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Original Article

NOTCH1 regulates the DNA damage response and sorafenib resistance by activating ATM in hepatocellular carcinoma

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Abstract: Objective: This study investigates the mechanism underlying sorafenib resistance in hepatocellular carcinoma cells (HCC), focusing on DNA damage repair (DDR) pathways to develop targeted therapeutic strategies. Methods: Bioinformatics analysis was used to screen genes associated with sorafenib resistance, which was further demonstrated by western blotting. Cell proliferation was determined using the EdU assay. The presence of binding sites between valproic acid (VPA) and NOTCH1 was analyzed by molecular docking. Comet and flow cytometry assays evaluated DNA damage and cell cycle arrest induced by VPA in sorafenib-resistant cells, with further mechanistic insights gained via western blotting and co-immunoprecipitation (Co-IP). Results: We found that NOTCH1/ATM axis plays a vital role in the prognosis of patients with liver cancer and in the behavior of sorafenib-resistant cells. HCC resistant to sorafenib exhibited enhanced cell proliferation ability. Moreover, overexpression of NOTCH1 in sorafenib-sensitive HCC cells significantly increased liver cancer cell proliferation. Conversely, silencing NOTCH1 expression in sorafenib-resistant HCC cell lines reduced their proliferative activity. Additionally, VPA enhanced the therapeutic efficacy against sorafenib-resistance cells by modulating NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling axis and homologous recombination (HR) activity. Conclusion: Targeting NOTCH1 and ATM is a promising strategy to overcome sorafenib resistance in HCC, particularly through the combined use of VPA and sorafenib.

Keywords: NOTCH1, ATM, RAD51, sorafenib resistance, valproic acid

Introduction

Sorafenib, a first-line targeted therapy, is commonly used to treat patients with advanced liver cancer. It inhibits tumor cell proliferation primarily by targeting RAF-1, B-Raf, and Ras/Raf/MEK/ERK signaling pathways. Additionally, it plays a vital role in anti-angiogenesis through its effects on hepatocyte factor receptor, FMS-like tyrosine kinase, vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, and platelet-derived growth factor receptor and other tyrosine kinases [1, 2]. The SHARP clinical trial demonstrated that sorafenib could extend median survival by 3 months compared with placebo when used as a first-line treatment for patients with advanced liver cancer [3]. However, the development of sorafenib resistance significantly limits the clinical application and poses a major challenge in the treatment of advanced liver cancer patients. Despite extensive research into the mechanisms of sorafenib resistance, the specific underlying mechanism remains elusive. Sorafenib resistance can be divided into primary and secondary types [4, 5]. Primary resistance is mainly related to the continuous activation of downstream EGFR and Ras/Raf/MEK/ERK signaling pathways, while secondary resistance involves changes in autophagy, epithelial-mesenchymal transition (EMT), tumor microenvironment, and the aberrant activation of carcinogenic signaling pathways [6, 7].

A deeper understanding of the molecular mechanisms responsible for sorafenib resistance could lead to more effective therapies for HCC patients. Our prior study has shown that NO-

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TCH1 is an important protein involved in sorafenib resistance [8, 9]. NOTCH1 is implicated in the oncogenesis of various tumors, including prostate cancer [9], acute T-lymphocytic leukemia [10], and breast cancer [11]. Interestingly, in certain tumors like bladder cancer [12] and head and neck squamous cell carcinoma [13], NOTCH1 exhibits tumor-suppressive effects. Our findings indicated abnormal expression of NOTCH1 in sorafenib-resistant HCC cells, yet the precise molecular mechanism remains to be explored. It has been reported that NOTCH1 may directly bind to the regulatory FATC domain of ATM in leukemia [14]. However, whether NOTCH1 induces sorafenib resistance via ATM in HCC has not been documented in existing literature.

ATM, a protein kinase, is capable of autophosphorylation and typically as inactive dimers in the nucleus and cytoplasm. Upon the occurrence of DNA double-strand breaks (DSBs), ATM is activated at Ser1981 by auto-phosphorylation. This activated form of ATM subsequently phosphorylates several key proteins involved in DNA repair, cell cycle arrest, and apoptosis, including CHK2, p53, and H2AX [15]. Once the repair of DNA double-strand damage fails, ATM-mediated activation of p53 acts on its downstream substrates such as cell cycle suppressor p21, leading to G1/M phase arrest and apoptosis. Additionally, ATM activation indirectly promotes DNA repair via two major DSB repair pathways, non-homologous end junction (NHEJ) and HR. ATM-dependent phosphorylation of 53BP1, which is recruited by chromatin damage markers, prevents DSB terminal resection and promotes NHEJ [16]. Moreover, ATM and CHK2 phosphorylate BRCA1 at DNA damage sites [17]. Through transcriptome sequencing analysis of sorafenib-sensitive Hun7 cells and sorafenib-resistant Huh7-SR cells, we have identified that ATM is activated in sorafenib-resistant Hun7 cells. However, whether ATM is involved in sorafenib resistance needs to be further investigated.

Drug combination is an effective strategy to combat sorafenib resistance. VPA, a broad spectrum antiepileptic widely used in clinical practice, also acts as a deacetylase inhibitor. Recent studies suggest that VPA plays potent anti-tumor effects across various tumors [18-20]. Our previous results demonstrated that VPA was able to inhibit the metastasis of sorafenib-resistant HCC cells by regulating NOTCH1 signaling pathway [8], but the underlying mechanisms remain to be elucidated.

In the current study, bioinformatics analysis revealed abnormal expression of NOTCH1 and ATM in sorafenib-resistant HCC cells, with survival analysis indicating that higher expression of these proteins correlate with shorter survival time in liver cancer patients. The results of western blot indicated NOTCH1/ATM signaling pathway hyperactivation in sorafenib-resistant HCC cells. Furthermore, Co-IP experiments results showed that there is an interaction between NOTCH1 and ATM, suggesting that VPA could be an inhibitor of HCC cell proliferation by regulating this pathway. Moreover, combining VPA with sorafenib promoted DNA damage through the NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling pathway and suppressed HR pathway to regulate RAD51-mediated DSB repair, thereby reversing sorafenib resistance.

Materials and methods

Human cell lines and reagents

SK-Hep1 and Huh7 cell lines were obtained from the Cell Bank, Chinese Academy of Science (Shanghai, China). Sorafenib-resistant HCC cell lines, SK-Hep1-SR and Huh7-SR, were developed by our research group. Sorafenib tosylate was sourced from MedChemExpress (USA). VPA was purchased from Sigma-Aldrich (China). The primary antibodies used were NOTCH1, ATM, p-ATM, p-BRCA1, p-CHK2, γ-H2AX, KU70, KU80, RAD51 (1:1000, Cell Signaling Technology, USA); β-Actin, GAPDH and β-Tubulin (1:5000, Santa Cruz Biotechnology, USA).

Western blotting

The experimental method is referred to the previous article [8].

EdU assay

The EdU assay was conducted according to the experimental procedures (Beyotime Biotechnology, China). Approximately 1.5×10^5 sorafenib-resistant HCC cells were seeded in 6-well plates and treated with drugs for 48 hours as per the experimental design. Post-treatment, cells were then incubated with EdU for 2 hours, fixed, permeabilized, and followed by nuclear staining. Photographs were taken with a fluorescence microscope.

Comet assay

For the comet assay, we employed a kit from Beyotime Biotechnology (China). Following a 48-hour drug treatment, cells were embedded in a low-melting agarose gel on slides. The cells were then lysed, unspun, subjected to alkaline electrophoresis, neutralized, and stained. Observations and photography were conducted under a fluorescence microscope.

siRNA interference

NOTCH1 siRNA1 (5'-GUGUCUGAGGCCAGCAAGATT-3'), NOTCH1 siRNA2 (5'-CAGGGAGCAUGUGUAACAUTT-3') and negative control (NC) siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from GenePharma (Shanghai, China). Approximately 1.5×10^5 cells per well were seeded in 6-well plates. Cells were transfected with 50 nmol/L siRNA using Lipofectamine 2000 (Invitrogen, USA) and cultured for 24 hours.

Cell cycle analysis

Cells were incubated with sorafenib, VPA, or a combination for 48 hours. Cells were digested and washed thrice with PBS. They were then incubated with 0.03% Triton X-100 and propidium iodide (PI) (BD, USA) for 15 mins, followed by flow cytometric analysis (FlowSight Amnis, Merck Millipore).

Co-IP

293T cells were transfected with NOTCH1 plasmids and lysed in 1% NP40 buffer containing protease and phosphatase inhibitors (ApexBio Technology, USA). The whole-cell lysate was centrifuged at 12000 rpm for 10 mins, and the supernatant was incubated with indicated 15 μL magnetic beads (Anti-FLAG® M2 Magnetic Beads, Sigma) at 4°C overnight. Magnetic beads were washed 3 times with lysis buffer. For Western blotting analysis, the samples were heated for 10 minutes in 80 μL 1× loading buffer.

Bioinformatical analysis

Bulk RNA sequencing (RNA-seq) comparing sorafenib-sensitivity and -resistant Huh7 cell lines was performed by Majorbio company. The differentially expressed genes was identified using DEseq2 [21] according to $P < 0.05$ and log2 FC > 1. Data from liver hepatocellular carcinoma (LIHC) in The Cancer Genome Atlas (TCGA) dataset were used to identify differential pathways and assess survival curves using Kaplan-Meier analysis [22].

Statistical analysis

Data were statistically analyzed with GraphPad Prism 9.5 software, and all experiments were replicated three times. Comparisons between two groups were performed using an unpaired t-test. One-way ANOVA was employed for multiple group comparisons, followed by Tukey's test. Patient survival analysis was conducted by Kaplan-Meier plot and evaluated by log-rank test. Results are presented as mean ± standard deviations (SD).

Results

NOTCH1/ATM is highly linked to the prognosis of liver cancer patients

Previous studies have implicated that NOTCH1 plays a very important role in mediating sorafenib resistance, although the exact mechanism involved remains unclear. To investigate this further, we performed RNA-seq analysis on previously established sorafenib-resistant Huh7-SR cells and their parental Huh7 HCC counterparts. Our analysis revealed abnormally expressed levels of NOTCH1 and ATM in Huh7-SR cells (**Figure 1A**). As elucidated in **Figure 1B**, there was a notable activation of the G2M DNA damage checkpoint pathway. Survival analysis using the data from the LIHC in TCGA indicated that higher expression of NOTCH1 and ATM correlated with reduced survival time in HCC patients. Moreover, simultaneous high expression of both genes was associated with the shortest survival times (**Figure 1C-E**). Gene set enrichment analysis demonstrated that pathways such as G2M CHECKPOINT, E2F TARGETS, MITOTIC SPINDLE and UV RESPONSE were highly enriched in sorafenib-resistant Huh7 cell lines (**Figure 1F**). Additionally, correlation analysis suggested that NOTCH1 expression could activate ATM and G2/M DNA damage checkpoint pathways (**Figure 1G** and **1H**). These findings indicate that the NOTCH1/ATM axis is crucial in sorafenib resistance and significantly associates with a dismal prognosis in liver cancer patients.

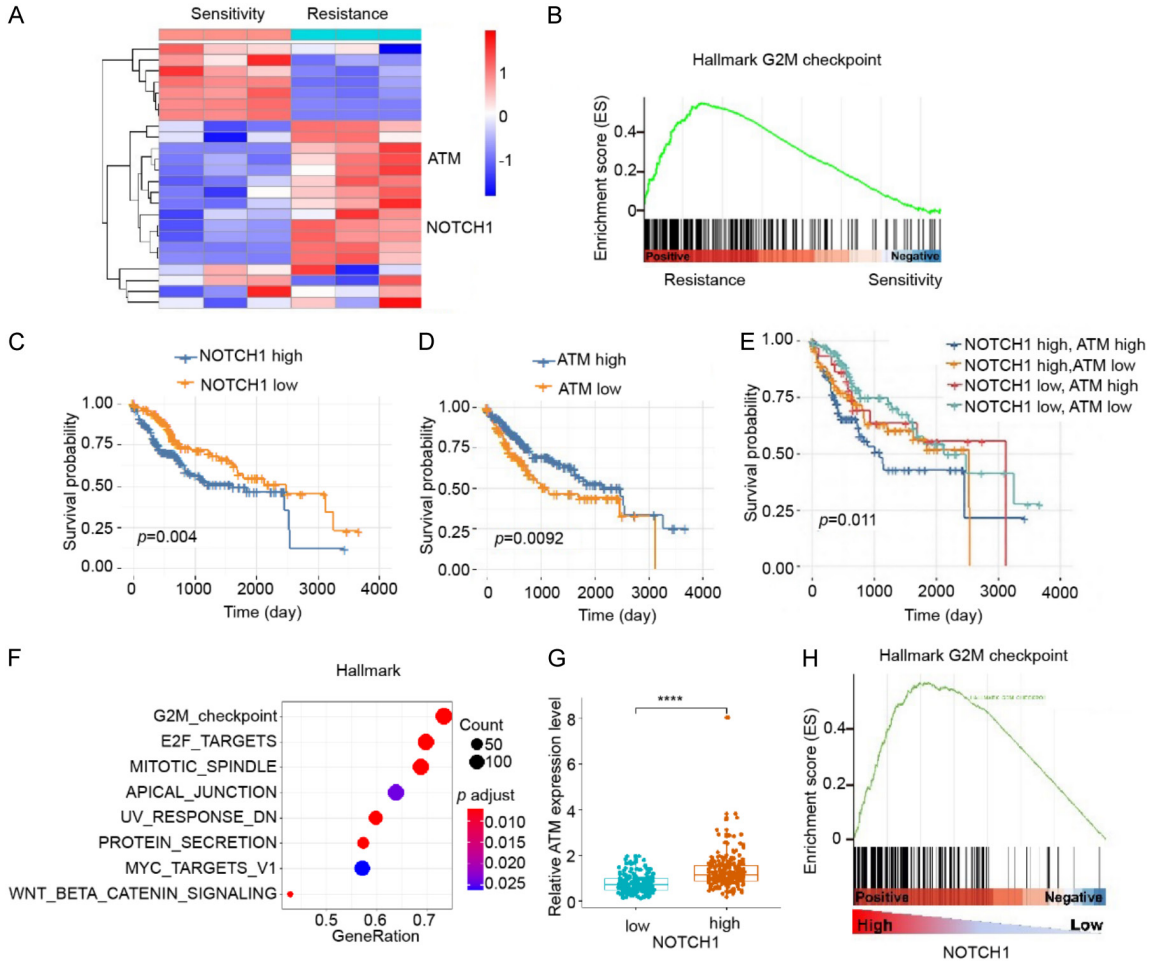

Figure 1. NOTCH1/ATM is significantly associated with the prognosis of liver cancer patients. A. Heatmap showed differentially expressed genes between sorafenib sensitivity and resistant Huh7 cell lines. B. Gene set enrichment analysis showed G2M checkpoint pathway between sorafenib sensitivity and resistant Huh7 cell lines. C. The survival curve between NOTCH1 high and low groups of liver hepatocellular carcinoma (LIHC) in The Cancer Genome Atlas (TCGA) dataset. D. The survival curve between ATM high and low groups of LIHC in TCGA dataset. E. The survival curve among NOTCH1 high ATM high, NOTCH1 high ATM low, NOTCH1 low ATM high and NOTCH1 low ATM low groups of LIHC in TCGA dataset. F. Gene set enrichment analysis showed differential pathways between sorafenib sensitivity and resistant Huh7 cell lines. G. The ATM expression between NOTCH1 high and low groups of LIHC in TCGA dataset. H. Gene set enrichment analysis showed G2M checkpoint pathway between NOTCH1 high and low groups of LIHC in TCGA dataset. ****P < 0.0001.

The NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling pathway is activated in sorafenib-resistant HCC cells

We have constructed two sorafenib-resistant HCC cell lines, SK-Hep1-SR and Huh7-SR, for subsequent studies. Notably, the EdU proliferation assay showed significantly increased proliferation in sorafenib resistant SK-Hep1-SR and Huh7-SR cells, indicating enhanced growth capabilities of this drug-resistant cell lines (**Figure 2A** and **2B**). According to the results of western blot analysis, it revealed activation of NOTCH1 and the downstream ATM-related proteins p-BRCA1, p-CHK2 and γ-H2AX in these sorafenib-resistant HCC cell lines (**Figure 2C**). Sorafenib was then administered to sensitive HCC cell lines SK-Hep1 and sorafenib-resistant HCC cell lines SK-Hep1-SR, respectively. The expression of ATM downstream signaling pathway γ-H2AX was significantly increased in sorafenib-resistant HCC cell lines, indicating the accumulation of DNA damage in SK-Hep1-SR cells (**Figure 2D**). Further, γ-H2AX also accumulates in resistant HCC cell lines with augmented concentrations of sorafenib (**Figure 2E**). These results implicate a significant role

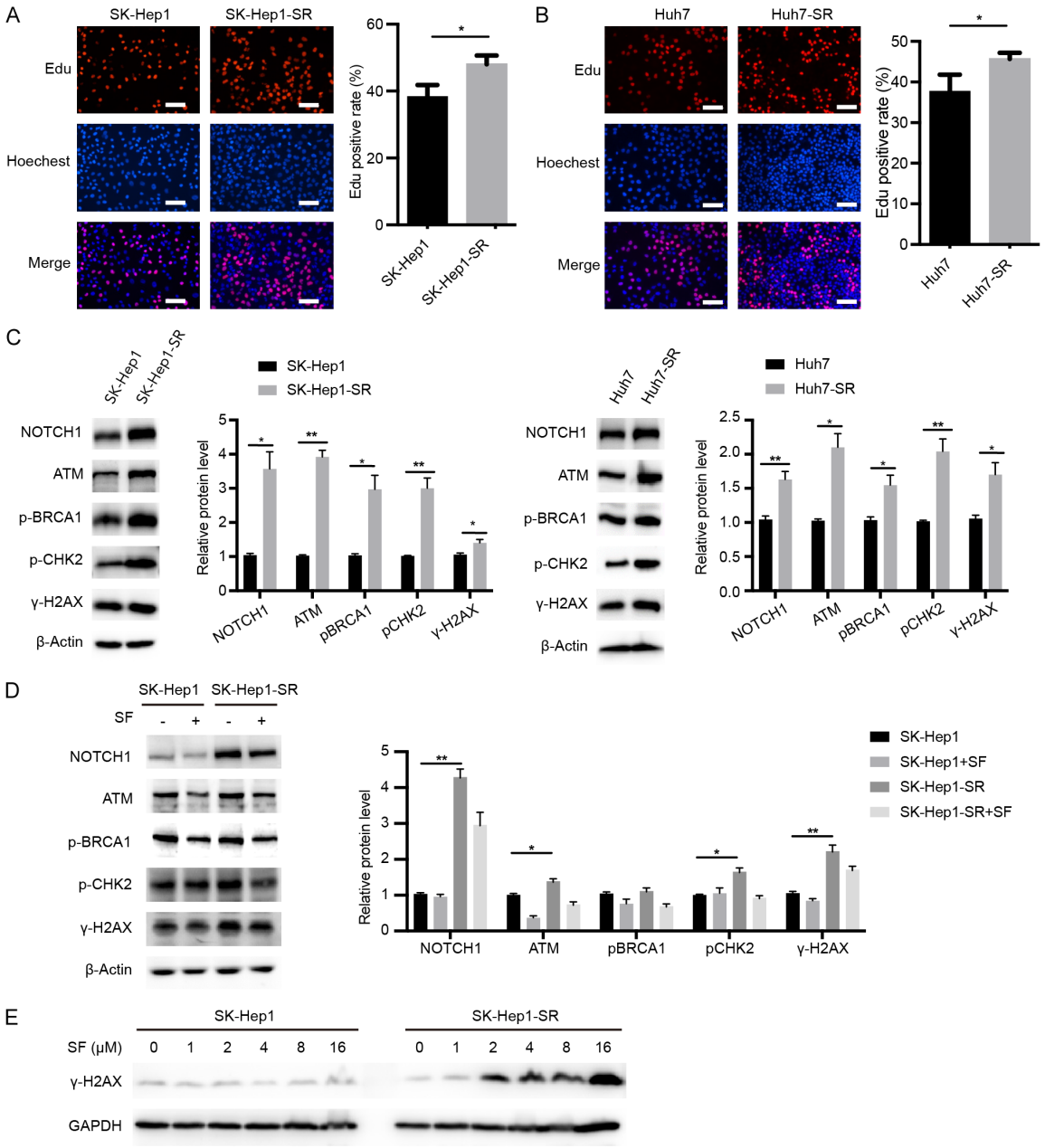

Figure 2. The NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling pathway is activated in sorafenib resistant hepatocellular carcinoma cell (HCC) cells. A, B. The cell proliferation capacity of SK-Hep1/SK-Hep1-SR and Huh7/Huh7-SR was detected by EdU assay. Scale bar = 100 μm. C. Expression of NOTCH1 and ATM-related proteins. D. The expression of NOTCH1 and ATM-related proteins was detected by western blot analysis of SK-Hep1 and SK-Hep1-SR with 5 μM sorafenib (SF), respectively. E. Cell lysate was prepared and immunoblotted for determining the expression level of γ-H2AX. *P < 0.05, **P < 0.01, n = 3.

for the NOTCH1/ATM signaling pathway in mediating sorafenib resistance.

The NOTCH1/ATM signaling pathway plays an important role in sorafenib resistant HCC cells

Activation of proteins associated with NOTCH1 and ATM signaling pathways was observed in sorafenib-resistant HCC cells, raising the question of their interaction. Firstly, by interfering NOTCH1 expression with siRNA, the phosphorylation levels of ATM and its downstream proteins p-CHK2 and γ-H2AX were found to be altered (**Figure 3A**). Co-IP experiments further confirmed a direct interaction between NOTCH1

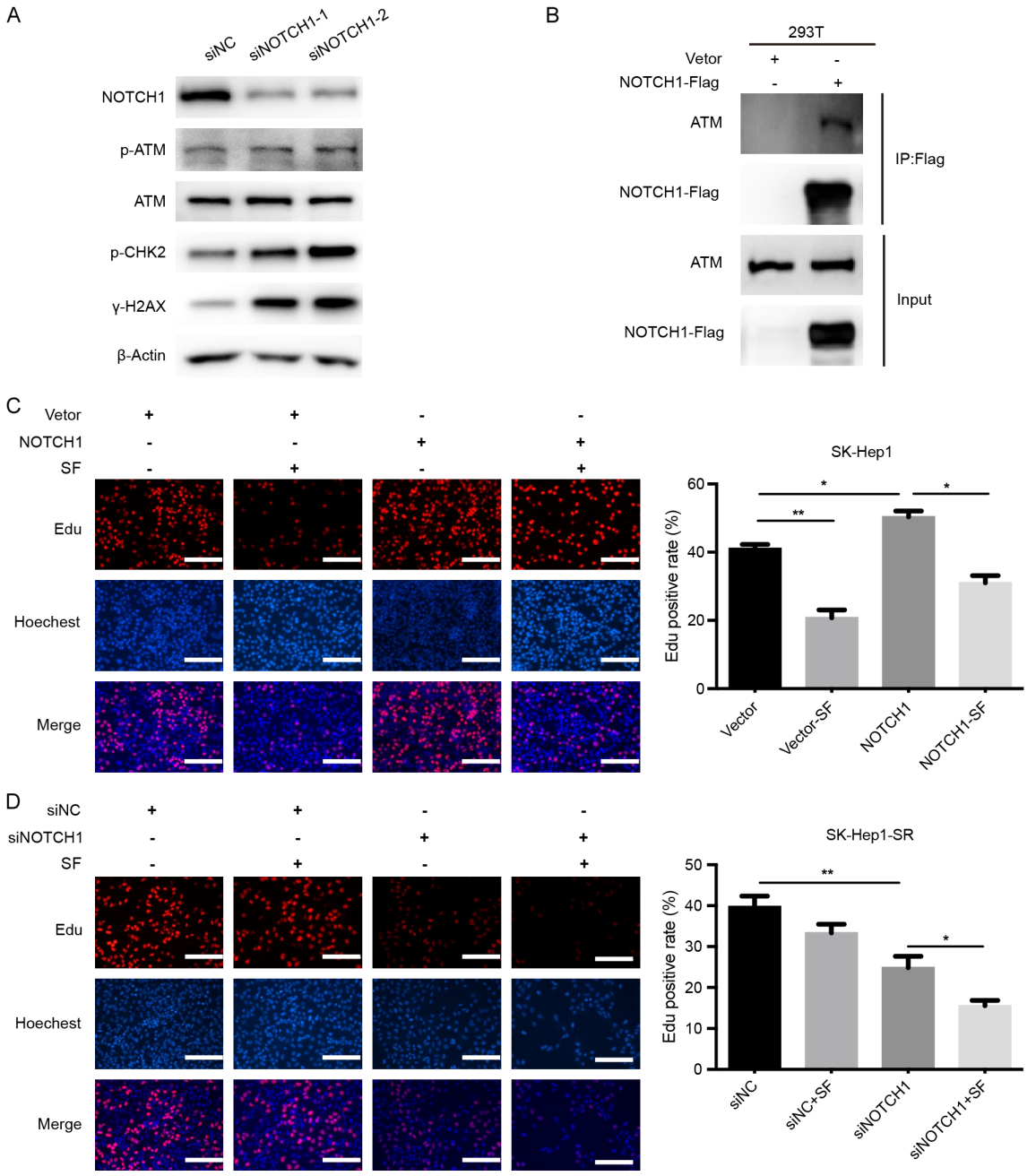

Figure 3. The NOTCH1/ATM signaling pathway plays an important role in sorafenib resistant hepatocellular carcinoma cell (HCC) cells. A. NOTCH1-siRNA1 and NOTCH1-siRNA2 were transfected into SK-Hep1-SR cells for 48 hours, and cell lysate was collected for western blot assay. B. The co-immunoprecipitation (Co-IP) experiment detected the interaction between NOTCH1 and ATM. C, D. EdU assay was used to detect the effect of NOTCH1 on cell proliferation. Scale bar = 200 μm. *P < 0.05, **P < 0.01, n = 3.

and ATM (**Figure 3B**). In addition, manipulation of NOTCH1 expression through overexpression in sensitive cells or knockdown in resistant cells showed corresponding changes in proliferation, as observed in subsequent EdU assays (**Figure 3C** and **3D**). These results indicate that NOTCH1 modulated sorafenib resistance predominantly through ATM signaling pathway.

VPA could inhibit the proliferation of HCC cells via regulating NOTCH1/ATM signaling pathway

In the previous study, we employed Molecular Operating Environment software to conduct virtual screening of drug molecular docking. The docking results revealed an optimal conformation where the carboxyl group energy of VPA

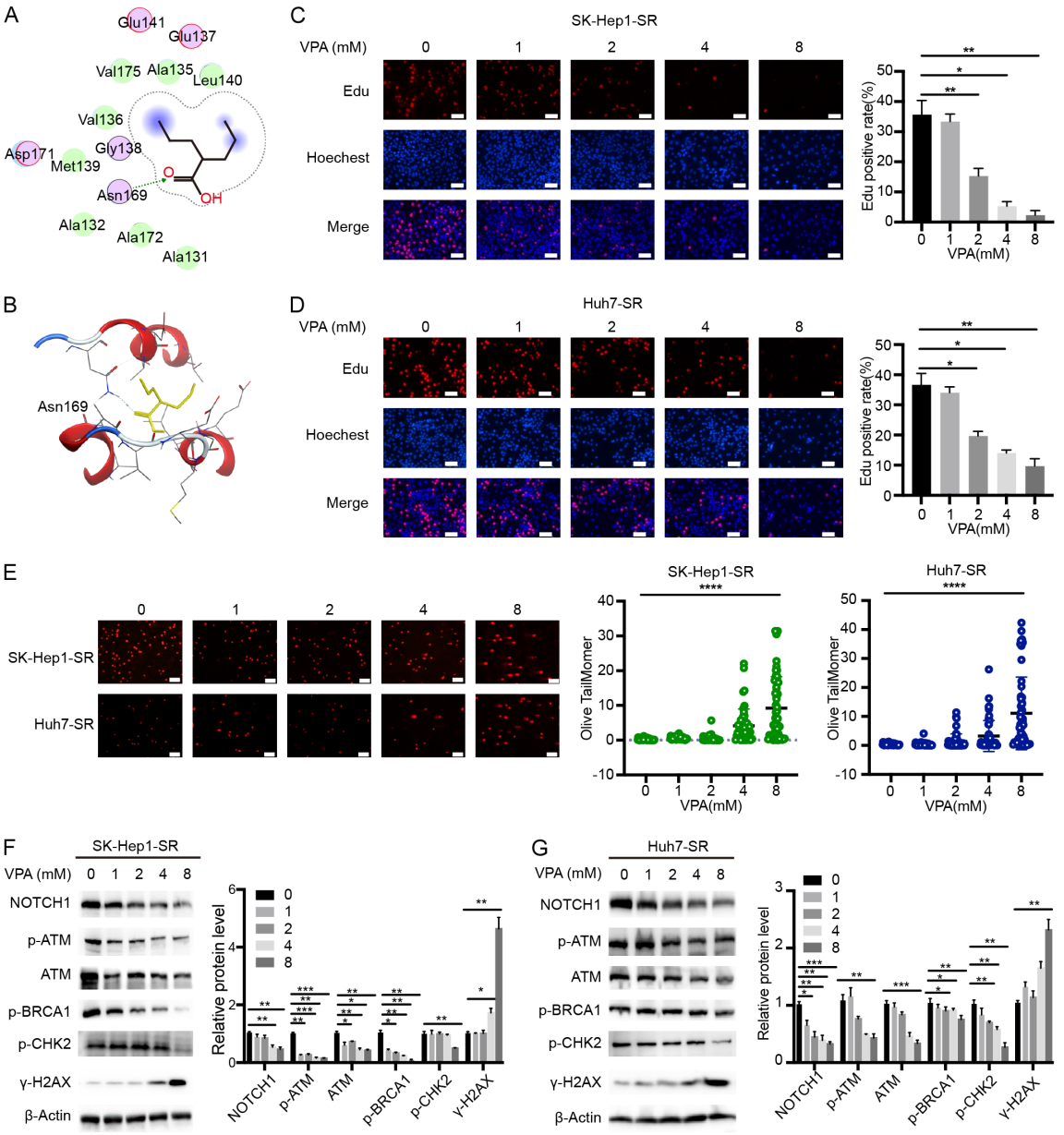

Figure 4. Valproic acid (VPA) could inhibit the proliferation of hepatocellular carcinoma (HCC) cells by regulating NOTCH1/ATM signaling pathway. A, B. Molecular docking simulation of VPA and NOTCH1 was carried out by Molecular Operating Environment software. C, D. EdU assay was used to detect the effect of VPA on the proliferation of SK-Hep1-SR and Huh7-SR cells. Scale bar = 100 μm. E. The effects of VPA on DNA damage in SK-Hep1-SR and Huh7-SR cells were detected by comet assay. Scale bar = 100 μm. F, G. SK-Hep1-SR and Huh7-SR cells were treated with 0, 1, 2, 4, 8 mM VPA for 48 hours, and the cell lysates were immunoblotted. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n = 3.

formed hydrogen bond with the ASN169 amino acid of NOTCH1 (**Figure 4A** and **4B**). This suggested that NOTCH1 may be a target of VPA. However, the regulation of ATM-related signaling pathways by VPA warrants further investigation. EdU experiments demonstrated that VPA restrained the proliferation of sorafenib resistant cells in a concentration-dependent manner (**Figure 4C** and **4D**). Additionally, the comet assay showed that VPA could induce DNA damage in these cells (**Figure 4E**). Western blot experiments indicated that VPA inhibited p-ATM and NOTCH1 expression and enhanced the expression of γ-H2AX, a marker of DNA damage downstream of ATM (**Figure 4F** and **4G**), in a concentration-dependent manner. These data

suggest that VPA could improve the sensitivity of sorafenib-resistant cells to sorafenib by modulating NOTCH1/ATM signaling pathway.

VPA combined with sorafenib promotes DNA damage and reverses sorafenib resistance by regulating NOTCH1/ATM/p-BRCA1/pCHK2/γ-H2AX signaling pathway

Given that VPA can induce DNA damage in sorafenib-resistant cells, we investigated whether combining VPA with sorafenib could intensify this effect, leading to the apoptosis of sorafenib and thereby overcoming resistance in HCC cells. EdU assays indicated that the combination of VPA and sorafenib effectively inhibited the proliferation of sorafenib-resistant cells (**Figure 5A** and **5B**). Cell cycle experiments revealed that this combination could block SK-Hep1-SR cells in G1/S phase (**Figure 5C**), potentially triggering the large number of apoptosis of sorafenib resistant cells. Comet experiments further demonstrated that the combined treatment could exacerbate DNA damage in sorafenib-resistant cells (**Figure 5D**). Additionally, western blot results showed that sorafenib combined with VPA may play an anti-tumor role by activating the NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling pathway (**Figure 5E** and **5F**). Collectively, these results suggest that VPA plus sorafenib could not only promote DNA damage but also effectively reverse sorafenib resistance.

VPA plus sorafenib could activate the ATM signaling pathway

We further validated our findings using ATM inhibitor KU60019. The results demonstrated that KU60019 variably inhibited ATM activity across different concentrations in sorafenib-resistant HCC cell lines (**Figure 6A**). Western blot results showed that 2 μM KU60019 could inhibit the phosphorylation of ATM protein expression and not increase the expression of γ-H2AX, without affecting the expression of NOTCH1 (**Figure 6A**). This indicated that NOTCH1 may act upstream of ATM. Despite ATM inhibition by 2 μM KU60019, the combination of VPA and sorafenib still enhanced ATM phosphorylation (**Figure 6B**), confirming that this drug combination robustly activated the ATM signaling pathway and was not reversed by ATM inhibitors.

VPA regulates RAD51-mediated DSB repair through HR pathway

DNA double strand breaks, typically repaired via HR and/or NHEJ, represent the most severe form of cellular damage. We found that RAD51-mediated DNA repair was active in sorafenib-resistant HCC cell lines, while the expression levels of two important NHEJ proteins, KU70 and KU80, remained unchanged (**Figure 7A**). Notably, increasing concentration of VPA corresponded with a decrease in RAD51 expression, with no similar alterations observed in KU70 and KU80 (**Figure 7B** and **7C**). Further results demonstrated that the combination of VPA and sorafenib inhibited DNA repair by targeting the RAD51-mediated HR pathway, leading to the apoptosis of sorafenib-resistant HCC cells (**Figure 7D** and **7E**). These findings suggest that the combined treatment disrupted the timely repair of DNA damage in sorafenib-resistant tumor cells by downregulating HR mediated by RAD51, thus exerting a cytotoxic effect.

Discussion

Our previous findings highlight NOTCH1 as a compelling therapeutic target for sorafenib resistance, although the precise molecular mechanism remains to be elucidated. Integrating bioinformatics analysis data with experimental data has shed light on the critical involvement of DDR pathway in sorafenib resistance, particularly emphasizing the significance of the NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signal axis. These results support that NOTCH1/ATM is a promising target in the treatment of sorafenib resistance.

The NOTCH1 signaling pathway is crucial for numerous biological processes, such as cell proliferation, cell differentiation, and tissue development [23, 24]. Our prior works have shown that aberrant activation of NOTCH1 signaling contributes to the proliferation and EMT in sorafenib-resistant HCC cells [8]. RNA-seq data suggested hyperactivation of NOTCH1 in sorafenib-resistant HCC cells, with concurrent upregulation of genes related to DNA damage repair, such as ATM, ATR, MSH2, MSH6, MLH1, etc. Survival analysis has validated that higher expression of NOTCH1 and ATM correlates with reduced survival times in liver cancer patients, particularly when both are highly expressed simultaneously. These findings underscored

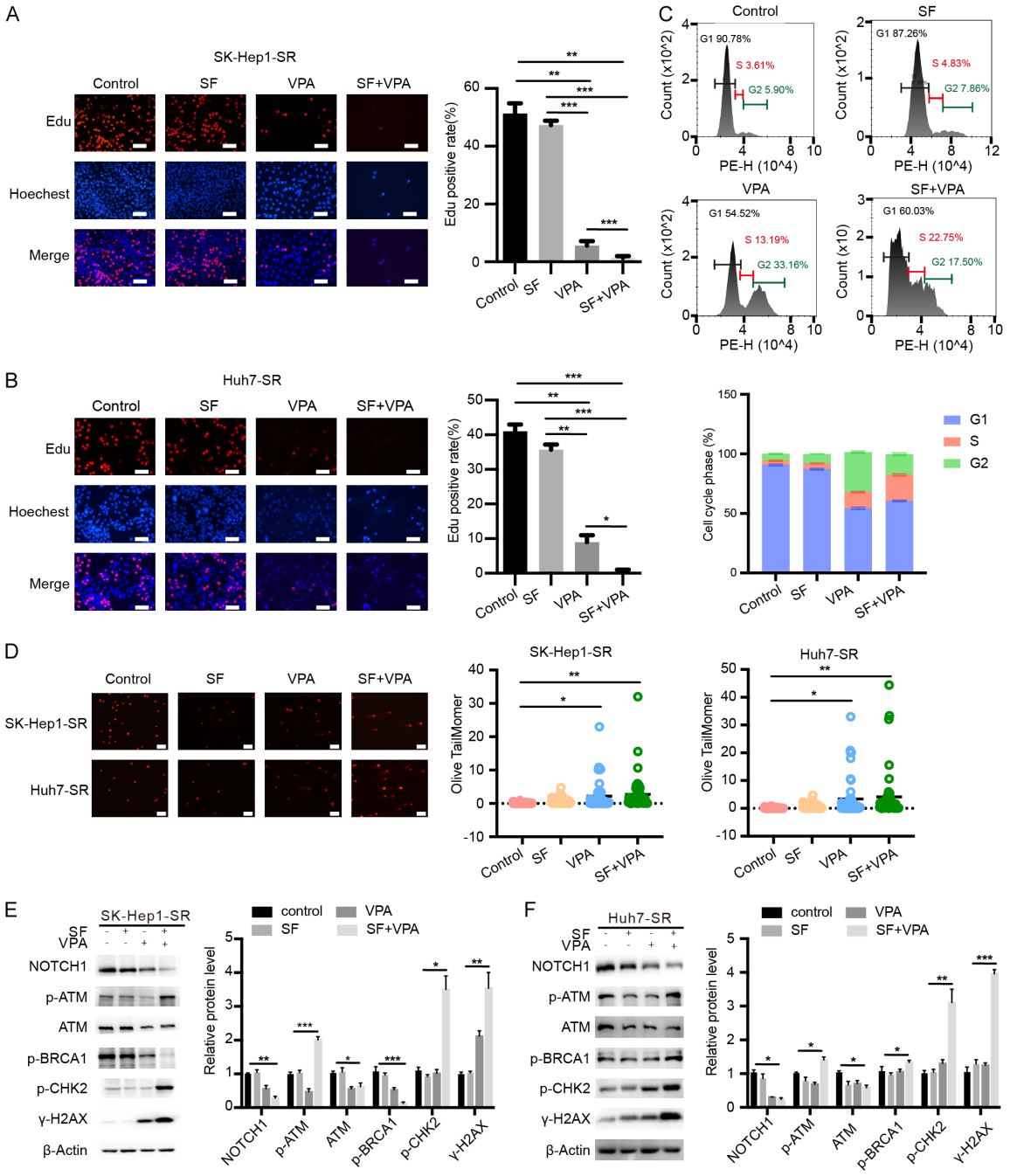

Figure 5. Valproic acid (VPA) combined with sorafenib (SF) promotes DNA damage and reverses sorafenib resistance by regulating NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling pathway. Cells were incubated with control, SF (5 μM), VPA (8 mM), or a combination for 48 hours. A, B. The proliferation capacity of the above four groups of cells was examined by EdU assay. Scale bar = 100 μm. C. Flow cytometry was used to detect the cycle changes of the four groups of cells. D. The Comet assay detected DNA damage in four groups of cells. Scale bar = 100 μm. E, F. Cell lysates were immunoblotted. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3.

the pivotal roles of NOTCH1 and ATM in mediating sorafenib resistance, highlighting their importance as targets for therapeutic intervention. Then, we used the constructed drug-resistant cell lines SK-Hep1-SR and Huh7-SR for verification. Consistent with previous results, NOTCH1 was abnormally expressed in high level in sorafenib-resistant cell lines, while ATM and its downstream protein p-BRCA1/p-CHK2/γ-H2AX were also markedly elevated. No-

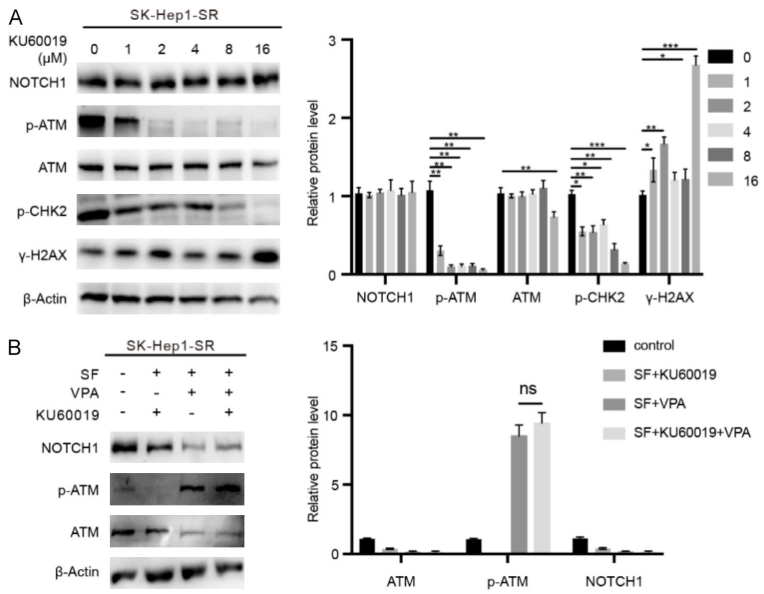

Figure 6. Valproic acid (VPA) combined with sorafenib (SF) could activate the ATM signaling pathway. A. SK-Hep1-SR cells were treated with 0, 1, 2, 4, 8, or 16 μM KU60019 for 48 hours, and the cell lysates were immunoblotted. B. Cells were incubated with KU60019 (2 μM), SF (5 μM), VPA (8 mM), or a combination for 48 hours, and the cell lysates were immunoblotted. ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001, n = 3.

tably, γ-H2AX accumulation increased with higher concentrations of sorafenib in these liver cancer cell lines. γ-H2AX is vital for damage recognition and the facilitation of repair, promoting chromosome integrity and activating a variety of DNA damage repair enzymes at damage sites [25, 26]. Further studies have highlighted that genes involved in DNA damage repair were abnormally expressed in sorafenib-resistant cell lines. Additionally, we have demonstrated a direct interaction between NOTCH1 and the ATM FATC domain [27], which was further substantiated through Co-IP experiments, confirming a functional linkage between NOTCH1 and ATM.

Numerous studies have identified that dysfunctional DNA repair proteins, such as BRCA1, RAD51, FEN1 and Polβ, are critical in the development and progression of HCC [28, 29]. The elevated activity of DNA repair has been linked to drug resistance, thereby reducing the effectiveness of chemotherapy drugs [30, 31]. Consequently, targeting key proteins within the DDR pathway offers a promising approach for both monotherapy and combination therapy strategies. For example, the dual inhibition of PARP and ATM has been shown to overcome trastuzumab resistance in HER2-positive breast cancer [32]. In our current study, we observed activation of the NOTCH1 protein alongside ATM and its downstream protein, p-BRCA1/p-CHK2/γ-H2AX. This suggested that simultaneous inhibition of NOTCH1 and ATM could represent a novel therapeutic strategy for treating patients with advanced liver cancer.

As a deacetylase inhibitor, VPA has been reported to have a broad spectrum of anti-tumor effects across various cancers [33-35], though the mechanisms underlying its anti-tumor action is unclear. Molecular docking studies have shown that VPA targets NOTCH1. Our previous studies have found that the combination of VPA and sorafenib promotes apoptosis in sorafenib-resistant cells in the G0/G1 phase. This arrest was mediated through the NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling axis, preventing DNA damage repair and inducing apoptosis, which increased the sensitivity of drug-resistant cells to sorafenib.

DSBs are one of the most severe types of DNA damage in eukaryotic cells, with the potential to induce apoptosis. The results of comet assay documented that the VPA and sorafenib together promoted the DNA strand break in sorafenib-resistant cells. There are two main ways to fix DSBs: the error-free HR and error-prone NHEJ [36]. Numerous studies have established that the lack of DNA repair protein is related to cancer development, while the augmented activity of DNA repair activity contributes to the occurrence and development of tumor drug resistance [37, 38]. HR is a critical mechanism for repairing the DSBs, which are among the most detrimental forms of cellular damage. Effective regulation of HR is critical for maintaining genomic stability [39]. Among various cancers, overactivation of HR enhances the resistance of tumor to DNA-damaging treatments. RAD51, a core HR protein, plays a key role in cancer development and survival [40]. Therefore, directly diminishing RAD51 activity and expres-

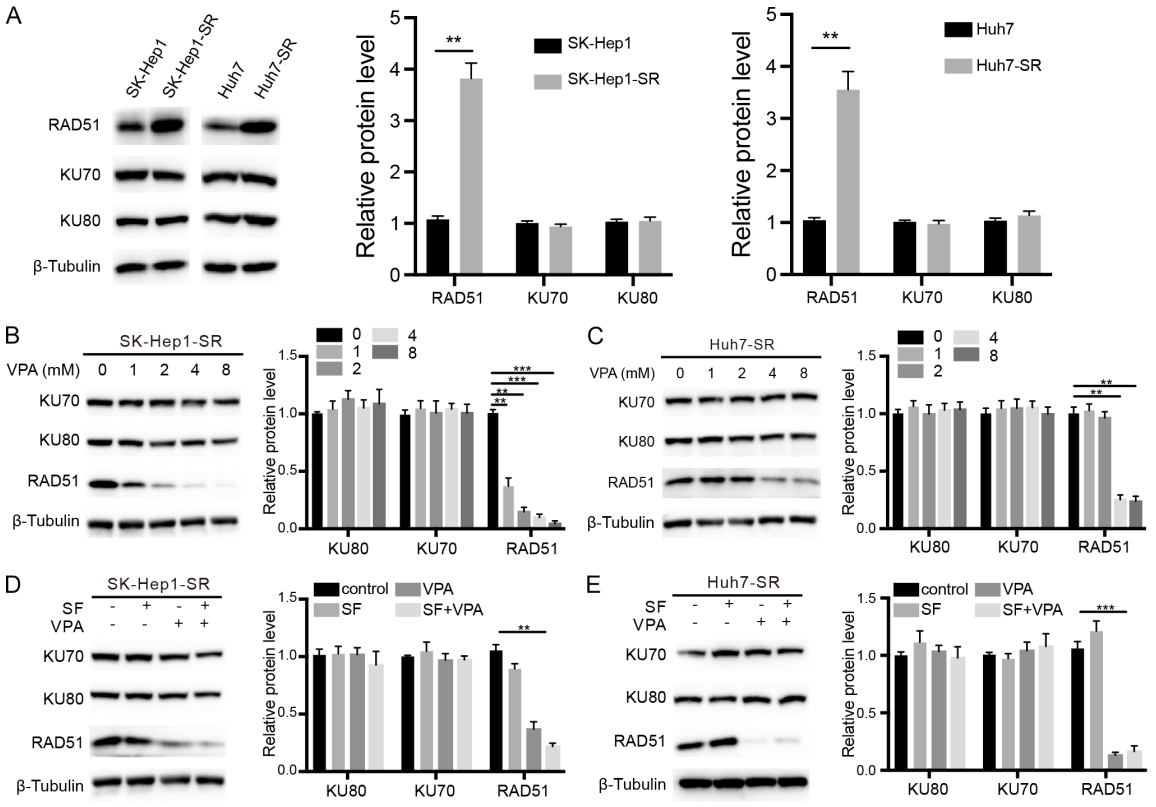

Figure 7. Valproic acid (VPA) regulates RAD51-mediated DNA double-strand break (DSB) repair through homologous recombinant (HR) pathway. A. SK-Hep1/SK-Hep1-SR and Huh7/Huh7-SR cells were collected and cell lysates were immunoblotted. B, C. SK-Hep1-SR and Huh7-SR cells were treated with 0, 1, 2, 4, or 8 mM VPA for 48 h, and the cell lysates were immunoblotted. D, E. SK-Hep1-SR and Huh7-SR cells were incubated with control, sorafenib (SF) (5 μM), VPA (8 mM), or a combination for 48 h, and the cell lysates were immunoblotted. **P < 0.01, ***P < 0.001, n = 3.

sion levels is a strategy to increase sensitivity of tumor cells to DNA damage therapies and counteract drug resistance. Our study observed that RAD51 was overactivated in sorafenib-resistant liver cancer cells, whereas KU70 and KU80, two important proteins regulating NHEJ, remained unaltered. This suggested a significant role for RAD51 in resistance to sorafenib. Moreover, the combination of VPA and sorafenib reduced the expression of RAD51 and inhibited DNA damage repair, suggesting a potential therapeutic strategy to overcome resistance in liver cancer.

However, our study has limitations. The binding region of NOTCH1 and ATM needs to be further explored, the cascade pathway of ATM regulating DNA damage-related proteins needs to be investigated, and the effectiveness of VPA combined with sorafenib needs to be validated in clinical patients.

Conclusion

We have identified that activation of the NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signal axis and heightened HR activity are instrumental in sorafenib resistance. VPA can promote the sensitivity of resistant cells to sorafenib by modulating NOTCH1/ATM/p-BRCA1/p-CHK2/γ-H2AX signaling axis and HR activity. In summary, our findings suggested that VPA combined with sorafenib holds promise as a therapeutic strategy for patients with advanced liver cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

Co-IP, co-immunoprecipitation; DDR, DNA damage repair; DSBs, DNA double-strand breaks; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma cell; HR, homologous recombinant; LIHC, liver hepatocellular carcinoma; NHEJ, non-homologous end junction; RNA-seq, RNA sequencing; TCGA, The Cancer Genome Atlas; VEGFR, vascular endothelial growth factor receptor; VPA, valproic acid.

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