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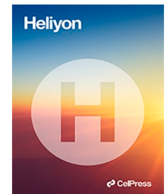

Research article

Co-overexpression of BRD4 and CDK7 promotes cell proliferation and predicts poor prognosis in HCC

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ABSTRACT

Aberrant expression of critical components of the *trans*-acting super-enhancers (SE) complex contributes to the continuous and robust transcription of oncogenes in human cancers. Small-molecule inhibitors targeting core-transcriptional components such as transcriptional bromodomain protein 4 (BRD4) and cyclin-dependent kinase 7 (CDK7) have been developed and are currently undergoing preclinical and clinical testing in several malignant cancers. By analysis of TCGA data and clinical specimens, we demonstrated that BRD4 and CDK7 were frequently overexpressed in human HCCs and were associated with the poor prognosis. Shorter survival and poorly differentiated histology were linked to high BRD4 or CDK7 expression levels. Interestingly, co-overexpression of BRD4 and CDK7 was a more unfavorable prognostic factor in HCC. Treatment with JQ1 or THZ1 alone exhibited an inhibitory impact on the proliferation of HCC cells, while JQ1 synergized with THZ1 showed a more pronounced suppression. Concurrently, a combined JQ1 and THZ1 treatment abolished the transcription of oncogenes *ETV4*, *MYC*, *NFE2L2*. Our study suggested that BRD4 and CDK7 coupled can be a valuable biomarker in HCC diagnosis and the combination of JQ1 and THZ1 can be a promising therapeutic treatment against HCC.

1. Introduction

The majority of instances of liver cancer (about 90 %) are hepatocellular carcinomas (HCC), which continue to pose a worldwide health problem due to their rising incidence and high mortality [1,2]. Activation of oncogenes and inactivation of tumor suppressor genes caused by epigenetic and genetic alterations frequently accompany the development of HCC [3]. Key oncogenic signaling pathways are activated as a result of the robust overexpression of multiple oncogenic drivers including ETV4, MYC, and AKT [4–6]. Emerging evidence indicates that the continuous and robust transcription of oncogenes in cancer cells is often driven by super-enhancers [4,7]. Super-enhancers (SE) are composed of typical enhancers densely loaded with mediator complexes, transcription factors and chromatin regulators [8], and are associated with cell fate-related genes in normal cells and oncogenes in cancer cells [9]. Critical mediator complexes members BRD4 and CDK7 are linked to SE-mediated transcription in malignancies [10,11]. BRD4 is essential for controlling chromatin remodeling and transcriptional activation, while CDK7 encourages effective transcriptional initiation and elongation. Emerging small-molecule inhibitors that target the SE have been demonstrated in clinical trials to exert a

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notable effect on cancer treatment [12,13]. Recent studies reported that the disturbance of the SE complex by co-targeting of BRD4 and CDK7 might be a promising therapeutic treatment against cancers including head and neck squamous cell carcinoma, pancreatic ductal adenocarcinoma (PDAC) and neuroblastoma [14–16]. However, whether BRD4 and CDK7 could be synergistic targets for the treatment of HCC remains largely unexplored.

Overexpression of BRD4 or CDK7 could drive tumor growth through the regulation of genes controlled by specific transcription factors or SE regions. BRD4 inhibitor JQ1 [17,18] or CDK7 inhibitor THZ1 [19,20] exerted the promising anticancer effects of by preferentially targeting the SE complex and influencing transcription of the carcinogenic gene. A recent study suggested that BRD4 and CDK7 were frequently overexpressed in human HCCs [21], and the inactivation of them with specific small-molecule inhibitors selectively repressed the expression of SE-associated oncogenes in HCC [22,23]. Therefore, we set out to test the idea that the simultaneous inhibition of BRD4 and CDK7, with JQ1 and THZ1, may work in concert to suppress HCC cell proliferation. The present study showed both BRD4 and CDK7 were overexpressed in HCC tissues and their co-overexpression associated with a worse prognosis of HCC. We also demonstrated that JQ1, in combination with THZ1, is an effective molecular targeting therapeutic strategy for inhibiting HCC cells proliferation through suppressing the expression of HCC driver genes.

2. Materials and methods

2.1. Data analysis from TCGA and GEO database

RNA sequencing data of 49 non-tumorous liver (NT) and 369 HCC samples as well as their clinical information were obtained from The Cancer Genome Atlas (TCGA). Similarly, the RNA sequencing data and corresponding clinical information were also downloaded from GEO (GSE14520) database. A paired or unpaired *t*-test was performed to compare the gene expression levels represented as FPKM values between the two groups. Patients were sorted according to FPKM values median of BRD4 and CDK7, and survival data were analyzed using the Kaplan-Meier method.

2.2. Tissue specimens and IHC staining

A total of 110 specimens were collected retrospectively from HCC patients with detailed clinical information including survival and follow-up data in the Zhongda Hospital affiliated with Southeast University (Nanjing, China). Patients who had undergone surgery, chemotherapy, radiation, or immunotherapy within the past six months were excluded. All patients were informed and agreed to participate in this research. This analysis was approved by the Ethics Committee of Zhongda Hospital affiliated with Southeast University, China (Ethics Approval No. 2021ZDKYSB032). Subsequently, 110 pairs of samples consisting of HCC tissues and corresponding adjacent non-tumor tissues were made into tissue microarrays (TMAs). TMAs were dewaxed in xylene and rehydrated in a series of gradient ethanol. Subsequently, the tissues were immersed in a sodium citrate antigen retrieval solution (pH 6.0) and heated at boiling temperature for 10 min to recover the antigen, and then incubated with 3 % H_2O_2 for 15 min to block endogenous peroxidases. After blocking the non-specific binding site with 3 % bovine serum albumin (BSA), the slides were incubated with corresponding primary antibody against BRD4 (1:200, Cat# ab128874, Abcam) and CDK7 (1:100, Cat# ab59987, Abcam) overnight at 4 °C. Subsequently, 50 min incubation with a secondary antibody (K5007, DAKO, Glostrup, Denmark) labeled with Horseradish Peroxidase (HRP) at room temperature was performed. After PBS washing, slides were developed using a freshly prepared DAB (3, 3′-diaminobenzidine) chromogenic reagent (G1211, Wuhan Servicebio, China) and were then counterstained with hematoxylin staining solution. Tissue immunostaining were observed under an optical microscope (Axiocam 105, Carl Zeiss, Germany), and the representative images were captured with Matrox Imaging (Carl Zeiss, Germany).

The staining was scored by double-blind method and was analyzed using the immunoreactive score (IRS) system. Final IRS score= (Score of the staining intensity) × (score of the percentage of positive cells). The staining intensity was scored on a scale of 0–3: 0 = negative, 1 = weak staining, 2 = moderate staining, 3 = intense staining. The percentage of positive cells was scored on a scale of 0–4: 0 if 0–5% were positive cells, 1 if 6–25 % were positive cells, 2 if 26–50 % were positive cells, 3 if 51–75 % were positive cells and 4 if 76–100 % were positive cells. The cutoff values were calculated according to Receiver Operating Characteristic (ROC). Based on the cutoff value of each marker, the patients were divided into low expression group and high expression group. The cut-off levels of BRD4 and CDK7 are 6.

2.3. Cell proliferation and colony formation assay

To measure cell proliferation, one day before being treated with inhibitors, 5×10^3 cells were seeded per well in a 96-well plate. 500 nM JQ1 (MedChem Express, Cat# HY-13030) or/and 10 nM THZ1 (MedChem Express, Cat# HY-80013) were added into the medium and replaced every 2 days. Proliferation of the HCC cells over the course of 1–4 days was determined by Cell Counting Kit-8 reagent (K1018, ApexBio) assay every 24 h. Data is representative of 3 independent experiments for each cell line.

For colony formation assay, 2×10^3 HepG2 or Hep3B cells were seeded per well in 6-well plates and kept under JQ1 (200 nM) or/and THZ1 (2 nM) treatment for 12 days. Colonies were fixed with 75 % alcohol and stained with crystal violet solution (C8470, Solarbio) then washed with water to remove the unincorporated stain. Data is representative of 3 independent experiments for each cell line.

2.4. RNA isolation and q-PCR

Total RNA was harvested and extracted using TRIZOL reagent (AG21102, Accurate Biology). 1 μg total RNA was reverse-transcribed with a HiScript III 1st Strand Synthesis (Vazyme) following the manufacturer's instruction. Gene expression was verified using SYBR Green Master Mix (Vazyme) on LineGene 9600 Plus Real-Time PCR Detection instrument. The primer sequences for *ETV4, MYC* and *NFE2L2* were as follows in Table S1.

2.5. Statistical analysis

The statistical analysis for each plot was described in figure legends. Data are presented as mean ± S.E.M. and are analyzed with Graphpad Prism version 7. Statistical significance between two groups was calculated using paired or unpaired *t*-test, while pairwise comparisons between multiple groups were analyzed statistically by one way ANOVA. P values of <0.05 were regarded as statistical significance.

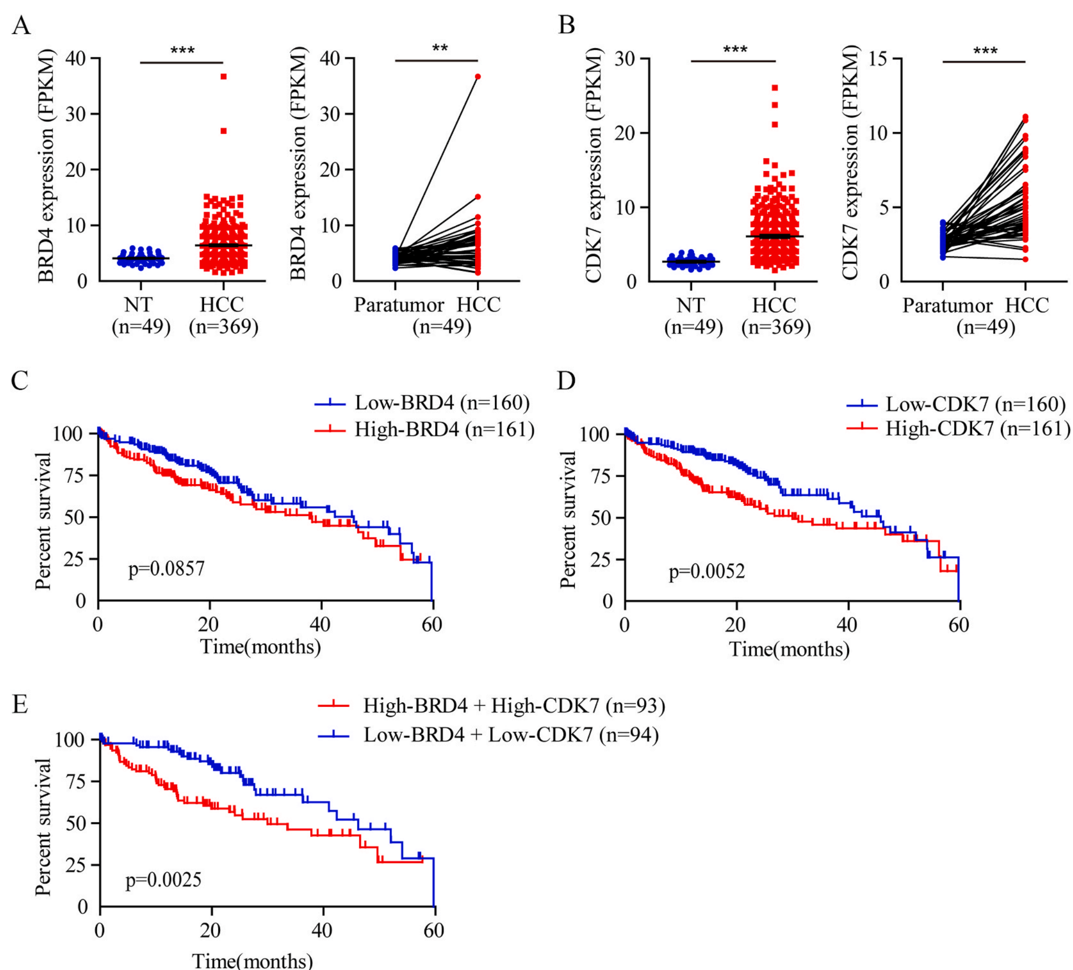

Fig. 1. The expression profiling, and survival analysis of BRD4 and CDK7 expression in TCGA HCC patients (A–B) Unpaired (left, n = 49 patients for normal livers; n = 369 patients for liver tumors) and paired (right, n = 49 patients) comparison of BRD4 (A) and CDK7 (B) mRNA levels, based on TCGA data. Statistical significance was determined by a two-tailed unpaired (left) or paired (right) *t*-test (**p < 0.01, ***p < 0.001). (C–D) HCC patients were divided into high-expression group and low-expression according to the expression values median of BRD4 and CDK7. Kaplan-Meier curves of 5-year overall survival of low-BRD4 group (n = 160) and high-BRD4 (n = 161) group; low-CDK7 group (n = 160) and high-CDK7 group (n = 161) were shown in C and D, respectively. (E) HCC patients were divided into simultaneously high expression group and simultaneously low expression group. Kaplan-Meier curves of 5-year overall survival of low-BRD4+low-CDK7 group (n = 94) and high-BRD4 + high-CDK7 group (n = 93) were shown in E. Statistical significance of C-E was determined by log-rank test.

3. Results

3.1. Expression of BRD4 and CDK7 in human HCCs and its correlation with a poor prognosis in patients with HCC based on TCGA dataset

Based on the RNA-sequencing (RNA-seq) data from TCGA, both BRD4 and CDK7 mRNA levels were upregulated in HCCs compared with normal tissues (Fig. 1A left and 1B left). 49 pairs of HCC patients showed overexpression of BRD4 and CDK7 in tumors compared to matched paracancerous tissues (Fig. 1A right and 1B right). Subsequently, using Kaplan-Meier plotter analysis, associations between gene expression levels and patient survival rates were displayed. The 5-year overall survival of HCC patients with high BRD4 or high CDK7 was less than that of individuals with low BRD4 or low CDK7 (Fig. 1C and D). Patients with co-overexpression of BRD4 and CDK7 had the worst prognosis ($p = 0.0025$, Fig. 1E), and this result was validated in other HCC cohort from the GEO database (GSE14520, Figs. S1A–C). However, there was no significant correlation between the expression levels of BRD4 or CDK7 and other clinical features, including lymph node metastasis, tumor stages and HCC biomarker alpha-fetoprotein (AFP) levels (Figs. S2A–F, $p > 0.05$). Taken together, co-overexpression of BRD4 and CDK7 could serve as an unfavorable factor and a prognostic marker for HCC.

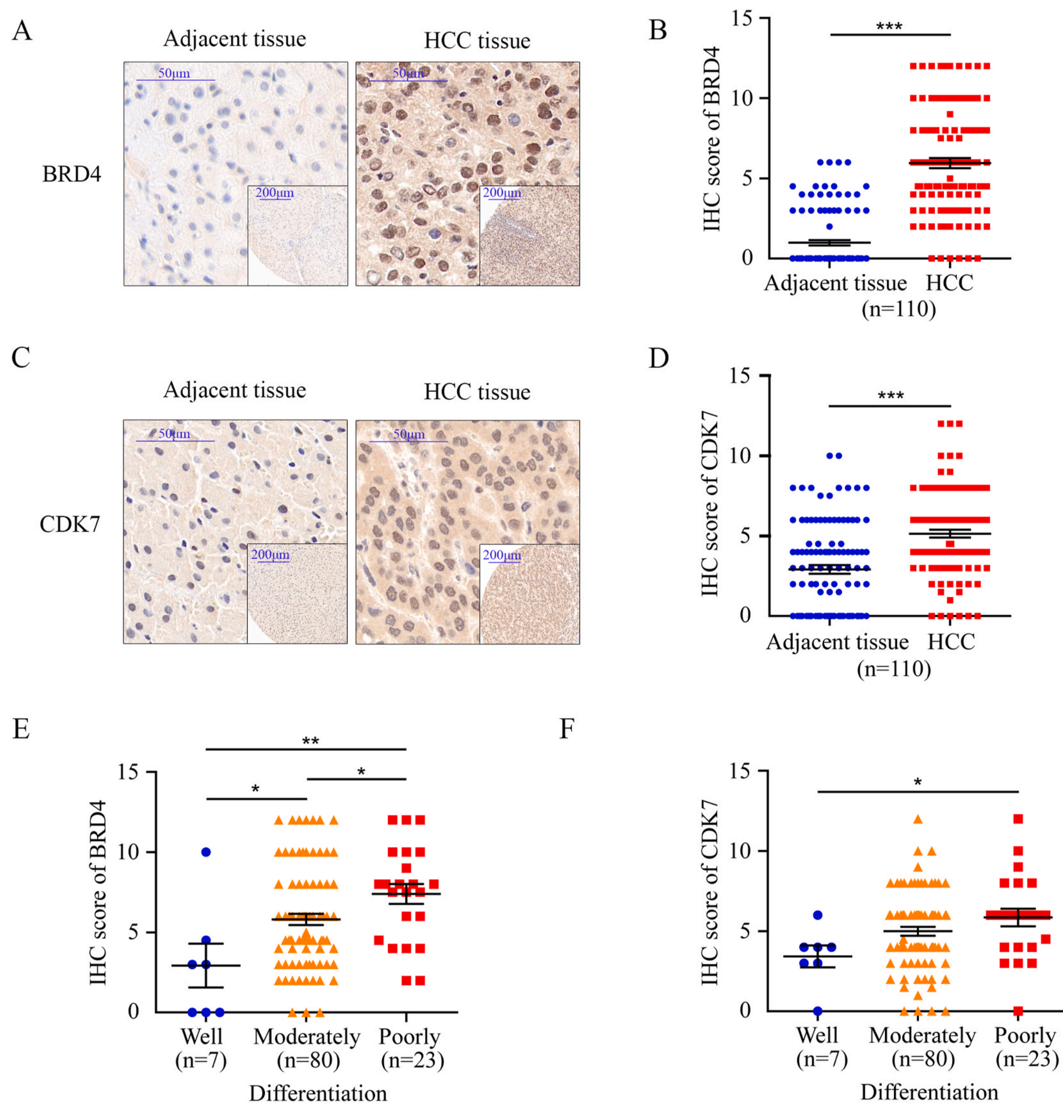

Fig. 2. Expression of BRD4 and CDK7 expression and its association with tumor differentiation grade in HCC patients (A–B) Representative immunohistochemical staining images (A) and quantification (B) of BRD4 in a HCC patient sample. (C–D) Representative immunohistochemical staining images (C) and quantification (D) of CDK7 in a HCC patient sample. Scale bars, 200 μm. n = 110 patients. (E–F) Associations of BRD4 and CDK7 expression in tissues with tumor differentiation grade were presented in E and F, respectively. Comparisons in B, D, E and F were made using a two-tailed unpaired *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.2. BRD4 and CDK7 are overexpressed and associated with poor differentiation in HCC

By immunohistochemical staining of BRD4 and CDK7 on sections of HCC patient samples, we also observed both BRD4 and CDK7 overexpression in tumor tissues compared with corresponding paracancerous tissues (Fig. 2A–D). We then assessed whether BRD4 or CDK7 expression was related to various clinicopathological features including tumor differentiation, lymph node metastasis, vascular invasion. We found that both BRD4 and CDK7 expressions was significantly associated with differentiation (Fig. 2E and F), but not correlated with vascular invasion (Figs. S3A–B) and lymph node metastasis (Figs. S3C–D). As shown in Fig. 2E and F, protein levels of BRD4 and CDK7 in HCC increased with a progressive loss of histological differentiation. Thus, we confirmed that BRD4 and CDK7 protein levels were elevated in HCC, and both high BRD4 group and CDK7 group had association with poor tumor differentiation.

3.3. Co-overexpression of BRD4 and CDK7 is an unfavorable prognostic factor in HCC patients

To further assess whether BRD4 or CDK7 expression is associated with the prognosis, we categorized patients based on the IHC scores of BRD4 or CDK7 and associated with the follow-up data. Kaplan-Meier analyses of overall survival probability of HCC patients showed that high BRD4 expression was strongly associated with reduced survival (Fig. 3A). Similar findings showed that increased CDK7 expression in this cohort was correlated with a worse outcome for patients (Fig. 3B). To address whether BRD4 and CDK7 co-operate as tumorigenic factors to predict prognosis in HCC, we further analyzed overall survival in relation to co-overexpression of BRD4 and CDK7. Combined analyses of BRD4/CDK7 expression demonstrated that HCC patients with BRD4/CDK7 co-overexpression had the lowest overall survival, whereas those who co-express modest levels of BRD4 and CDK7 had the best prognosis (Fig. 3C). These findings implied that the co-overexpression of BRD4 and CDK7 was a poor prognostic indicator in HCC patients.

3.4. Synergistic effects of JQ1 and THZ1 against HCC cell proliferation

Small-molecule inhibitors JQ1 and THZ1 to target BRD4 and CDK7 respectively have been used to suppress tumor growth in preclinical experiments [24,25]. Our results above suggested that both BRD4 and CDK7 were substantially overexpressed in human

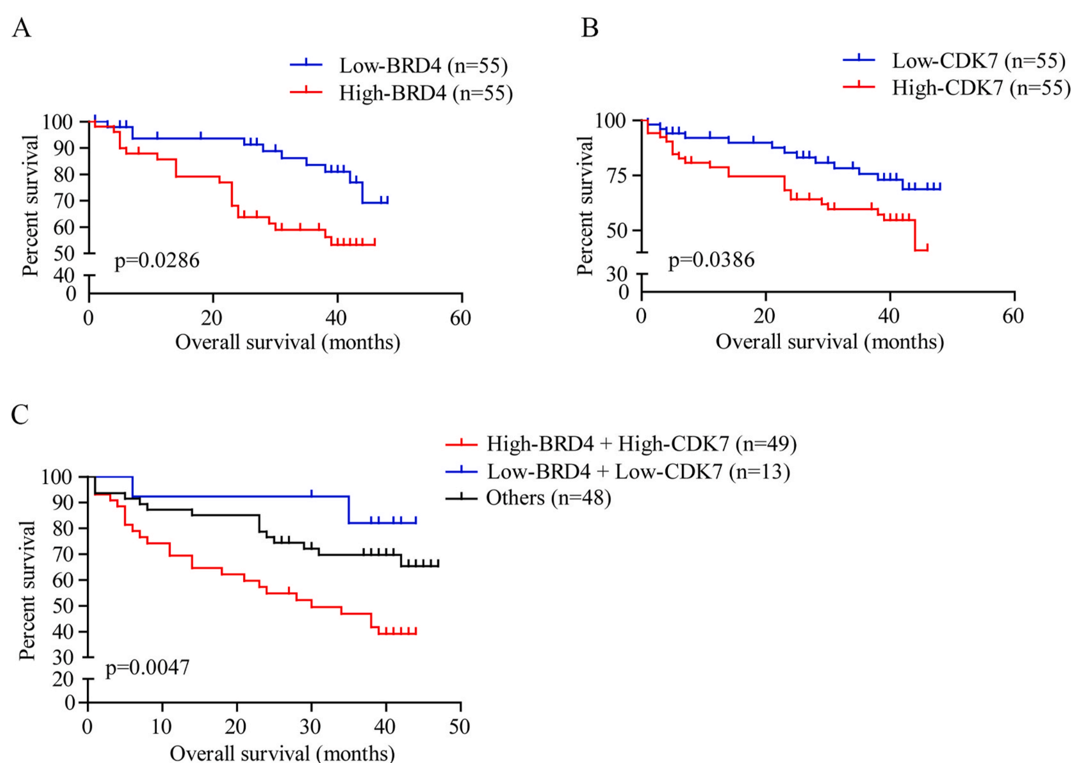

Fig. 3. Kaplan-Meier curves representing patient survival and BRD4 and CDK7 expression (A) Kaplan-Meier curves of overall survival of HCC patients with high BRD4 expression (red line, n = 55) vs. low BRD4 expression (blue line, n = 55). (B) Kaplan-Meier survival estimates comparing high (red line, n = 55) and low CDK7 expression (blue line, n = 55) in HCC. (C) Overall survival estimations of HCC patients stratified by combined tumor BRD4 and CDK7 expression status are shown in C. The whole cohort was divided into three expression groups IHC scores: patients with high expression of BRD4 and CDK7 (red line, n = 49), patients with low expression of BRD4 and CDK7 (blue line, n = 13) and other patients (black line, n = 48). Statistical significance was determined by a log-rank test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

HCCs and were significantly correlated with a poor prognosis in patients with HCC. We supposed that concurrently perturbation of BRD4 and CDK7 would be an attractive approach for HCC therapy. To determine whether BRD4 had a synergistic effect with THZ1 on inhibition of HCC cell proliferation, we treated HCC cells with BRD4 inhibitor JQ1, CDK7 inhibitor THZ1 or both. In HepG2 cells,

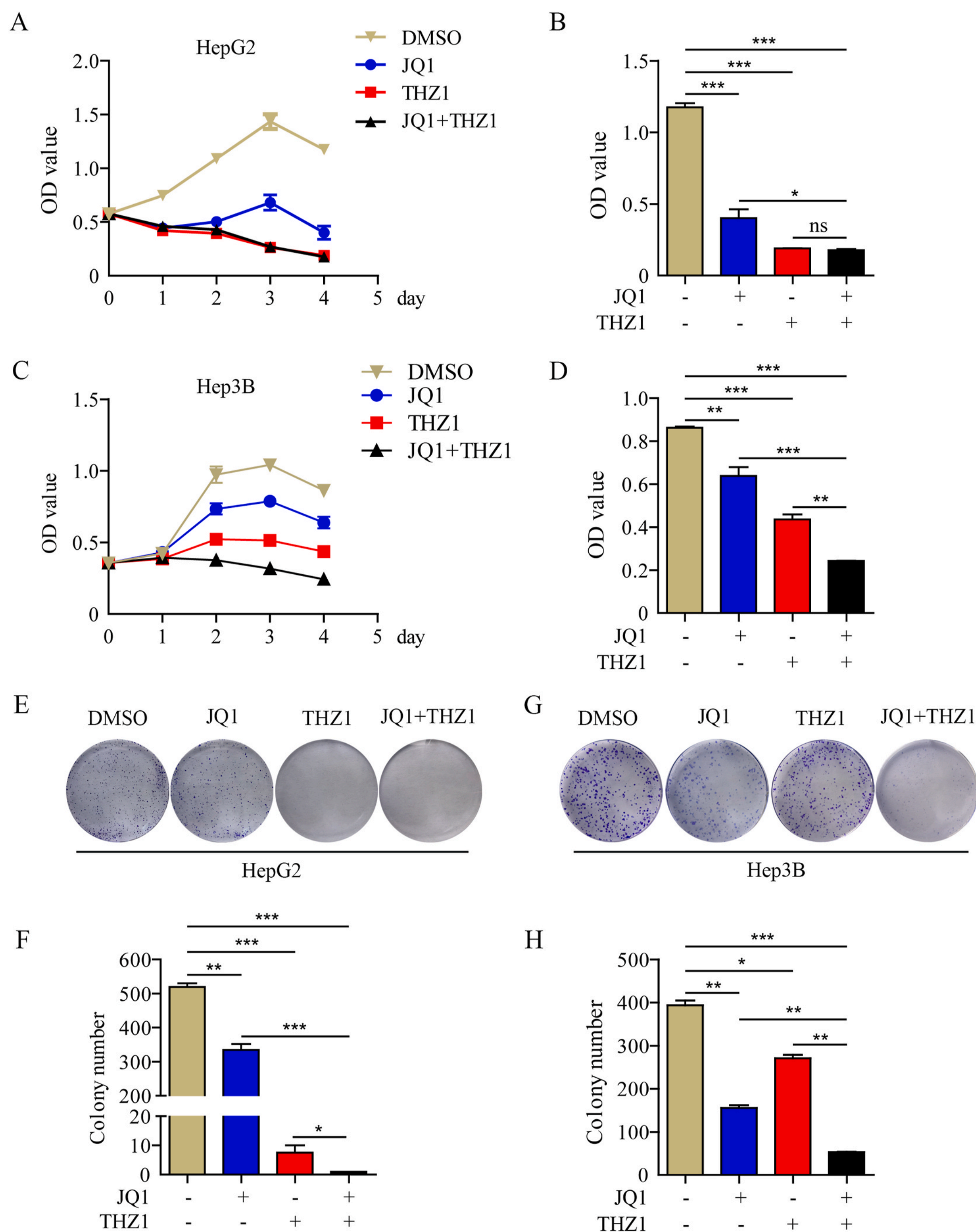

Fig. 4. Effect of JQ1 and THZ1 combination on HCC cell proliferation
(A) Cell proliferation (CCK-8 assay) for HepG2 cells during 4 days to investigate the effects of JQ1 (500 nM), THZ1 (10 nM), JQ1+THZ1. (B) The mean OD values of HepG2 cells on the fourth day following JQ1 or THZ1 treatment or dual treatment was presented with a histogram. (C) Cell proliferation assay for Hep3 cells, days 1–4, investigating the effects of JQ1, THZ1, JQ1+THZ1. (D) Hep3B cell proliferation on the fourth day following JQ1 or THZ1 treatment or dual treatment was determined by the mean OD values. (E–H) HepG2 (E) and Hep3B (G) cells were treated with JQ1 (200 nM) or THZ1 (2 nM) alone or their combination for 14 days. The colony formation of HCC cells was photographed. Quantification analysis of colony formation was performed by counting clone numbers, which is shown in F and H. Each value in graph was the mean ± SE from three independent experiments. Comparisons between two groups were made using a two-tailed unpaired *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001).

co-treatment with JQ1 and THZ1 led to a more significant reduction in cell proliferation than treatment with JQ1 alone (Fig. 4A and B). In Hep3B cells, dual treatment with JQ1 and THZ1 had a more significant inhibitory effect in cell proliferation than using JQ1 or THZ1 alone (Fig. 4C and D). Synergistic inhibition of cell proliferation with the JQ1 and THZ1 combination therapy was also observed in Huh7 and PLC cells (Figs. S4A–D). These results indicated that combination of JQ1 and THZ1 was proved more effective in inhibiting HCC cells growth.

Given the effect of combined treatment of JQ1 and THZ1 on short-term cell viability of HCC cells, we focused on long-term colony formation of HCC cells when treated with JQ1, THZ1 or both. Long-term clonogenic assays revealed that the co-treatment of JQ1 and THZ1 resulted in a considerable decrease in clone formation, meanwhile the JQ1 or THZ1 alone treatment had a lesser impact (Fig. 4E–H). Taken together, these findings documented that co-suppression of BRD4 and CDK7 resulted in a strong inhibition of HCC cell proliferation in both short-term viability assays and long-term clonogenic assays, confirming their strong synergistic restraint on tumor growth.

3.5. Joint inhibition of JQ1 and THZ1 on oncogene expression

It has been shown that the inhibition of CDK7 and BRD4 attenuated cancer growth through the preferential repression of SE-associated oncogenes, such as *MYC*, *NFE2L2* and *ETV4* [26–28]. We next investigated the regulation of these oncogenes in connection to inhibitors-JQ1, THZ1 or both. With real-time q-PCR, we examined that combinatorial treatment of JQ1 and THZ1 resulted in a greater downregulation of the expression of *MYC*, *NFE2L2* and *ETV4* than JQ1 or THZ1 alone in HepG2 and Hep3B cells (Fig. 5A–C). These results suggested that the dual suppression of BRD4 and CDK7 significantly reduced expression of key oncogenes when compared to inhibiting BRD4 and CDK7 individually.

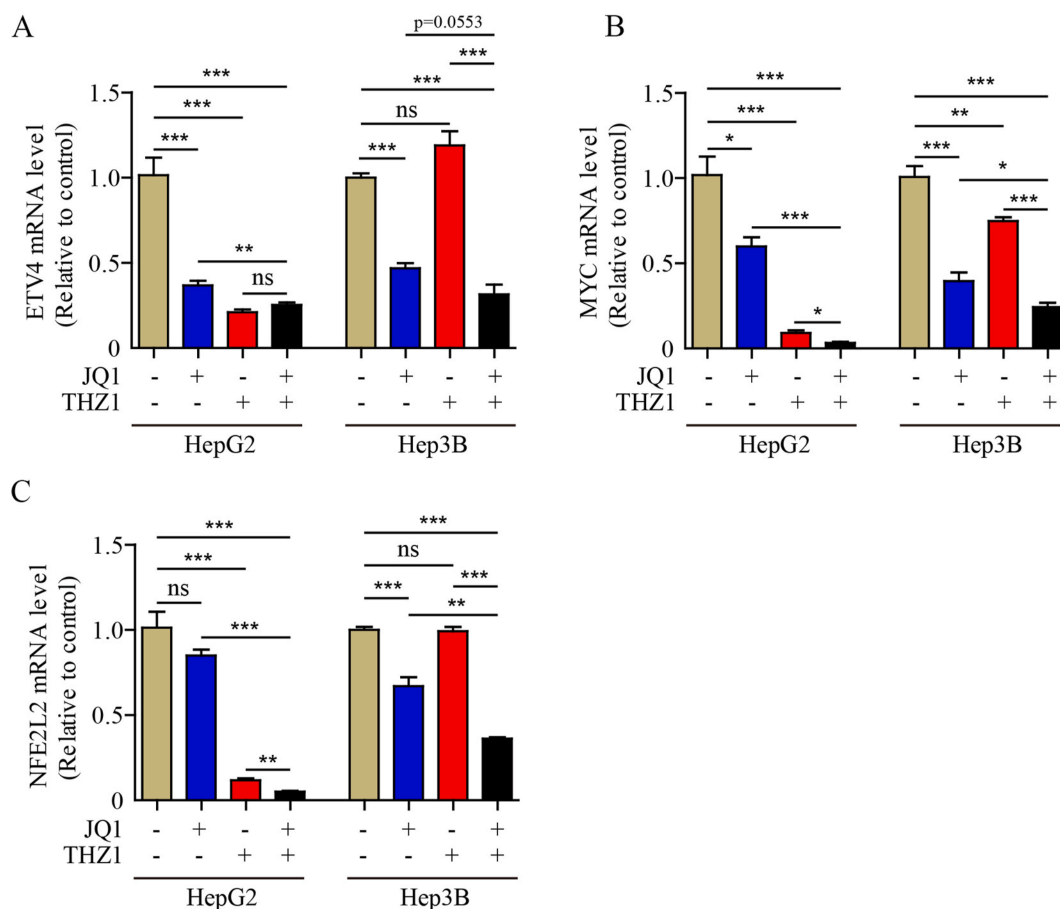

Fig. 5. Effect of JQ1 and THZ1 combination on oncogene expression (A–C) HepG2 and Hep3B cells were treated with JQ1 (500 nM) or THZ1 (10 nM) alone or their combination for 24 h. RNA was extracted and the relative mRNA levels of ETV4 (A), MYC (B), NFE2L2 (C) shown as bar graph was determined by q-PCR (two-tailed unpaired *t*-test). GAPDH was used as an internal control. Data represent mean ± SEM of more than three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

4. Discussion

SE-associated regulators BRD4 and CDK7 have been shown to possess an oncogenic role in various cancers due to its positive functions as a modulator of transcription factor activity for Pol II progression. They are frequently overexpressed in many kinds of cancers including esophageal squamous cell carcinoma, breast cancer, gastric cancer and hepatocellular carcinoma [21,23,29–33]. In the current work, we have demonstrated that overexpression of BRD4 and CDK7 are both associated with poorly differentiated tumors and poor prognosis in HCC. Notably, there is a functional difference between BRD4 and CDK7 for the regulation of SE-associated genes transcription, despite the fact that BRD4 and CDK7 are both globally essential for Pol II progression through early stages of transcription cycles. BRD4 focuses on the recruitment and stabilization of functionally relevant mediator complexes by controlling chromatin remodeling [34], while CDK7 acts as both an effector CDK, that phosphorylates Pol II and other targets within the transcriptional machinery, and a CDK-activating kinase (CAK) to activate other essential CDK [35]. In fact, BRD4 and CDK7 collaborate on transcription elongation by regulating activity of positive transcription elongation factor-b (P-TEFb) [36] and phosphorylation of the RNA polymerase II (Pol II) C-terminal domain (CTD) [37]. In our study, the patients with co-overexpression of BRD4 and CDK7 had poorer prognosis than others, despite correlation of overall survival status with poorly-differentiated appeared to occur independently. Our findings demonstrate that co-expression pattern of BRD4 and CDK7 could serve as a prognostic marker for HCC.

Both BRD4 and CDK7 are positive regulators of SE-mediated transcription and can been potential epigenetic therapeutic target. BRD4 inhibitor JQ1 and CDK7 inhibitor THZ1 are currently applied in preclinical and clinical trials for treatment of multiple tumors [38–40]. However, disadvantages such as short half-life, poor bioavailability and dose-dependent cytotoxicity have hampered their clinical application. Felice Ho-Ching Tsang et al. showed that genetically removed CDK7 and BRD4 alone significantly attenuated cell proliferation and colony formation in both HepG2 and Huh7 cells [21]. In this study, combinational therapeutic targeting of BRD4 and CDK7 by simultaneously treatment with JQ1 and THZ1 synergistically induced anticancer effects in HCC (Figs. 3 and 4). It was notable that short-term combined treatment of JQ1 and THZ1 by using HepG2 and Huh7 cells did not show synergistic effect on limiting HCC cell proliferation when compared to treatment with THZ1 alone. As shown in Fig. S5A, the expression level of CDK7 in Huh7, HepG2 and Hep3B is lower than that in PLC, while the expression level of BRD4 in Hep3B and PLC cells is relatively high. Therefore, we speculated different response to JQ1 and THZ1 between these HCC cells might be due to difference of BRD4 and CDK7 expression level in each cell line. However, the intricate collaboration between BRD4 and CDK7 to regulate transcription of genes seems to make this synergy reasonable and conceivable. The important crosstalk between BRD4 and CDK7 is not only because of their functional complementation in regulation of SE-associated genes [41,42] but also due to mutual regulation between each other. CDK7 interacts with and phosphorylates BRD4, potently inhibiting BRD4 kinase activity, and the CDK inhibitor directly inhibited BRD4 recruitment to activate chromatin globally [37,43]. Our results indicated that a combined therapy with JQ1 and THZ1 would be beneficial to inhibiting HCC cells growth in vitro. The effect of their combination therapy on mice bearing subcutaneous xenograft need to be further confirmed.

Inhibiting BRD4 or CDK7 could result in loss of SEs function with consequent transcription elongation defects and block of oncogenes expression including *MYC* and *MYCN* [19,44,45]. In our previous work, we illustrated that SE-associated gene *ETV4* promoted HCC cell migration and invasion [4]. In addition, Hoang Kieu Chi Ngo et al. showed that Nrf2 (coded by *NFE2L2* gene) was a critical driving force for hepatocellular carcinogenesis [46]. Therefore, SE-associated genes *MYC*, *ETV4* and *NFE2L2* were selected as proposed key candidate oncogenes in HCC to confirm. We believed that the heightened anticancer effects may be caused by the synergism created by the simultaneous inhibition of BRD4 and CDK7 in the SE complex. In our results, *MYC*, *ETV4* and *NFE2L2* mRNA levels were significantly decreased upon co-treatment with JQ1 and THZ1 in both Hep3B cells and HepG2 cells, while the expressions of *ETV4* and *NFE2L2* were not regulated by THZ1 in Hep3B cells. Our study suggests that combination of JQ1 and THZ1 significantly suppressed the mRNA level of oncogenes *MYC*, *ETV4* and *NFE2L2*. Further study is necessary to evaluate other known oncogenes in HCC, such as *CTNNB1*, *NRAS* and *YAP* [47,48].

5. Conclusion

Overexpression of BRD4 and CDK7 were both associated with poorly differentiated tumors and poor prognosis in HCC. HCC patients with concurrent overexpression of BRD4 and CDK7 showed a worse prognosis compared with others, which indicated that co-overexpression of BRD4 and CDK7 could be a feasible biomarker for HCC prognosis. Combined inhibition of BRD4 and CDK7 by JQ1/THZ1 combination effectively inhibited the growth of HCC cells and the expression of SE-associated oncogenes. Our findings suggested the potential effectiveness of JQ1-THZ1 combination strategy for treating HCC.

Ethics statement

This study was reviewed and approved by the Ethics Committee of Zhongda Hospital affiliated with Southeast University, China (Ethics Approval No. 2021ZDKYSB032). All patients were informed and agreed to participate in this research.

Funding

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Data availability statement

In this study, any data in this study can be obtained from the corresponding author upon reasonable request. Publicly available datasets were analyzed. These are available on The Cancer Genome Atlas (https://portal.gdc.cancer.gov/) and GEO (https://www.ncbi.nlm.nih.gov/).

CRediT authorship contribution statement

Xinxiu Li: Writing – review & editing, Writing – original draft, Data curation. **Chuqian Zheng:** Data curation. **Yue Liu:** Formal analysis. **Hui Sun:** Formal analysis. **Yanyan Qian:** Writing – review & editing, Formal analysis. **Hong Fan:** Writing – review & editing, Resources, Project administration, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24389.

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