phenylindole; HCC, hepatocellular carcinoma.

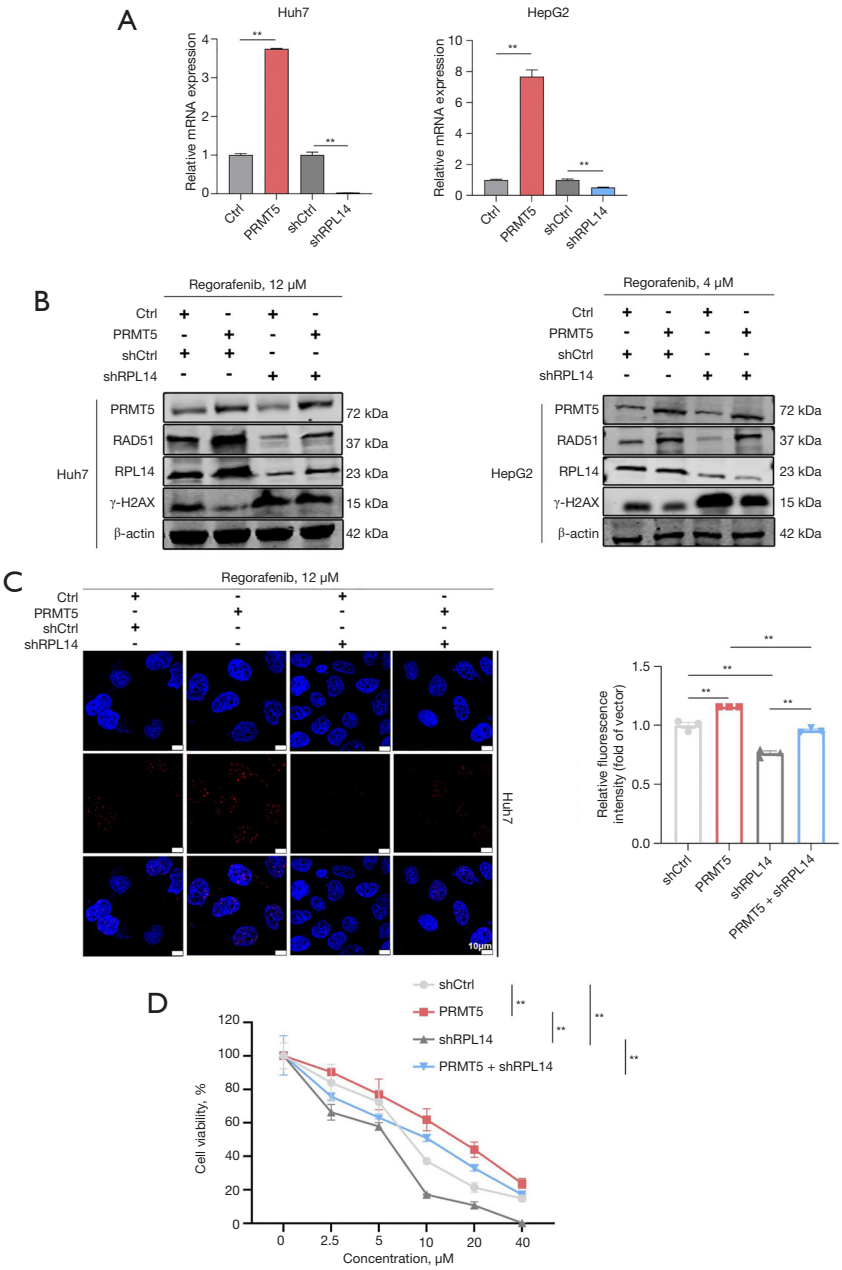

Figure 5 PRMT5 regulates DNA damage repair signaling pathway through RPL14. (A) qPCR detection of mRNA expression after overexpression of PRMT5 in Huh7 and HepG2 cells with concomitant knockdown of RPL14. (B) WB for expression of DNA damage marker γ-H2AX and DNA repair marker RAD51 in cells of regorafenib-treated PRMT5 overexpression, RPL14 knockdown, PRMT5 overexpression, and concomitant RPL14 knockdown, and control stably transformed Huh7 and HepG2 cell lines. (C) Indirect immunofluorescence assays were performed to verify the focus formation and quantitative fluorescence analysis of DNA repair marker RAD51 in the nuclei of PRMT5 overexpression, RPL14 knockdown, PRMT5 overexpression with concomitant RPL14 knockdown, as well as in the nuclei of control stably transfected Huh7 cell lines. Data in the graphs are expressed as mean ± SE and three fields of view were selected for quantitative analysis in each group. (D) Different concentrations of regorafenib were used to treat PRMT5 overexpression, RPL14 knockdown, and PRMT5 overexpression with simultaneous RPL14 knockdown, and control stably transformed Huh7 cell lines, and their cell survival was examined. The data in the graphs are expressed as mean ± SE, with three replicates per group. Scale bar 10 μm. **, P<0.01. qPCR, quantitative real-time polymerase chain reaction; WB, western blot; SE, standard error.

a marked decline in comparison to that of the PRMT5 overexpression alone group (*Figure 5C*).

In conclusion, our findings suggest that PRMT5 plays a role in regulating the DNA damage repair signaling pathway, which is in line with the transcriptome sequencing results presented in the initial section. Moreover, the experimental results corroborated that PRMT5-mediated repair following DNA damage in both types of HCC cells were markedly reduced after RPL14 knockdown. The veracity of this conclusion was once again validated in the indirect immunofluorescence assay described above. Consequently, we can conclude that PRMT5 exerts its regulatory influence over the DNA damage repair signaling pathway through RPL14.

However, the enhancement of HCC cell survival by PRMT5 with RPL14 has not been previously documented in the scientific literature. Accordingly, a CCK8 cytotoxicity assay was employed to ascertain the impact of PRMT5 and RPL14 on HCC cell survival and the interrelationship between them. Stably transformed Huh7 cell lines overexpressing PRMT5, RPL14, or a combination of both were treated with different concentrations of regorafenib and observed for 48 hours. The results demonstrated that PRMT5 overexpression enhanced HCC cell survival, whereas RPL14 knockdown impeded the augmented survival of HCC cells. Furthermore, the increase in cell survival was markedly slower following RPL14 knockdown in PRMT5-overexpressing cells than in the PRMT5-overexpressing group. Consequently, we concluded that PRMT5 reduced the sensitivity of HCC cells to regorafenib via RPL14 (*Figure 5D*).

In summary, our experiments confirmed that PRMT5 modulates the DNA damage repair signaling pathway through RPL14. Furthermore, the CCK8 cytotoxicity assay has also confirmed that PRMT5 regulates the DNA damage repair signaling pathway through RPL14. RPL14 reduces the sensitivity of HCC cells to regorafenib.

High expression of PRMT5 and RPL14 in HCC is associated with poor prognosis in patients

PRMT5 promotes the progression and deterioration of HCC through multiple pathways, and the expression of RPL14 in HCC tissues also reflects the malignant characteristics of HCC cells. To further clarify the relationship between PRMT5 and RPL14 expression and patient prognosis, we analyzed HCC data in the TCGA public database and found that the expression of PRMT5 and RPL14 in HCC was higher than that in adjacent tissues (*Figure 6A*, Figure S1G). The analysis on the GEPIA website showed that there was a positive correlation between the expression of PRMT5 and RPL14 in HCC (*Figure 6B,6C*). Therefore, we believe that PRMT5 and RPL14 are highly expressed and positively correlated in liver cancer tissues.

By analyzing the HCC survival data in the TCGA public database, it was found that the disease-free survival, progression-free survival, and overall survival of patients with high expression of PRMT5 and RPL14 were shorter than those of patients with low expression (*Figure 6D-6F*). Therefore, we believe that the high expression of PRMT5 and RPL14 in HCC patients predicts a poor prognosis, which is closely related to the poor prognosis of patients.

Since there are no literature reports on the expression of PRMT5 and RPL14 in clinical HCC samples, we selected tumor tissues and adjacent liver tissues from 14 patients with HCC in EHBH. WB analysis showed that 13 cases (13/14) of HCC tissue samples had higher expression of PRMT5 compared with adjacent tissues; RPL14 expression was higher in 11 (11/14) HCC tissue samples; however, the expression of PRMT5 and RPL14 in 10 (10/14) HCC tissue samples was higher than that in the adjacent tissues (*Figure 7A*). In addition, the expression of RPL14 protein in 106 patients' liver cancer tissues and their adjacent liver tissues was detected by immunohistochemical technology. The results showed that the expression of RPL14 in tumor tissues was higher than that in their adjacent liver tissues, which was consistent with the results in the TCGA database (*Figure 7B,7C*). Data from 106 tumor tissues were then analyzed quantitatively, and survival analysis was performed in combination with clinical data. The results showed that when RPL14 was highly expressed, patient survival was shorter, and the prognosis was worse (*Figure 7D*).

In summary, in this part, we first confirmed that PRMT5 and RPL14 were highly expressed in HCC tissues at mRNA and protein levels with positive correlation, and the expression was also associated with poor patient prognosis using the TCGA database and clinical samples. This was subsequently validated in an independent cohort, and the same conclusion was reached. The above results were consistent with those of the previous sections, which reaffirmed that PRMT5 regulates the DNA damage repair pathway through RPL14 and provided theoretical support for a new strategy of combining regorafenib with PRMT5 and RPL14 as a potential target for the treatment of refractory HCC.

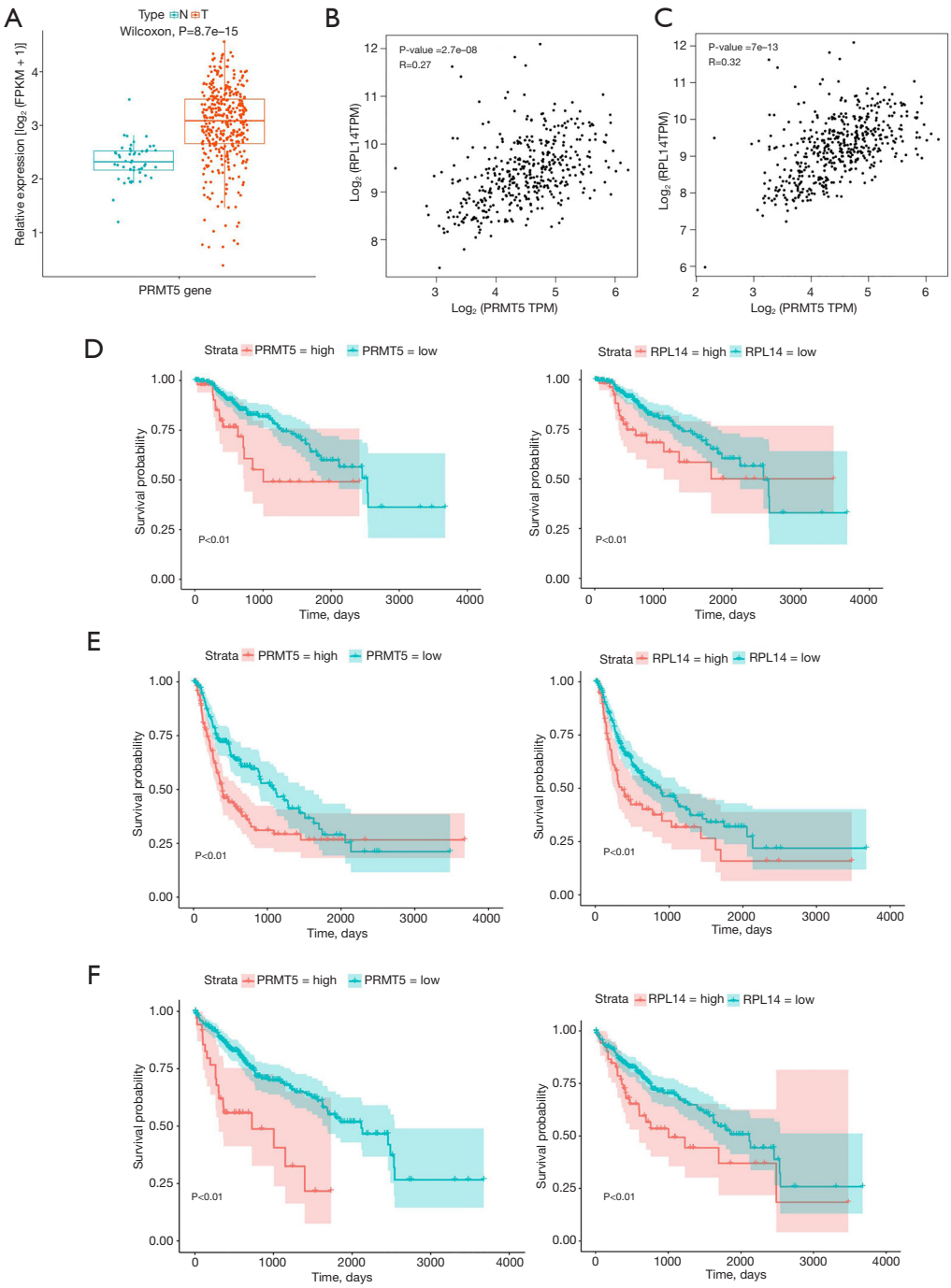

Figure 6 High expression of PRMT5 and RPL14 in HCC is associated with poor prognosis in patients. (A) The expression of PRMT5 in HCC was detected in the TCGA database. (B) Spearman test was used to analyze the relationship between PRMT5 and RPL14 in HCC on the GEPIA online website. (C) Pearson test was used to analyze the relationship between PRMT5 and RPL14 in HCC on the GEPIA online website. (D) The relationship between the expression of PRMT5 and RPL14 and the disease-free survival of HCC patients was analyzed in the TCGA database. (E) The relationship between the expression of PRMT5 and RPL14 and the progression-free survival of HCC patients was analyzed in the TCGA database. (F) The relationship between the expression of PRMT5 and RPL14 and the overall survival of HCC patients was analyzed in the TCGA database. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; TPM, transcripts per million; GEPIA, Gene Expression Profiling Interactive Analysis.

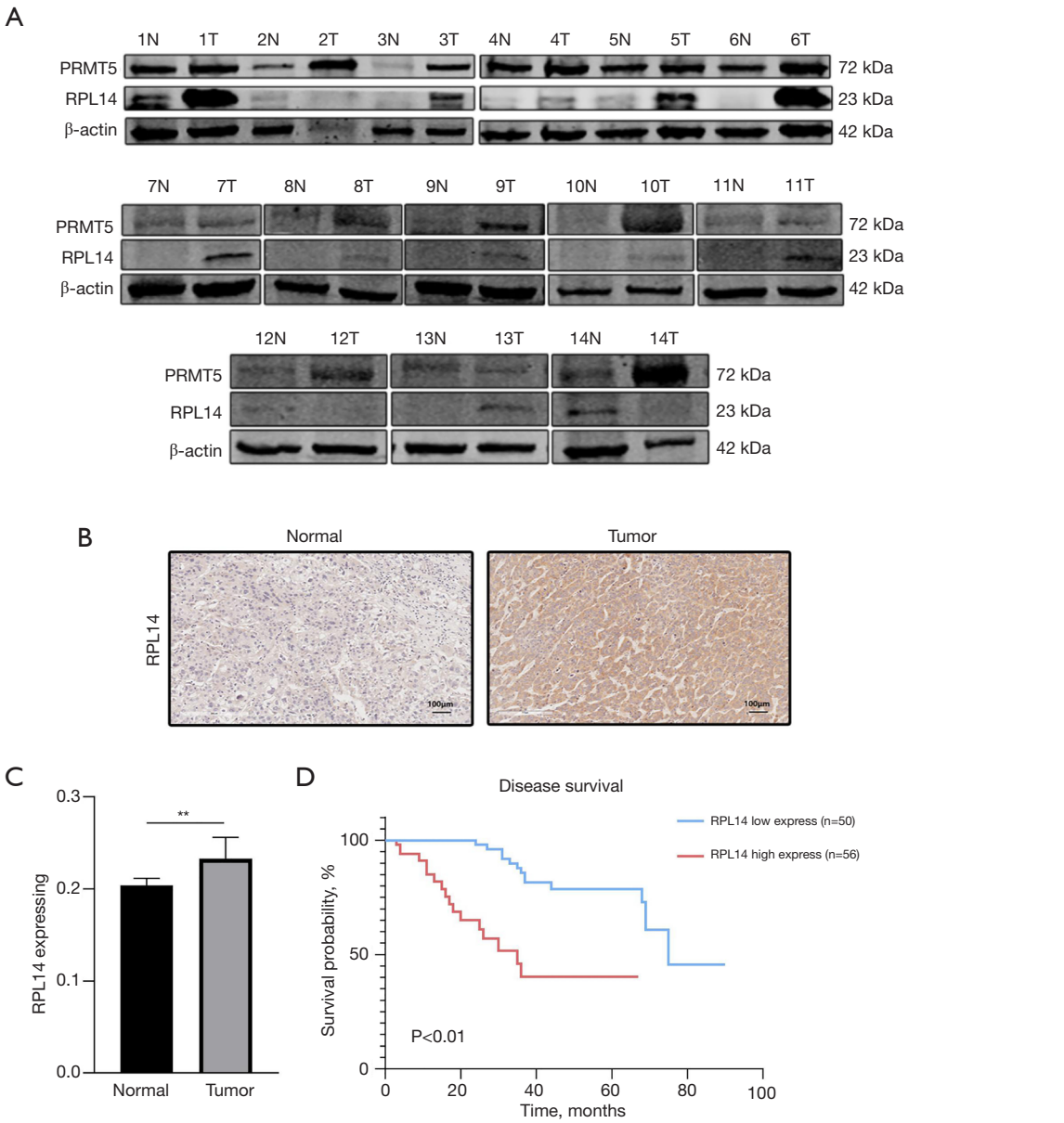

Figure 7 Independent cohort tested PRMT5 and RPL14 expression in HCC tissues and prognostic relevance. (A) The expressions of PRMT5 and RPL14 in tumor tissues and adjacent tissues were detected by WB. (B) The expression of RPL14 in tumor tissues and adjacent tissues was detected by immunohistochemistry. (C) RPL14 expression histogram of 106 tumor tissues and adjacent tissues. (D) The high and low expression of RPL14 in 106 cancer tissues and the prognosis curve. Scale bar 100 μm. **, P<0.01. HCC, hepatocellular carcinoma; WB, western blot.

Discussion

Despite advancements in the understanding of HCC genetics and therapies, the high incidence of HCC remains a significant global health burden. The intricate molecular pathogenesis of HCC is inextricably linked to a diverse mutational landscape. The genetic heterogeneity of HCC tumors is frequently the underlying cause of primary resistance to initial drug therapy in many patients and their tumors. Furthermore, the development of drug resistance may occur or be exacerbated following exposure to the same drug therapy. For example, mutations and overexpression of

genes in major signaling pathways that promote cell growth, proliferation, angiogenesis, and epithelial-mesenchymal transition and prevent apoptosis may underlie resistance to molecularly targeted agents. Tumor cells can also resist anti-tumor drugs by activating cell survival pathways and preventing apoptosis. This is achieved by blocking reactive oxygen species production and by promoting epithelial-mesenchymal transition. Furthermore, additional factors, including the tumor microenvironment, altered drug metabolism, and DNA damage repair, contribute to the development of drug resistance in HCC. Therefore, identifying novel therapeutic targets and elucidating the molecular mechanisms underlying HCC progression are crucial for improving treatment outcomes. In this study, we explored the role of PRMT5 in modulating the sensitivity of HCC cells to regorafenib and identified RPL14 as a key mediator of this process.

PRMT5 plays a critical role in various cancer processes, including DNA damage repair (18,19). Our findings demonstrated that PRMT5 enhanced DNA repair in HCC cells, thereby reducing their sensitivity to regorafenib. Transcriptomic analysis revealed that PRMT5 overexpression upregulates genes involved in DNA repair pathways, including RAD51, while decreasing markers of DNA damage like γ-H2AX. This suggests that PRMT5 promotes resistance to regorafenib by facilitating DNA repair.

PRMT5-overexpressing Huh7 cells and control cells were treated with a 12 μM concentration. The results demonstrated that 698 differential genes were up-regulated, and 685 differential genes were down-regulated in the PRMT5-overexpressing group as compared to the control group. Additionally, the results of the GO analysis indicated that there was an increase in the activation of signaling pathways related to DNA damage repair in the PRMT5-overexpressing group cells. In the regorafenib-treated PRMT5 overexpressing Huh7 cells, elevated levels of RAD51 protein and reduced levels of γ-H2AX protein were observed, indicating that PRMT5 may be implicated in and facilitate the repair of DNA damage after regorafenib-induced DNA damage in HCC cells.

One of the key mechanisms underlying the resistance of tumor cells to antitumor drugs is the abnormal activation of the DNA damage repair system (17). Our study aimed to determine whether PRMT5 could reduce the sensitivity of HCC cells to regorafenib by facilitating DNA repair. Using mass spectrometry, we identified several RPL family members interacting with PRMT5, particularly RPL14, which has been less studied in HCC. Analysis of the TCGA database showed that RPL14 expression was elevated in HCC compared to adjacent normal liver tissues, indicating its potential oncological significance.

Further examination of RPL14 revealed discrepancies between its mRNA and protein levels in normal liver tissues, likely due to post-transcriptional regulation or post-translational modifications. Mass spectrometry and immunoprecipitation experiments confirmed that PRMT5 catalyzes the symmetric dimethylation of RPL14. Additionally, PRMT5 overexpression elevated RPL14 levels in Huh7 and HepG2 cells. Treatment with a protein synthesis inhibitor (CHX) extended RPL14's half-life, while a proteasome inhibitor reduced its ubiquitinated degradation. This demonstrates that PRMT5 stabilizes RPL14 via symmetric dimethylation.

The combination of regorafenib with programmed cell death protein 1 (PD-1) inhibitors has been shown to extend survival in HCC patients (20). In our study, we observed that regorafenib increased γ-H2AX expression, indicating enhanced DNA damage in HCC cells. Notably, RPL14 expression was elevated in regorafenib-treated Huh7 cells, while RPL14 knockdown slowed cell proliferation, decreased viability, and increased sensitivity to regorafenib in both Huh7 and HepG2 cells. These effects were dose- and time-dependent, suggesting that RPL14 promotes cell survival and inhibits DNA damage caused by regorafenib.

Our findings further suggest that PRMT5-facilitated DNA repair through RPL14 plays a key role in HCC resistance to regorafenib. PRMT5 overexpression reduced γ-H2AX levels, while RPL14 knockdown reversed this effect, increasing DNA damage. RAD51 levels were similarly affected, with PRMT5 overexpression enhancing RAD51 expression and RPL14 knockdown reducing it. The CCK8 assay confirmed that PRMT5 enhances HCC cell viability during regorafenib treatment, while RPL14 knockdown reduces survival. This points to a potential mechanism where PRMT5 regulates DNA damage repair via RPL14. In the final part of our study, TCGA data revealed that PRMT5 and RPL14 were significantly upregulated in HCC patients, correlating with poor prognosis, including reduced disease-free and overall survival. This was further validated in clinical samples from our hospital, where most HCC samples exhibited higher PRMT5 and RPL14 expression compared to adjacent liver tissues. High RPL14 expression was associated with shorter survival, reinforcing the role of PRMT5 and RPL14 in HCC progression and regorafenib resistance.

PRMT5 was under alternative splicing (AS) in HCC patients after radiotherapy and generated a splicing isoform PRMT5-ISO5. It has been reported that interventional aadiology (IR)-induced elevation of PRMT5-ISO5 levels can delay tumor progression in spontaneous HCC. In this process, the induction of IR is dependent upon alterations in the levels of serine and arginine-rich splicing factor 3 (SRSF3) and heterogeneous nuclear ribonucleoprotein H 1 (HNRNPH1) at the level of PRMT5-ISO5 transcription. The binding efficiency of SRSF3 and HNRNPH1 to PRMT5 pre-mRNA may be a factor influencing the changes in the level of PRMT5-ISO5 in response to IR treatment in different HCC cells. The findings of this study indicate that the exogenous expression of truncated PRMT5 is insufficient for the restoration of colony-forming ability following IR treatment. This observation is consistent with the hypothesis that a lack of PRMT5 is responsible for this phenomenon. These findings lend support to the notion that diminished PRMT5 expression or augmented PRMT5-ISO5 levels can render cells more susceptible to IR treatment. Consequently, the combined targeted inhibition of PRMT5 or RPL14 during IR therapy to increase PRMT5-ISO5 levels, thereby enhancing tumor killing and regression and thus improving patient prognosis, represents a novel avenue of investigation.

Our findings indicate that the sensitivity of HCC to regorafenib can be enhanced by the co-targeting of PRMT5 and RPL14, which may limit metastasis and drug resistance. Concurrently, the combination of PRMT5 inhibitors with other pharmaceutical agents, including chemotherapy, immune checkpoint inhibitors, and other drugs, may prove efficacious through the synergistic targeting of cancer cells via disparate mechanisms. This approach could enhance therapeutic efficacy, circumvent drug resistance, and mitigate the potential adverse effects associated with higher doses of individual drugs.

However, there are limitations in this study. It is unclear whether PRMT5 affects sensitivity to other HCC therapies, such as sorafenib, lenvatinib, or apatinib. The specific modification sites of RPL14 by PRMT5 and the detailed mechanism of PRMT5-regulated ubiquitination inhibition remain unexplored. Additionally, our study was limited by the small number of clinical samples, all related to hepatitis B (HBV) infection. Future studies should include larger sample sizes and explore the roles of PRMT5 and RPL14 in different HCC subtypes and other etiologies. We aim to extend our research by testing various HCC-targeted drugs and establishing more diverse HCC models.

Conclusions

PRMT5 promotes DNA damage repair through RPL14 and leads to regorafenib resistance in HCC. RPL14 plays a critical role in enhancing PRMT5-driven DNA damage repair and reducing drug sensitivity and has been identified as a potential therapeutic target for overcoming regorafenib resistance in HCC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-737/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-737/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-737/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the ethics committee of EHBH (No. EHBHKY2024-K011-P001), and conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all the patients.

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