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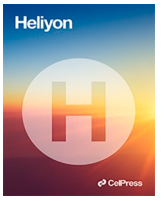

Research article

AXL inhibition prevents RPA2/CHK1-mediated homologous recombination to increase PARP inhibitor sensitivity in hepatocellular carcinoma

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A B S T R A C T

Homologous recombination defects (HRD) render cells fail to repair DNA double-strand break (DSB), which causes synthetic lethality in these cells with punch by poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi). Here, we reveal a receptor tyrosine kinase, AXL, whose inhibition leads to HRD in hepatocellular carcinoma (HCC) cells. AXL is upregulated in HCC tumors, which is positively correlated with low survival rates. AXL knockdown or AXL inhibition by bemcentinib reduces HR efficiency in HCC cells, and AXL plays its role in HR repair through its kinase activity. Furthermore, we find that AXL interacts with RPA2, enhancing the recruitment of RPA2 to DNA damage sites. Mechanistically, AXL promotes the tyrosinization of RPA2 at tyrosine 9, promoting the phosphorylation of CHK1, thereby strengthens the HR repair ability in HCC cells to resist DNA damage. In conclusion, our results reveal that AXL is a promising therapeutic biomarker for HCC patients, and present that targeting AXL-RPA2-CHK1 pathway together with PARP inhibitor will be effective therapeutic strategy in HCC.

1. Introduction

As the most prevalent primary liver cancer, Hepatocellular carcinoma (HCC) represents over 90 % of liver malignancies and presents a significant global health challenge due to its increasing incidence worldwide [1,2]. Current treatment options for HCC encompass surgical resection, liver transplantation, radiofrequency thermal ablation, and targeted therapy [3]. However, the aggressive nature and high recurrence rate of HCC contribute to a survival rate (5-year) of less than 15 % for advanced cases [4]. Despite the emergence of targeted therapies like tyrosine kinase inhibitors, their benefits are limited [5,6]. Consequently, there is a pressing need for novel therapeutic approaches for HCC.

HCC arises in the setting of chronic inflammation [7], leading to persistent replication stress, DNA damage accumulation, and a strong reliance on compensatory DNA damage repair mechanisms for cell survival [8]. PARP1 (Poly(ADP-ribose) polymerase 1) is an enzyme that facilitates ADP-ribose transferring from nicotinamide adenine dinucleotide to protein substrates, and it is activated by DNA single-as well as double-strand breaks (SSBs and DSBs) [9,10]. PARP inhibitors (PARPi) trap PARP enzymes at sites of damaged DNA, leading to the formation of DNA-PARP complexes as well as causing severe cytotoxicity in cells [11–13]. PARPi exploit synthetic lethality in cells with deficiencies in homologous recombination (HR) repair and are currently utilized in the treatment of breast and ovarian cancer patients with HR deficiencies [14,15]. However, in the case of HCC, key HR regulators like BRCA1 and BRCA2 show low mutation rates as well as abnormal expression levels, constraining the effectiveness of PARPi treatment [16]. Therefore, there is a pressing need to identify new targets that mimic the role of BRCA1/2 in HR but are more suitable for HCC. This will broaden the

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applicability of PARPi in specific HCC patients who could benefit from this therapeutic approach.

The focus of this study is to inquire into the function of the receptor tyrosine kinase AXL in HCC. Our findings indicate that AXL is amplified in HCC tissues and is related to poor prognosis in HCC patients. Specifically, we find that silencing or inhibiting AXL with bemcentinib reduces HR repair efficiency in HCC cells, making them more susceptible to DNA damage inducers such as neocarzinostatin (NCS) and the PARP inhibitor Olaparib. Interestingly, it is observed that AXL enhances HR repair by acting on its 558 Lysine site, which is crucial for its kinase activity, leading to CHK1 phosphorylation in HCC cells. Additionally, the research reveals that AXL interacts with RPA2, facilitating RPA2 recruiting to DNA damage area. Mechanistically, AXL promotes the tyrosinization of RPA2 at tyrosine 9, enhancing CHK1 phosphorylation and fortifying the HR repair capacity in HCC cells to combat DNA damage. These results underscore the major role of AXL in the HR repair in the HCC and propose that targeting the AXL-RPA2-CHK1 pathway concurrently with PARP inhibitors could serve as an effective therapeutic approach for HCC.

2. Results

2.1. AXL upregulation is associated with poor prognosis in HCC patients

To explore the role of AXL in HCC, we examined the levels of AXL mRNA in primary HCC tissue samples and normal hepatic tissue samples from two datasets (GSE14323 and GSE54503). Our analysis revealed a significant upregulation of AXL mRNA levels in HCC tissue samples compared to normal hepatic tissue samples (Fig. 1A and B). Furthermore, the clinical data analysis from the GSE10141

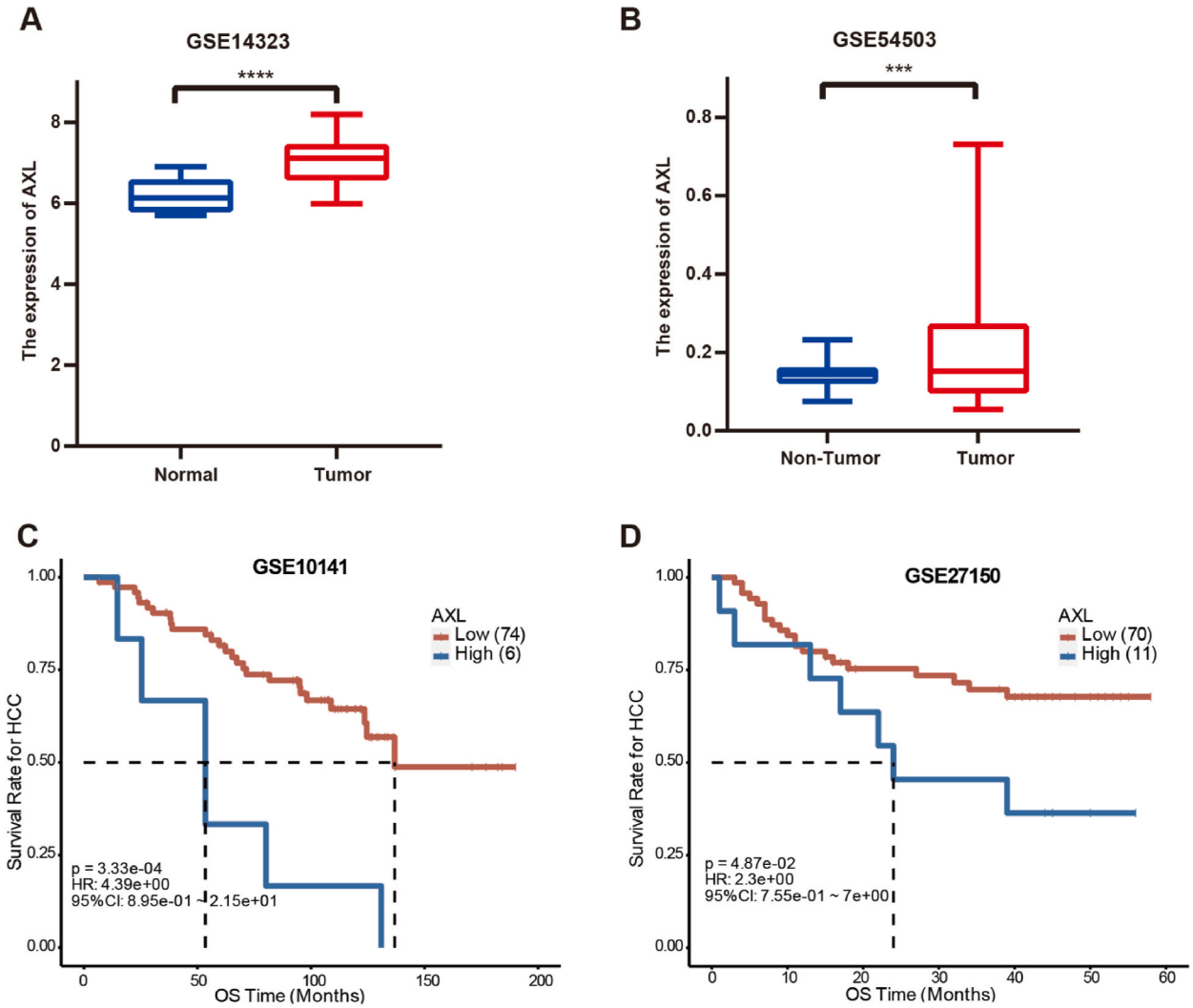

Fig. 1. Fig. 1. **Overexpression of AXL in HCC, correlating with unfavorable prognosis in patients with HCC. (A–B)** Variation in AXL mRNA expression levels in normal liver and HCC tissues as per GSE14323 (A) and GSE54503 datasets (B). ***P < 0.001, ****P < 0.0001. **(C–D)** Survival curves for AXL^{high} and AXL^{low} HCC patients based on the GSE10141 (C) and GSE27150 datasets (D).

and GSE27150 datasets indicated that HCC patients with AXL amplification exhibited shorter overall survival rates (Fig. 1C and D). These findings indicate that AXL is highly expressed in HCC tissues and is positively relevant with a poorer prognosis in HCC patients.

2.2. AXL promotes HR repair in HCC cells

For investigating the function of AXL in the response to DNA damage, we compared viability of AXL-knockdown cells to control cells when exposed to the DNA damage agent NCS. Our findings revealed a significant decrease in the survival of AXL-depleted cells compared to control cells in response to NCS (Fig. 2A). Immunoblotting analysis demonstrated that AXL depletion reduced the phosphorylation of CHK1, rather then CHK2, in HCC cells following NCS treatment (Fig. 2B). Additionally, our ChIP analysis indicated that AXL was recruited to the site-specific DSBs in HCC cells (Fig. 2C). Furthermore, AXL-depleted cells exhibited increased sensitivity to the PARP inhibitor Olaparib (Fig. 2D). We also examined the impact of AXL knockdown on HR and NHEJ repair efficiency in HCC cells, and observed a decrease in HR repair efficiency, with no change in NHEJ repair efficiency, in AXL knockdown cells (Fig. 2E and F). We also found that the cell cycle phases in AXL-depleted cells were almost the same as the control cells (Fig. 2G), indicating that the effect of AXL on HR repair is not cell cycle dependent. Moreover, AXL knockdown caused a great reduction in the formation of RAD51 foci following NCS treatment in HCC (Fig. 2H and I). These findings strongly suggest that AXL enhances HCC cells' HR repair ability. In summary, our results designate that AXL plays a crucial role in enhancing HR repair in HCC cells.

2.3. AXL enhances HCC cell HR ability dependent on its kinase activity

We proceeded to investigate whether AXL's kinase activity was necessary for the HR repair process. Studies previously showed that the AXL K558R mutant makes AXL kinase inactive [17]. Then the AXL Knockdown cells were overexpressed with AXL wild-type (WT) as well as the AXL K558R mutant, respectively. It showed that AXL WT, rather than the AXL kinase inactive mutant, rescued the reduced phosphorylation of CHK1 upon AXL knockdown (Fig. 3A). Moreover, AXL WT, rather than the AXL kinase inactive mutant, reversed the increased sensitivity of AXL knockdown cells to NCS and Olaparib (Fig. 3B and C). Additionally, the AXL kinase dead mutant failed to enhance HR efficiency and RAD51 recruitment upon DNA damage (Fig. 3D–F). Bemcentinib (R428, BGB324), a

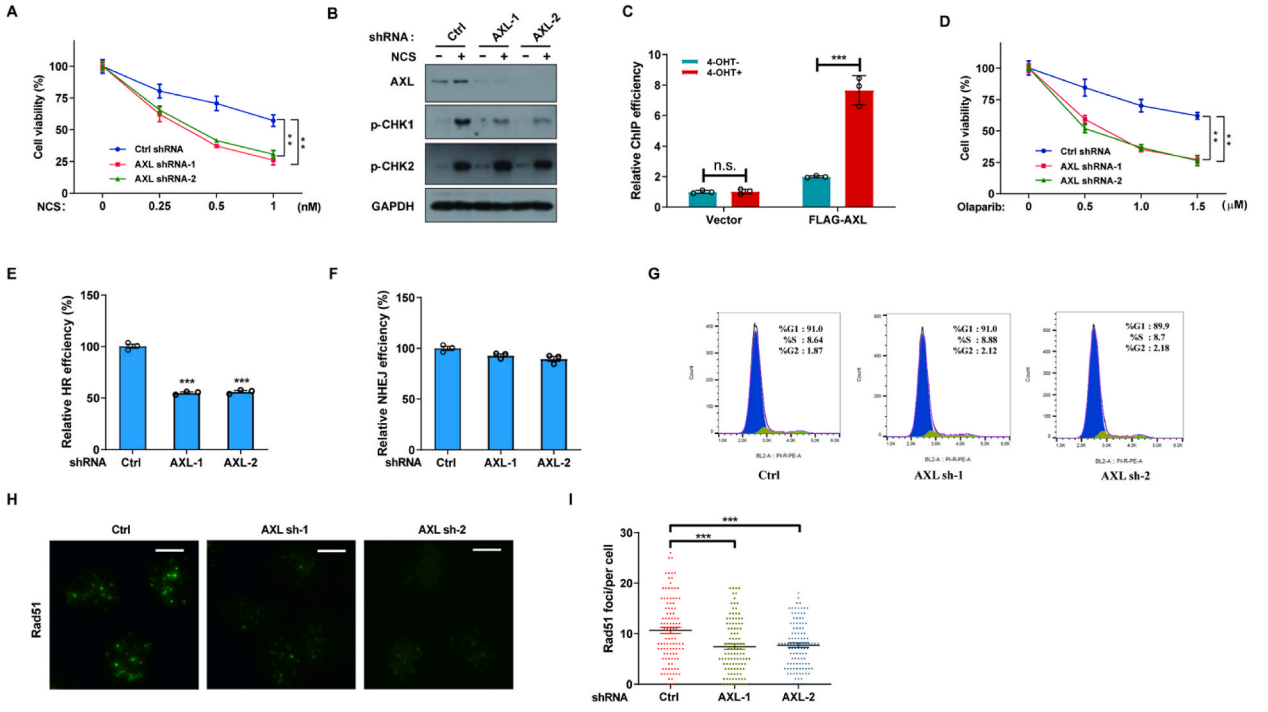

Fig. 2. AXL promotes HR repair in HCC cells. (A) The response of PLC/PRF/5 cells (Ctrl, shAXL#1, and shAXL#2) to neocarzinostatin (NCS) was evaluated using CCK8 assays. **(B)**Analysis of CHK1 and CHK2 phosphorylation levels (Ctrl, shAXL#1, and shAXL#2) treated with 0.5 nM NCS. **(C)** ER-*Asi*SI PLC/PRF/5 cells were transfected with empty vector or Flag-AXL, followed by induction of DSBs with 4-OHT. ChIP-qPCR was utilized to detect Flag-AXL accumulation at *Asi*SI-induced DNA damage sites. **(D)** Evaluation of the sensitivity of PLC/PRF/5 cells (Ctrl, shAXL#1, and shAXL#2) to Olaparib using CCK8 assays. **(E–F)** Transfection of PLC/PRF/5 cells (Ctrl, shAXL#1, and shAXL#2) with DR-GFP (E) or EJ5-GFP (F) in conjunction with pCBA-I-SceI and mCherry. After 2 days, flow cytometric analysis was performed. **(G)** Cell cycle analyses of Ctrl and AXL knockdown PLC/PRF/5 cells. **(H)** Detection of RAD51 foci in PLC/PRF/5 cells treated with 0.5 nM NCS, both before and 6 h post-treatment, by immunofluorescence. Representative images of RAD51 foci in PLC/PRF/5 cells, scale bar = 10 μm. Note: ***$p < 0.001$. **(I)** A scatter plot illustrating focus signals per cell after three independent experiments is provided.

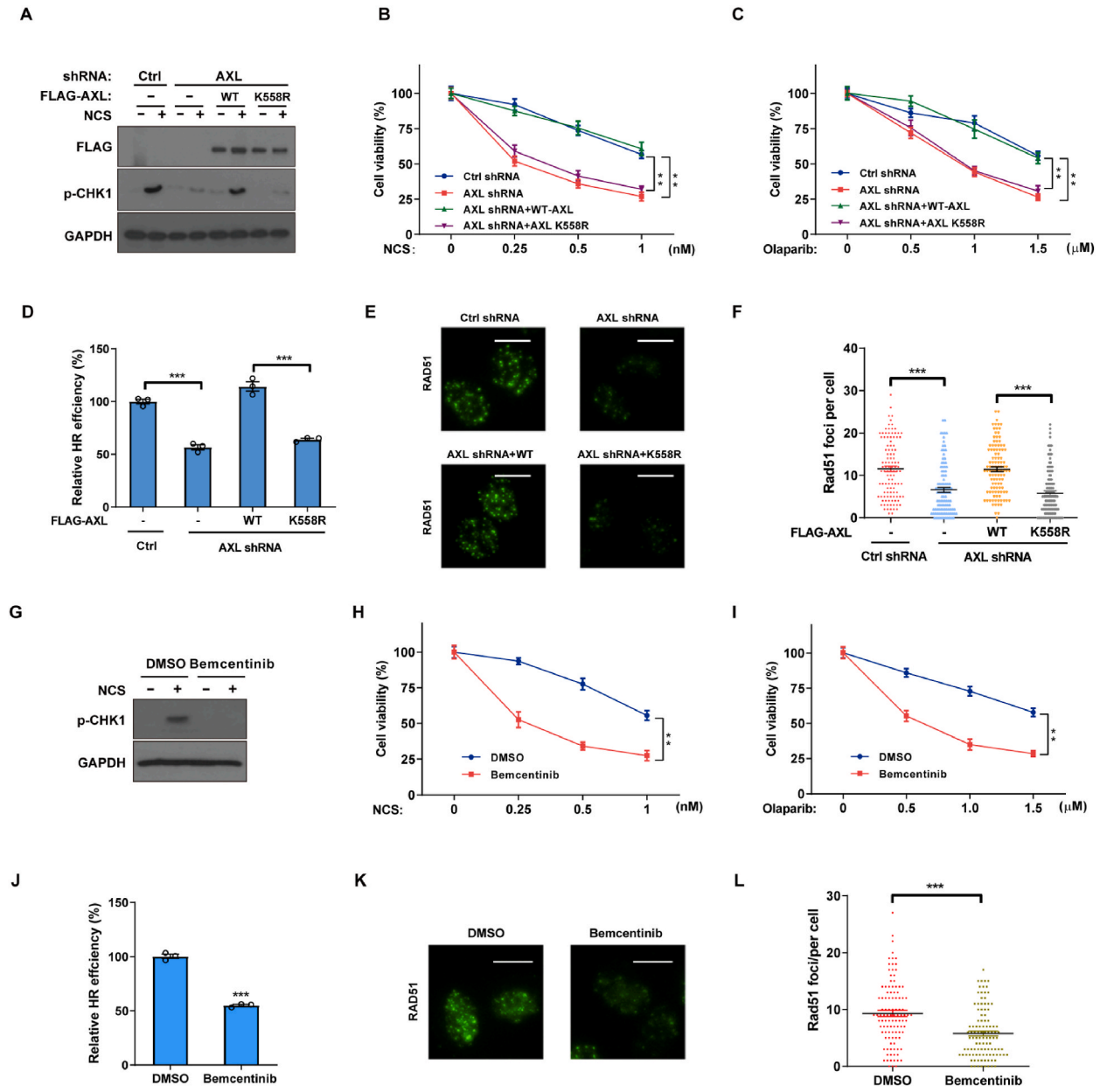

Fig. 3. AXL enhances HCC cell HR ability dependent on its kinase activity. **(A)** AXL-deficient PLC/PRF/5 cells were rescued with either AXL wild-type (WT) or AXL K588R mutants, followed by analysis of CHK1 phosphorylation levels post treatment without/with 0.5 nM NCS. **(B–C)** Cell viability of specified PLC/PRF/5 cells (Ctrl, shAXL, WT rescue, and K558 rescue) in response to NCS (B) and Olaparib (C) was evaluated through CCK8 assays. **(D)** AXL-WT and AXL-K558 rescued PLC/PRF/5 cells were transfected with DR-GFP. Subsequently, flow cytometric analysis was conducted 48 h later. **(E–F)** AXL-WT and AXL-K558 rescued PLC/PRF/5 cells were exposed to 0.5 nM NCS. Representative images of RAD51 foci in the specified PLC/PRF/5 cells are depicted, scale bar = 10 μm (E). The detection of RAD51 foci before or 6 h after NCS treatment using immunofluorescence (F). **(G)** Following treatment with NCS and AXL inhibitor bemcentinib, phosphorylation levels of CHK1 in PLC/PRF/5 cells were analyzed. **(H–I)** Cell viability of PLC/PRF/5 cells under the combined treatment of bemcentinib with NCS (H) or Olaparib (I) was determined. **(J)** HR repair capability of PLC/PRF/5 cells upon bemcentinib treatment was assessed. **(K–L)** Representative images of RAD51 foci in the specified PLC/PRF/5 cells were captured (K). RAD51 foci induced by NCS in PLC/PRF/5 cells with bemcentinib treatment were recorded via immunofluorescence (L). Remarks: ***$p < 0.001$.

selective orally bio-available small molecule AXL kinase inhibitor [18], was found to reduce CHK1 phosphorylation and increase the sensitivity of HCC cells to NCS and Olaparib (Fig. 3G–I). Furthermore, AXL inhibition by Bemcentinib resulted in decreased HR repair ability and RAD51 foci formation upon DNA damage (Fig. 3J–L). Collectively, these findings elucidate that AXL's ability to enhance HR repair in HCC cells is dependent on its kinase activity.

2.4. AXL interacts with and recruits RPA2 to DNA damage sites

After confirming AXL's involvement in the ATR-CHK1 signaling pathways, we proceeded to investigate its potential interaction with the regulators in this pathway. Our findings revealed the presence of RPA2 in AXL complexes (Fig. 4A) and the existence of AXL in RPA2 complexes (Fig. 4B), suggesting that RPA2 could be a functional binding partner of AXL. Subsequently, we examined whether AXL could influence the recruitment of RPA2 upon DNA damage. As depicted in Fig. 4C and D, AXL knockdown resulted in a reduction of RPA2 foci following NCS treatment. Similarly, treatment with the AXL inhibitor, Bemcentinib, yielded a comparable effect to AXL knockdown, impeding the recruitment of RPA2 to DNA damage area (Fig. 4E and F). Collectively, our results provide evidence that AXL interacts with RPA2, facilitating RPA2 recruiting to DNA damage area.

2.5. AXL promotes the tyrosinization of RPA2 at tyrosine 9, promoting the phosphorylation of CHK1

AXL tyrosine kinase phosphorylates the C-terminal tyrosine residues, activating multiplex intracellular signaling [19,20], we were curious about the impact of AXL on the tyrosinization of RPA2. In HCC cells, inhibition of AXL by Bemcentinib or depletion of AXL resulted in reduced tyrosinization of RPA2 (Fig. 5A and B). Interestingly, the RPA2 T9A mutant, but not the T4A mutant, showed a decrease in protein tyrosinization (Fig. 5C), suggesting that tyrosinization of RPA2 specifically occurs at tyrosine 9. Furthermore, the RPA2 T9A mutant failed to rescue the reduction in CHK1 phosphorylation upon RPA2 knockdown (Fig. 5D). Functionally, the RPA2

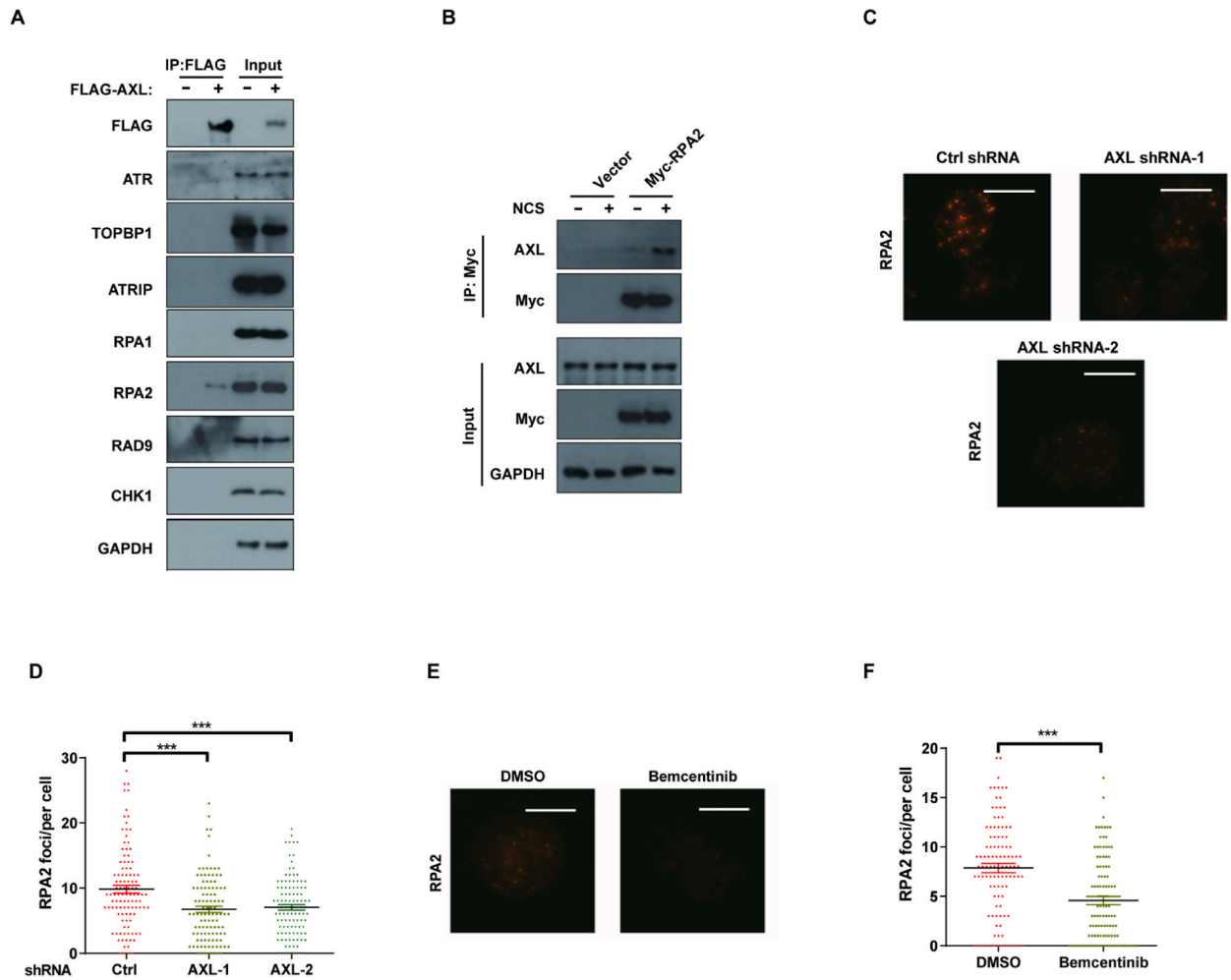

Fig. 4. AXL interacts with RPA2, enhancing the recruitment of RPA2 to DNA damage sites. (A) PLCPRF/5 cells over-expressing FLAG-AXL were lysed and pull down with indicated antibody, followed by boiling and blotting with the specified antibodies. **(B)** PLCPRF/5 cells over-expressing myc-RPA2 were lysed and pull down by anti-myc antibody, followed by boiling and blotting with the specified antibodies. **(C–D)** Immunofluorescence (C) was used to observe the RPA2 foci (D) induced by NCS in AXL-knockdown PLC/PRF/5 cells, scale bar = 10 μm. **(E–F)** Immunofluorescence (E) was utilized to detect the RPA2 foci (F) induced by NCS in PLC/PRF/5 cells treated with bemcentinib, scale bar = 10 μm. Remarks: ***$p < 0.001$.

T9A mutant exhibited loss of function in synthetic lethality with NCS and Olaparib (Fig. 5E and F), impaired HR repair (Fig. 5G), and reduced RAD51 recruitment upon DNA damage (Fig. 5H and I). These findings indicate that AXL promotes the tyrosinization of RPA2 at tyrosine 9, leading to enhanced CHK1 phosphorylation and upregulation of HR repair, thereby conferring increased resistance to DNA damage in HCC cells.

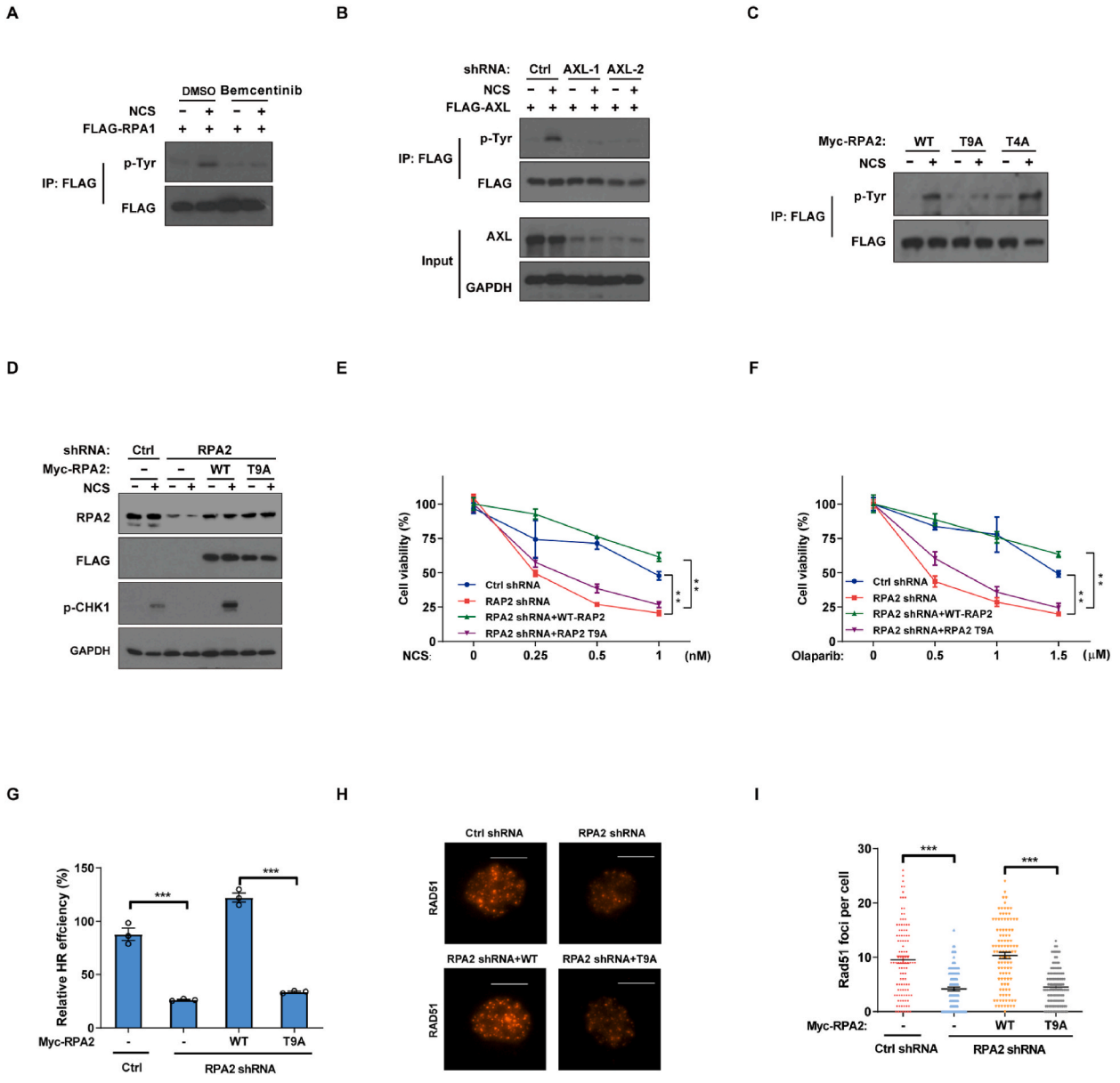

Fig. 5. AXL promotes the tyrosinization of RPA2 at tyrosine 9, promoting the phosphorylation of CHK1. (A) PLCPRF/5 cells that overexpressed FLAG-RPA2 were subjected to bemcentinib treatment, followed by cell lysis and immunoprecipitation using anti-FLAG antibody. The lysates were then boiled and probed with specific antibodies. **(B)** In PLCPRF/5 cells over-expressing FLAG-RPA2 along with AXL knockdown, cell lysis was performed, followed by protein pulldown by anti-FLAG antibody. The samples were boiled and probed with specific antibodies. **(C)** RPA2-knockdown PLC/PRF/5 cells were rescued by RPA2 WT and T9A mutant, followed by cell lysis and antibody probing. **(D)** The RPA2-knockdown PLC/PRF/5 cells were rescued by RPA2 WT and T9A mutant, the samples were lysed and blotted with indicated antibodies. **(E, F)** RPA2-knockdown PLC/PRF/5 cells were rescued by RPA2 WT and T9A mutant, and then their sensitivity to NCS (E) and Olaparib (F) was assessed. **(G)** RPA2 knockdown PLC/PRF/5 cells were rescued by RPA2 WT and T9A mutant, followed by determination of the HR repair efficiency of the cells. **(H, I)** RPA2 knockdown PLC/PRF/5 cells were rescued by RPA2 WT and T9A mutant, and subsequently, immunofluorescence (H) was used to examined the RAD51 foci (I) in the cells, scale bar = 10 μm. Remarks: ***$p < 0.001$.

2.6. AXL inhibition raise the raise the therapeutic effect of Olaparib for HCC in vivo

The results presented above provide evidence that inhibiting AXL leads to HRD and enhances sensitivity of HCC cells upon DNA damage inducers. This prompted us to investigate the efficacy of combining AXL inhibition with Olaparib in an in vivo setting. NOD SCID mice with PLC/PRF/5 xenografts were generated as well as treated with Olaparib in combination with Bemcentinib. The control group received DMSO along with Olaparib. As depicted in Fig. 6A–C, we found that inhibiting AXL by Bemcentinib enhances the therapeutic efficacy of Olaparib for HCC in vivo, indicating that the combination treatment of Olaparib and Bemcentinib holds promise as a therapeutic strategy for HCC.

3. Discussion

BRCA1/2 dysfunction results in HR deficiency and caused DNA damage, which sensitize cells to PARP inhibitors. BRCA1/2 mutation has been associated with the increased risks of breast and ovarian cancer among women, but it is rarely occurred in HCC patients [21]. Therefore, strategies targeting HR-related genes have been developed to synergistically enhance the efficacy of PARP inhibitors in HCC cancer. For example, BRD4 inhibition induces synthetic lethality in ARID2-deficient hepatocellular carcinoma by BRCA1 and RAD51 transcription [22]; combination of androgen receptor (AR) inhibitor enzalutamide (Enz) and Olaparib might be effective for advanced HCC [23]. However, the known HR-related targets were still not satisfactory and need to be identified in the future to development more effectively therapeutic approach to suppress HCC progression. Though the survival rates of young-onset breast cancer patients who carry a BRCA mutation are similar with the non-carriers, evidence also showed that mutation of homologous recombination repair (HRR) genes may has effect of the OS of certain cancers. For example: The HRR gene carriers in pancreatic ductal adenocarcinoma (PDAC) show longer OS compared to non-carriers. In addition, it was reported that RPA2 proteins expression is associated with adverse survival rates in stage II and stage III colon cancer patients [24]. Because our date showed that AXL plays a critical role in HR repair by directly interacting with RPA2 protein, it is possible that AXL affecting the overall survival rate of liver patients at least partly due to the regulation of RPA2. However, previous date showed that AXL upregulation is associated with tumor growth and metastasis through downstream signaling pathways including JAK/STAT, PI3K/AKT, and NF-kB pathway [25]. Further research is necessary to delve deeper into the biological mechanisms underlying the correlation between AXL dysregulation and lower overall survival in HCC cancer.

Despite the challenges in the application of PARPi in HCC, mounting evidence demonstrates that aiming at HR in HCC could be a potential therapeutic approach. Initially, scientists observed an upregulation of HR in HCC when compared to normal liver tissue using the Rosa26HR gene-edited mouse model, suggesting a beneficial role of HR in HCC development [26]. Understanding the mechanisms by which the HCC cells acquire enhanced HR capability is crucial for characterizing potential targets to sensitize HCC cells to DNA damage therapies. In HER2-positive breast cancer cell lines, an up-regulation in the expression of total AXL as well as phosphorylated AXL was observed, and AXL inhibition could reverse the resistance to lapatinib in these cells [27]. Additionally, in lung cancer, AXL were found to enhance EGFR inhibition resistance [28,29]. AXL overexpression also provided protection against growth inhibition by imatinib in chronic myeloid leukemia cell lines, while silencing AXL in these cells resulted in apoptosis in these cell lines [30]. Notably, recent studies have linked AXL expression with radio-resistance in HNSCC, and the inhibition of AXL enhanced sensitivity to radiotherapy and chemotherapy [31]. In esophageal cancer, AXL overexpression was related to resistance to DNA damage-induced cellular responses as well as cisplatin-induced apoptosis [32]. Upregulation of AXL was also observed in cisplatin-resistant ovarian cancer, while inhibition of AXL in these cells had negligible effect on cell growth [33]. Silencing AXL in a panel of lung as well as breast cancer cell lines sensitized them to the mitotic inhibitors, demonstrating that the inhibition of the AXL could sensitize mesenchymal tumors to drugs that causes DNA damage as well as cell cycle inhibition [34]. However, the role of AXL is still not fully interpreted. In this context, we examined the differences in AXL expression levels between HCC and normal tissues. We initially showed that AXL was highly amplified in HCC tissues, and upregulated AXL expression was relaxant with a bad outcome for HCC patients, suggesting that AXL upregulation plays a promoting role in HCC progression.

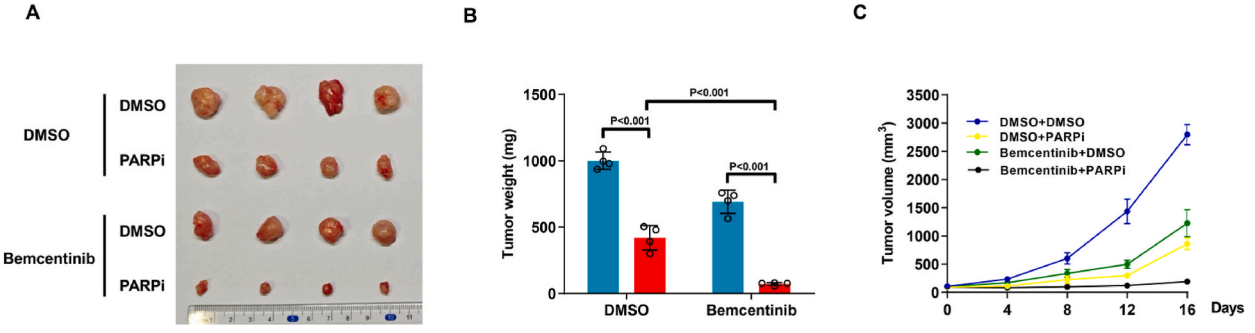

Fig. 6. Blocking AXL enhances the therapeutic efficacy of Olaparib for HCC in vivo. (A–C) Tumor growth of PLC/PRF/5 cells was assessed in xenografts treated with Olaparib plus DMSO or Olaparib plus bemcentinib (n = 4). Images of mouse xenograft tumors (A), changes in tumor weight over 16 days (B), and the volumes of xenograft tumors (C) were depicted.

Building on the evidence implicating AXL in DDR, our research illustrates that silencing of AXL suppresses the HR repair capacity and promotes the sensitivity of HCC cells to Olaparib, both in vitro as well as in vivo. These findings shed light on the mechanisms behind the upregulation of HR in HCC cells and their resistance development to PARPis. They also suggest that a combined approach targeting AXL depletion as well as using PARPis may offer a useful strategy to enhance therapeutic efficacy in AXL-expressing HCC. Considering bemcentinib as a specific AXL inhibitor, a combination therapy with bemcentinib could significantly expand the application of PARPis in HCC patients. Furthermore, as CHK1 phosphorylation plays a pivotal role in cellular DNA damage response [35], our research shows that AXL promotes CHK1 phosphorylation, leading us to hypothesize that AXL-mediated CHK1 phosphorylation could lead to decreased genomic stress levels. RPA2, a direct upstream target of CHK [36], is crucial in cellular response to DNA damage, and we noted a notable increase in tyrosine 9-phosphorylated RPA2 in HCC cells. Additionally, our findings demonstrate that tyrosine 9 tyrosinization of RPA2 promotes CHK1 phosphorylation in HCC cells, thus identifying the AXL-RPA2-CHK1 repair axis in HCC.

In summary, our study uncovers a new connection between AXL signaling and response to DNA damage. We demonstrate that AXL acts as a prognostic factor in HCC, emphasizing the potentially significant role of AXL-mediated HR proficiency in HCC. Functionally, we have demonstrated that silencing AXL or inhibiting it with bemcentinib suppresses HR as well as sensitizes HCC cells to drug therapies, containing NCS and the PARP inhibitor Olaparib. Mechanistically, we provide evidence that AXL interacts with and promotes the tyrosinization of RPA2 at tyrosine 9 in HCC cells, thereby promoting CHK1 phosphorylation and enhancing HR repair capability to counteract DNA damage in HCC cells. Aiming at this pathway in HCC patients may provide benefits from PARPis treatment.

4. Materials and methods

4.1. Cell lines and cell culture

PLC/PRF/5 (Resources:ATCC, Cat No:CL-0415, RRID:CVCL_0485) and 293FT (Resources:ATCC, Cat No:CL-0313, RRID:CVCL_6911) used in this project were obtained from Procell Life Science Technology Co., Ltd. (China). The cells were maintained in DMEM medium (Gibco) together with 10 % FBS (Gibco) as well as 1 % penicillin/streptomycin (P/S) cocktail (Invitrogen). The culture plates were put in a 37 °C constant temperature incubator.

4.2. Constructs

Specific shRNAs designed to target AXL or RPA2 were inserted into the pLKO.1-puro vector (Addgene). The sequences for shNTC, shAXL, and shRPA2 can be found in the Supplementary Information. For overexpression of the specified proteins, the coding sequence for indicated gene was inserted into the pLenti-CMV-blast vector (Addgene). The primers used for overexpression constructs are listed in the Supplementary Information.

4.3. Immunoblotting and antibodies

For Immunoblotting, the following primary antibodies were used: Anti-AXL (sc-166269, 1:2000) and Anti-RPA2 (sc-56770, 1:2000) were procured from Santa Cruz; Anti-RPA1 (A300–241A, 1:5000) was obtained from Bethyl Laboratories. Additionally, Anti-FLAG (F1804, 1:2000) was purchased from Sigma; anti-pT68 Chk2 (2661,1:1000), anti-pS345 Chk1 (2348, 1:1000), Anti-ATR (2790, 1:1000), and anti-Myc Tag (2276, 1:2000) were sourced from CST; and anti-GAPDH (60004–1-lg, 1:2000).

4.4. NHEJ and HR reporter assay

NHEJ reporter (EJ5-GFP, Addgene) or HR reporter (DR-GFP, Addgene) together with pCherry (Addgene) and pCBA-I-SceI (Addgene) were transfected into cells. Cells were collected for flow cytometric analysis after 48 h. The efficiency of NHEJ or HR was normalized.

4.5. Colony formation

A total of 1000 cells expressing the specified constructs were seeded into individual wells of six-well plates and allowed to incubate for 24 h before being exposed to the designated doses of indicated drugs for 10–14 days. The resulting colonies were subsequently stained with GIEMSA (5 %) for counting.

4.6. DNA resection measurement

ER-*Asi*SI cells expressing Flag-AXL were collected after treating with 1 μM 4-OHT for DNA extraction using DNAzol reagent (Invitrogen). A total of 500 ng DNA was digested with *Bsr*GI restriction enzyme at room temperature overnight. Subsequently, about 20 ng of the digested samples were utilized as templates for QPCR.

4.7. Immunofluorescence

Cells were fixed as well as permeabilized with acetone: methanol (1:1) at −20 °C for 0.5 h. Following this, cells were incubated with primary antibodies (RPA2, sc-56770, 1:500 and RAD51, ab133534, 1:500) at 4 °C overnight and then incubated with secondary antibodies (room temperature, 0.5 h). The number of foci was counted by Image-Xpress confocal and ImageJ.

4.8. Immunoprecipitation (IP)

Immunoprecipitation assay was conducted as previously described [37]. In brief, Cells were lysed (0.02M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.6 % Nonidet P-40 with protease inhibitors) as well as centrifuged at 15,000×*g* for 25 min. The supernatant was subjected to indicated antibodies alone with protein A/G-Sepharose beads (Amersham Biosciences) and rotated for12 h t at 4 °C. Beads were then washed by NETN buffer three times, and samples were boiled with 50 μl 1x SDS loading buffer as well as immunoblotted with indicated antibodies.

4.9. Tumor xenograft

Parental PLC/PRF/5 cells were injected into 8-week-old male athymic nude mice. Every mouse received a 100 μl mixture of 2×10^6 cells with 30 % growth factor reduced Matrigel (BD Biosciences). Mice with tumors of approximately 100 mm^3 were randomly divided into groups: the combination treatment group received Olaparib (50 mg/kg) along with Bemcentinib (20 mg/kg). The control group received DMSO with Olaparib. Tumor volume was measured every 4 days using calipers, and mice were sacrificed on day 16 after treatment.

Statistics

All statistical analyses were conducted by SPSS version 21.0 software (SPSS Inc., Chicago, USA). Paired t-tests were used to evaluate the level of AXL between tumor and normal tissues. Overall survival (OS) was defined from the diagnostic date to death or loss of follow-up. Significant differences in OS were assessed by log-rank test. Data in bar as well as line graphs are presented as mean ± SD of three independent experiments. Statistical significance is represented in figures by: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Ethics statement

All procedures involving the study were approved by the Research Ethics Committee of China-Japan Friendship Hospital (2023-KY-064-1).

CRediT authorship contribution statement

Kai-Min Li: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Ligong Deng:** Validation, Software, Resources, Formal analysis. **Li-Jun Xue:** Methodology, Investigation. **Chang Tan:** Resources, Formal analysis. **Shu-Kun Yao:** Writing – review & editing, Visualization, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36283.

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