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ORIGINAL ARTICLE

Aurora B facilitates cholangiocarcinoma progression by stabilizing c-Myc

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Abstract

Background: Cholangiocarcinoma (CCA), a malignancy that arises from biliary epithelial cells, has a dismal prognosis, and few targeted therapies are available. Aurora B, a key mitotic regulator, has been reported to be involved in the progression of various tumors, yet its role in CCA is still unclarified.

Methods: Human CCA tissues and murine spontaneous CCA models were used to assess Aurora B expression in CCA. A loss-of-function model was constructed in CCA cells to determine the role of Aurora B in CCA progression. Subcutaneous and liver orthotopic xenograft models were used to assess the therapeutic potential of Aurora B inhibitors in CCA.

Results: In murine spontaneous CCA models, Aurora B was significantly upregulated. Elevated Aurora B expression was also observed in 62.3% of human specimens in our validation cohort (143 CCA specimens), and high Aurora B expression was positively correlated with pathological parameters of tumors and poor survival. Knockdown of Aurora B by siRNA and heteroduplex oligonucleotide (HDO) or an Aurora B kinase inhibitor (AZD1152) significantly suppressed CCA progression via G2/M arrest induction. An interaction between Aurora B and c-Myc was found in CCA cells. Targeting Aurora B significantly reduced this interaction and accelerated the proteasomal degradation of c-Myc, suggesting that Aurora B promoted the malignant properties of CCA by stabilizing c-Myc. Furthermore, sequential application of AZD1152 or Aurora B HDO drastically improved the efficacy of gemcitabine in CCA.

Conclusions: Aurora B plays an essential role in CCA progression by modulating c-Myc stability and represents a new target for treatment and chemosensitization in CCA.

KEYWORDS
Aurora B kinase, Cholangiocarcinoma, c-Myc, combination therapy, targeted therapy

Ke Liu, Xuxuan Zhou, and Fei Huang contributed equally to this work.

1 | INTRODUCTION

CCA, a highly aggressive and molecularly heterogeneous tumor, accounts for 10%–20% of primary liver cancers, making it the second most common hepatobiliary malignancy.[1-2] The occurrence and mortality rate of CCA have been increasing steadily in the last several decades.[3-4] Currently, surgical resection remains the mainstay of CCA treatment; most patients are unable to undergo radical therapy since they are diagnosed at an advanced stage.[5-7] Standard systemic chemotherapy using cisplatin and gemcitabine only has a limited therapeutic effect.[8] In 2020, the Food and Drug Administration (FDA) approved pemigatinib for the treatment of advanced CCA patients harboring FGFR2 gene rearrangements or fusions.[9-10] Nonetheless, there are limited options for targeted therapy of CCA due to its unclear pathogenesis, which makes this malignant tumor a serious clinical challenge.[11] Hence, it is essential to understand the molecular mechanisms of CCA to develop an effective therapeutic strategy.

Aurora B kinase is a mitotic serine/threonine protein kinase belonging to the Aurora kinase family along with Aurora A and C kinases.[12,13] Aurora B encodes a passenger protein necessary for the regulation of the mitotic checkpoint, cytokinesis, and chromosomal segregation.[14] Overexpression of Aurora B is often associated with drug resistance and cell metastasis in cancers including non-small cell lung cancer, colorectal cancer, and clear cell renal cell carcinoma.[15,16] Therefore, Aurora B has been identified as an effective drug target, which has led to the design and synthesis of small molecule inhibitors. Preliminary studies have shown that AZD1152, a specific Aurora B inhibitor, is effective against different solid tumors, including colon cancer, breast cancer, and lung cancer.[17,18] In addition to preclinical studies, AZD1152 has also been examined in various clinical trials, suggesting that it is expected to be developed as an antitumor drug.[19,20] This compound is currently undergoing phase III clinical trials to verify its safety and effectiveness.[21] However, the role of Aurora B in CCA is still unknown. Previous studies showed that c-Myc-overexpressing cancer cells are vulnerable to Aurora B inhibitors (e.g. AZD1152).[22] Dysregulation of c-Myc has been reported in CCA.[23] Its regulatory role in both CCA occurrence and progression indicates that inhibiting the expression or activity of c-Myc is a promising strategy for treating CCA. However, due to the undruggable protein structure of c-Myc, approaches to inhibit its function have met with little success.[24,25] Therefore, impairment of the pathway that regulates c-Myc degradation may be a crucial strategy for preventing the oncogenic activation of c-Myc in cancer cells.[26]

Gemcitabine resistance has been regarded as the main cause of chemotherapeutic failure in CCA.[27] Combination therapy is a promising treatment to improve chemotherapy resistance. From a mechanistic perspective, gemcitabine resistance is caused by changes in drug metabolism or attenuation of gemcitabine-induced cell death.[28] In recent years, Aurora kinase has been shown to regulate drug resistance in different malignant tumors, including melanoma, glioblastoma, lung cancer, and liver cancer.[29,30] However, there is a lack of research on the association between Aurora B and drug response in CCA. Therefore, it is necessary to consider combination therapy to improve the traditional chemotherapy regimen for CCA.

The present study suggested that Aurora B was upregulated in human CCA, which correlated with the pathological characteristics of CCA and predicted poor survival. Aurora B induces CCA cell proliferation and invasion by modulating c-Myc. Furthermore, these findings reveal that sequential treatment with Aurora B inhibitors and gemcitabine in CCA is crucial for the inhibition of proliferation. Thus, we speculate that Aurora B is a putative tumor promoter in CCA and can serve as a prognostic biomarker and therapeutic target for CCA.

2 | METHODS

2.1 | Clinical CCA specimens

Human CCA tissues, including cancerous tissues (intratumor) and associated paracarcinoma tissues (peritumor), were obtained from 143 patients who had undergone curative liver resection at The First Affiliated Hospital of Sun Yat-sen University between January 2015 and December 2020 (detailed patient information is shown in Table S1). In addition, 10 normal hepatic tissues obtained from patients who underwent resection due to benign hepatic lesions were used as normal controls. The use of the clinical specimens for research purposes was approved by the Jinan University Ethics Committee and IEC for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-Sen University (ethics approval number: [2021]678) and was performed in accordance with the *Declaration of Helsinki*. Written informed consent was obtained from each patient at each institution. The difference among categorical variables were compared with the chi-square test.

2.2 | Animals

Four- to six-week-old male BALB/c nude mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Eight- to nine-week-old male SD rats and 5- to 7-week-old male BALB/c mice were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China). All experimental animals were monitored in an SPF (specific pathogen-free) grade Laboratory Animal Center of Jinan University. The experimental animal protocol was reviewed and approved by the Institutional Animal Ethical Committee, Laboratory Animal Center of Jinan University and followed the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health. The animals were anesthetized using inhaled isoflurane gas and sacrificed by CO_2 asphyxiation.

2.3 | Establishment of spontaneous CCA murine models

The thioacetamide (TAA)-induced spontaneous CCA rat model was constructed as previously reported.[31,32] Sprague–Dawley (SD) rats (male, 350±20g) received 0.03% TAA (Sigma, USA) in drinking water.

Visible and whitish ICC tumors were recorded at the 24th week after TAA administration. The establishment of the diethylnitrosamine–left and median bile duct ligation (DEN-LMBDL) model of CCA was performed as reported. To achieve tumor development in mice, we subjected 7-week-old male BALB/c mice to two separate weekly intraperitoneal injections of 100 mg/kg DEN. Then, LMBDL was performed in all experimental mice. After two weeks, DEN (25 mg/kg) was administered by oral gavage once a week. The total duration of the experiment lasted 28 weeks, and CCA was successfully established.[33]

2.4 | CCA mouse xenograft models

To establish subcutaneous xenograft tumors, HuCCT1 cells (2×10^6) were suspended in 200 μL of PBS with Matrigel (1:1) and then injected subcutaneously into the right back tissues of 4-week-old male BALB/c nude mice. When the tumor volume reached approximately 100 mm^3, tumor-bearing mice were randomly divided into three groups. The control group received a vehicle control, the positive control group was administered gemcitabine (25 mg/kg) by intragastric injection every other day, and the treatment group was administered AZD1152 (25 mg/kg) by intragastric injection every other day. The experiment was terminated when the tumor volume of the vehicle group reached about 1000 mm^3. Tumor volume was measured every 3 days and calculated as $(a\times b^2)/2$, where a and b are the longest and shortest tumor diameters, respectively.

For the establishment of the orthotopic CCA model, BALB/c nude mice were anesthetized, and CCA cell lines (HuCCT1) were suspended in PBS with Matrigel solution (PBS: Matrigel = 1:1). Then, 100 μL (approximately 1×10^6 cells) of cell suspension was injected into the subcapsular region of the middle lobe of the mouse liver.[34] For the treatment, Aurora B HDO (10 mg/kg) was injected into mice through the tail vein twice a week. Gemcitabine (25 mg/kg) administered by intragastric injection was used as the positive control.

All other methods are available in the "Supporting materials".

3 | RESULTS

3.1 | Aurora B is upregulated in both human CCA and murine CCA

To detect the expression of Aurora B in CCA, we first analyzed the mRNA expression of Aurora B in 36 primary CCA tissues and 9 nontumor tissues from the CCA dataset of The Cancer Genome Atlas (TCGA). The expression of Aurora B was significantly upregulated in CCA tissues compared to nontumor tissues. A similar result was also observed in Gene Expression Omnibus (GEO) datasets (GSE26566) (Figure 1A). Next, we evaluated the protein levels of Aurora B in human CCA cells and normal human biliary epithelial cells. Experimental data indicated that the expression of Aurora B was markedly higher in most CCA cell lines than in the normal biliary epithelium cell line, HIBEpiC, where Aurora B was undetectable (Figure 1B). We further explored the expression of Aurora B in human tissues, including 143 CCA samples, by immunohistochemical staining. The results showed that Aurora B was upregulated in 62.3% (89/143) of human CCA samples and that the expression level of Aurora B in CCA was significantly higher than that in normal ducts (NDs) (Figure 1C,D). To further verify Aurora B expression in CCA, we next constructed two murine spontaneous induction models: a TAA-induced rat CCA model and a DEN–LMBDL-induced mouse CCA model. In the TAA model, the rats were supplied with TAA (300 mg/L) in the drinking water, and CCA evolved as a consequence of biliary hyperplasia. After 24 weeks of TAA supplementation, we found that Aurora B expression in CCA lesions (CK19 positive) was higher than that in normal tissues (Figure 1E). DEN is currently the most widely used chemical to establish murine HCC. It induces liver damage and stimulates the dysplasia of liver cells and bile duct epithelial cells[35]; when coupled with LMBDL, it induces CCA (Figure 1F). Similar data were also collected in the DEN–LMBDL-induced mouse model (Figure 1G). These data collectively indicated that the upregulation of Aurora B is a common event in CCA and may be associated with tumor progression.

3.2 | Overexpression of Aurora B is associated with poor prognosis in CCA patients

To investigate the impact of Aurora B expression on clinical pathological features in CCA patients, 143 CCA patients were divided into two groups based on the expression level of Aurora B: the high-expression group (IHC score ≥1.5, $n=60$) and the low-expression group (IHC score <1.5, $n=83$). We then analyzed the correlation between Aurora B expression and clinical parameters, including gender, age, HBsAg, CA199, tumor number, tumor size, tumor staging, vascular invasion, and so on (detailed information is listed in Table S1). Among the clinical parameters listed in Table S1, although not all parameters were statistically significant, several aggressive clinical pathological features, including larger tumor volume ($p=0.028$), CA125 expression ($p=0.027$), advanced TNM staging ($p=0.032$), positive lymph node metastasis ($p=0.018$), and multiple tumors ($p=0.045$), were correlated with Aurora B expression. They were more strongly correlated in the Aurora B high-expression group than in the low-expression group (Table 1), indicating that high Aurora B expression is associated with aggressive tumor growth and metastasis and may be involved in tumor progression in CCA. The prognostic implication of Aurora B in CCA was explored next. Kaplan–Meier analysis showed that a high level of Aurora B expression was correlated with poor overall survival ($p<0.001$) and lower disease-free survival ($p<0.05$) in our cohort (Figure 2A). Similar results were observed in TCGA data containing 18 CCA cases (Figure 2B). These results collectively indicated that Aurora B could serve as a biomarker in CCA progression and predict the prognosis of CCA patients.

3.3 | Aurora B promotes the proliferation and invasion of CCA cells in vitro

To assess the function of Aurora B in the progression of CCA, Aurora B was transiently knocked down with siRNA in the CCA cell lines

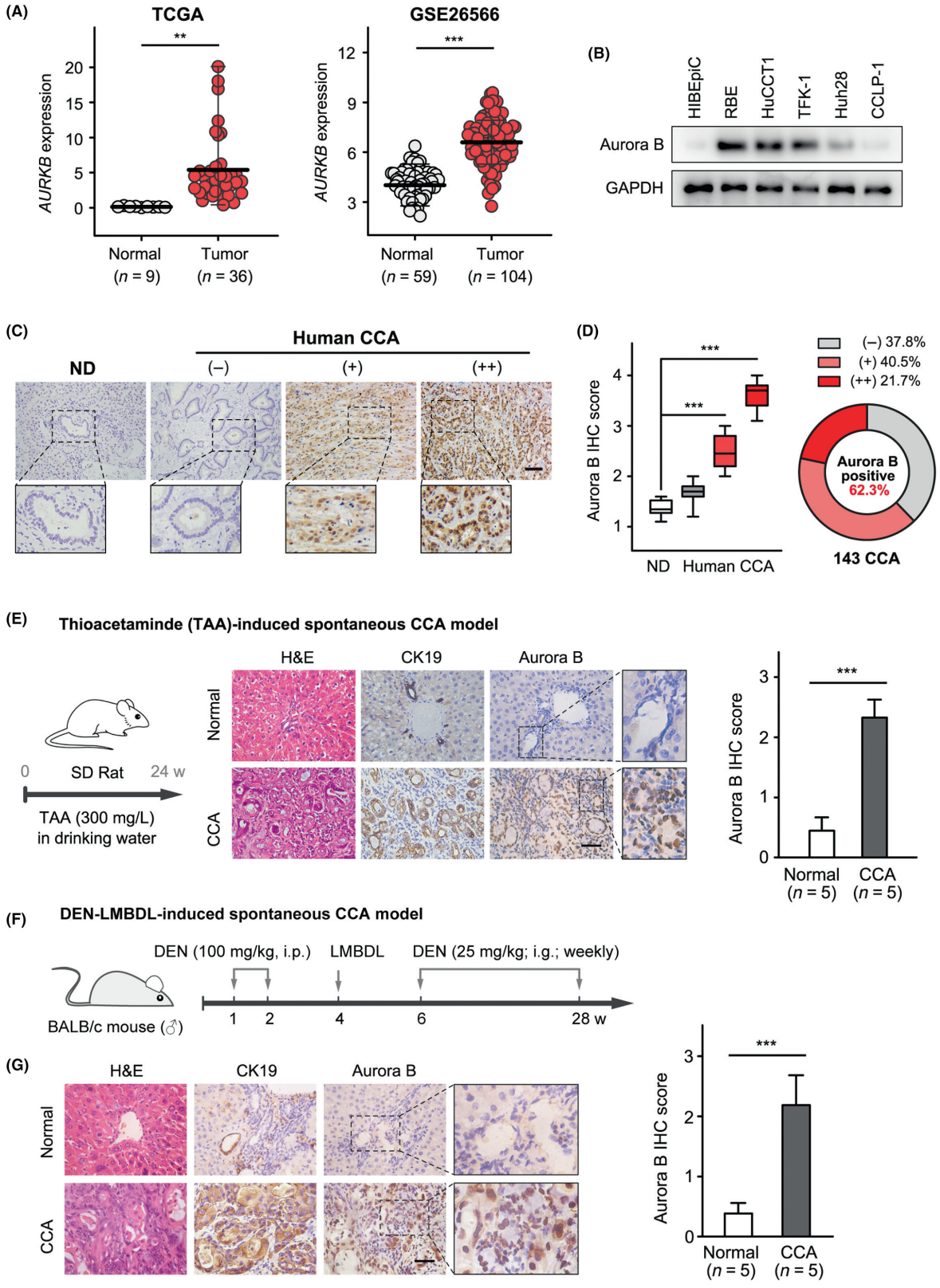

FIGURE 1 Aurora B expression is upregulated in CCA. (A) Relative mRNA level of *Aurora B* expression among adjacent nontumor and CCA tissues (data from TCGA and GSE26566). $^{**}p<0.01$; $^{***}p<0.001$. (B) Aurora B protein expression in a normal biliary epithelium cell line and five CCA cell lines as detected by Western blotting. (C) Representative images of human CCA tissues stained with Aurora B. (D) statistical analysis of their IHC scores. Negative expression (–, 0 ≤ IHC score <1); weak expression (+, 1 ≤ IHC score <2); strong expression (++, 2 ≤ IHC score <3). Scale bar: 50 μm, $^{***}p<0.001$. (E) Schematic of the TAA rat CCA model (left). Immunohistochemical staining of Aurora B in normal tissues and CCA tissues in the TAA-induced spontaneous CCA model (middle) and statistical analysis of the Aurora B IHC score (right). Scale bar: 50 μm, $^{***}p<0.001$. (F), Schematic of the experimental design of the DEN–LMBDL-induced spontaneous CCA model. (G) Immunohistochemical staining of Aurora B in normal tissues and CCA tissues in the DEN–LMBDL-induced spontaneous CCA model and statistical analysis. Scale bar: 50 μm, $^{***}p<0.001$.

TABLE 1 The correlation between Aurora B expression and clinical parameters of 143 CCA patients.

Characteristics	Aurora B		
	Low	High	*p* value
Age (y)			
≤60	40 (27.97%)	36 (25.17%)	0.163
>60	43 (30.07%)	24 (16.78%)	
Gender			
Male	44 (30.77%)	35 (24.48%)	0.528
Female	39 (27.27%)	25 (17.48%)	
HBsAg			
Positive	31 (21.68%)	27 (18.88%)	0.358
Negative	52 (36.36%)	33 (23.08%)	
CA199 (U/mL)			
<37	34 (23.78%)	21 (14.69%)	0.456
≥37	46 (32.17%)	37 (25.87%)	
ND	3 (2.10%)	2 (1.40%)	
CA125 (U/mL)			
<35	61 (42.66%)	34 (23.78%)	**0.027***
≥35	19 (13.29%)	24 (16.78%)	
ND	3 (2.10%)	2 (1.40%)	
CEA (ng/mL)			
<5	59 (41.26%)	35 (24.48%)	0.095
≥5	21 (14.69%)	23 (16.08%)	
ND	3 (2.10%)	2 (1.40%)	
Tumor size (cm)			
≤5	54 (37.76%)	28 (19.58%)	**0.028***
>5	29 (20.28%)	32 (22.38%)	
Tumor number			
Single	64 (44.76%)	37 (25.87%)	**0.045***
Multiple	19 (13.29%)	23 (16.08%)	
Vascular invasion			
Yes	27 (18.88%)	22 (15.38%)	0.607
No	56 (39.16%)	38 (26.57%)	
Perineural invasion			
Yes	31 (21.68%)	19 (13.29%)	0.482
No	52 (36.36%)	41 (28.67%)	
Lymph node metastasis			
Yes	18 (12.59%)	24 (16.78%)	**0.018***
No	65 (45.45%)	36 (25.17%)	
Tumor differentiation			
I–II	41 (28.67%)	35 (24.48%)	0.281
III–IV	30 (20.98%)	17 (11.89%)	
ND	12 (8.39%)	8 (5.59%)	
TNM stages			
I–II	55 (38.46%)	29 (20.28%)	**0.032***
III–IV	28 (19.58%)	31 (21.68%)	

Bold is used to indicate a *p*-value less than 0.05

Note: Our cohort, 143 patients from The First Affiliated Hospital Sun Yat-sen University.

Abbreviation: ND, not determined.

*$p < 0.05$ was considered as statistically significant (chi-square test).

RBE and HuCCT1, which have high endogenous Aurora B levels (Figure 3A). CCK-8 and EdU assays showed that the proliferation rates were remarkably suppressed in Aurora B-knockdown RBE and HuCCT1 cells compared with control cells (Figure 3B,C). Clinical relevance analyses showed that the expression of Aurora B is related to lymph node metastasis, so we assessed whether Aurora B can regulate the metastasis and invasion of CCA. Compared to the control cells, the Aurora B-knockdown cells exhibited markedly inhibited cell migration and invasion, as shown by Transwell assays (Figure 3D). Wound healing assays showed that Aurora B knockdown decreased the motility of RBE and HuCCT1 cells (Figure 3E). EMT plays crucial roles in the metastasis and invasion of CCA cells. Our data demonstrated a significant reduction in mesenchymal phenotype markers, including N-cadherin and vimentin, in Aurora B-silenced RBE and HuCCT1 cells. In contrast, the expression of E-cadherin, an epithelial phenotype marker, was markedly upregulated, suggesting that Aurora B knockdown reversed the epithelial phenotypes and suppressed EMT in RBE and HuCCT1 cells (Figure 3F). Altogether, these findings demonstrate that Aurora B has oncogenic activities in CCA.

3.4 | Pharmacological inhibition of Aurora B suppresses the malignant characteristics of CCA in vitro and in vivo

To evaluate the treatment potential of Aurora B inhibitors against CCA, we treated the RBE and HuCCT1 cells with AZD1152, a specific Aurora B kinase inhibitor. CCK8 assays indicated that AZD1152 effectively suppressed the cell proliferation in a concentration-dependent manner (Figure S1A), but revealed a non-time-dependent profile, implying a reversible inhibitory mechanism. Colony formation assay indicated that AZD1152 inhibited anchorage-independent growth of CCA cells (Figure S1B). Furthermore, to assess whether AZD1152 administration can lead to the suppression of Aurora B kinase activity in human tumor xenografts, a CCA xenograft model based on the HuCCT1 cell line was established in athymic nude mice. Gemcitabine is the standard first-line systemic treatment in patients with advanced CCA,[8] we used gemcitabine administration as a positive control. Mice bearing HuCCT1 CCA xenografts were exposed to vehicle, gemcitabine (25 mg/kg) or AZD1152 (25 mg/kg) every other day. The subcutaneous tumors were measured every 3 days, and the mice were euthanized when the tumor volume reached approximately 1000 mm^3. The treatment with AZD1152 showed a better therapeutic effect compared to treatment with gemcitabine (Figure 4A,B). To further explore the role of AZD1152 in inhibiting the metastasis of CCA cells, both Transwell and wound healing assays were conducted. Cells treated with AZD1152 showed significantly lower migration and invasion rates than control cells (Figure S1C,D), and the suppression of Aurora B upregulated the expression level of E-cadherin while downregulating the expression of Vimentin and N-cadherin compared to that in the control group (Figure S1E). In addition, IHC staining of subcutaneously transplanted tumors further confirmed that AZD1152 suppressed phosphorylated Aurora B and the growth of CCA, as verified by a decrease in Ki-67-positive CCA cells, and it also suppressed EMT in CCA xenografts (Figure S2A).

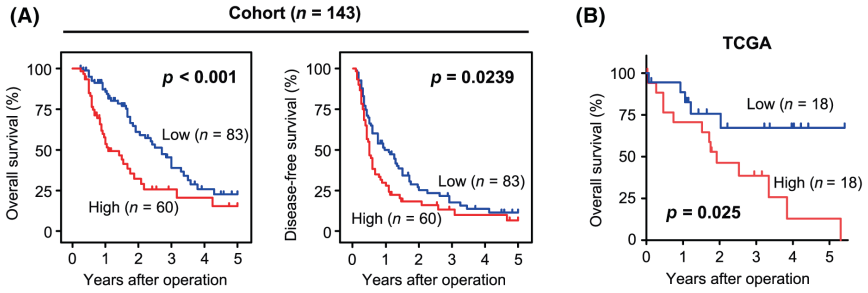

FIGURE 2 Upregulation of Aurora B is associated with poor prognosis of CCA. (A) Overall survival curves and disease-free survival curves of CCA patients from our cohort ($n = 143$) with high ($n = 60$) or low Aurora B ($n = 83$) expression levels. (B) Overall survival curves of CCA patients from the TCGA database with high ($n = 18$) or low Aurora B ($n = 18$) expression levels.

Recently, antisense oligonucleotides (ASOs) have been extensively employed as targeted therapeutic tools in drug development.[36,37] This study designed a cholesterol-conjugated DNA/RNA heteroduplex oligonucleotide targeting Aurora B (Aurora B HDO) (Figure 4C). In vitro experiments demonstrated that Aurora B HDO enters cells rapidly (Figure S2B) and significantly reduces the expression of Aurora B in CCA cells in a dose-dependent manner (Figure 4D). Existing studies have demonstrated that cholesterol in the bloodstream is primarily absorbed and transported into cells through endocytosis of low-density lipoprotein receptor (LDLR) on the surface of hepatocytes. Based on this knowledge, we hypothesized that cholesterol-conjugated Aurora B HDO could also accumulate in CCA if LDLR is also expressed, leading to a decrease in Aurora B mRNA levels (Figure 4E). Consistent with our hypothesis, high expression of LDLR was observed in rat, mouse, and human CCA tissues compared to normal or peripheral liver tissue (Figure 4F), which led to a significant accumulation of Aurora B HDO in CCA cells in an orthotopic CCA model using the HuCCT1 and RBE cell lines (LDLR-positive, data not shown) (Figure 4G). Additionally, compared to the gemcitabine treatment group, Aurora B HDO significantly inhibited orthotopic HuCCT1 xenograft growth, with reduced Aurora B expression (Figures 4H and S2C). These data indicated that targeted therapy has more potential than traditional chemotherapy against Aurora B-positive CCA.

3.5 | Inhibition of Aurora B blocks cell cycle progression and promotes apoptosis in CCA cells

Aurora B plays an essential role in chromosome alignment during mitosis. To evaluate whether the increase in tumor growth mediated by Aurora B is caused by cell cycle enhancement, cell cycle analysis was conducted after treatment of REB and HuCCT1 cells with AZD1152 for 24 h. Compared with the DMSO control, AZD1152 treatment reduced the cell fraction in the G0/G1 phase and elevated the cell fraction in G2/M (Figure 5A). Inhibition of Aurora B kinase led to abnormal mitotic exit and cytokinesis failure, thereby inducing the formation of polyploidy cells. Immunofluorescence results showed that AZD1152 induced polyploidy in RBE and HuCCT1 cells, while few polyploidies were detected in control cells (Figure 5B). The mechanism underlying the effect of Aurora B on the G2/M phase transition was then elucidated. Given that CDK1/Cyclin B1 is an important factor for the G2/M transition during the cell cycle, we speculated that CDK1/Cyclin B1 is involved in Aurora B-modulated cell cycle distribution. This was evidenced by the fact that AZD1152 treatment reduced CDK1 and Cyclin B1 expression in CCA cells (Figure 5C). Because AZD1152 has been shown to induce polyploidy in cells, the effect of AZD1152 on apoptosis was further examined. The flow cytometry findings demonstrated that AZD1152 treatment for 24 h induced CCA cell apoptosis (Figure 5D). The cleavage of two apoptosis markers, caspase 3, and PARP after AZD1152 treatment further confirmed the induction of apoptosis in CCA cells (Figure 5C). Altogether, these findings demonstrate that Aurora B is an essential molecule responsible for CCA apoptosis and cell cycle distribution.

3.6 | Aurora B-mediated stability of c-Myc promotes CCA progression

It has previously been shown that c-Myc is highly expressed in CCA tumor cell2.[23] Aurora B stabilizes c-Myc in T-cell acute lymphoblastic leukemia by regulating phosphorylation at Ser67. c-Myc then activates AURKB transcription, which creates a positive feedback loop, thus triggering a cascade of oncogene interactions.[38] To dissect the antitumor activity of Aurora B and c-Myc association in CCA, coimmunoprecipitation was performed using RBE and HuCCT1 cell lysates, which demonstrated the direct interaction between endogenous Aurora B and c-Myc in CCA cells; after Aurora B activity was inhibited using AZD1152, this interaction was reduced (Figure 6A). Next, Aurora B was knocked down in RBE and HuCCT1 cells with Aurora B HDO, and Aurora B silencing reduced the steady-state levels of c-Myc protein (Figure 6B). However, depletion of Aurora B failed to induce a substantial decrease in c-Myc mRNA level (Figure 6C), implying that Aurora B regulates c-Myc via a posttranscriptional mechanism. The cycloheximide (CHX) chase assay revealed that inhibition of Aurora B significantly promoted the half-life degradation of endogenous c-Myc in RBE and HuCCT1 cells (Figure 6D), which further supported the idea that inhibition of Aurora B downregulates c-Myc

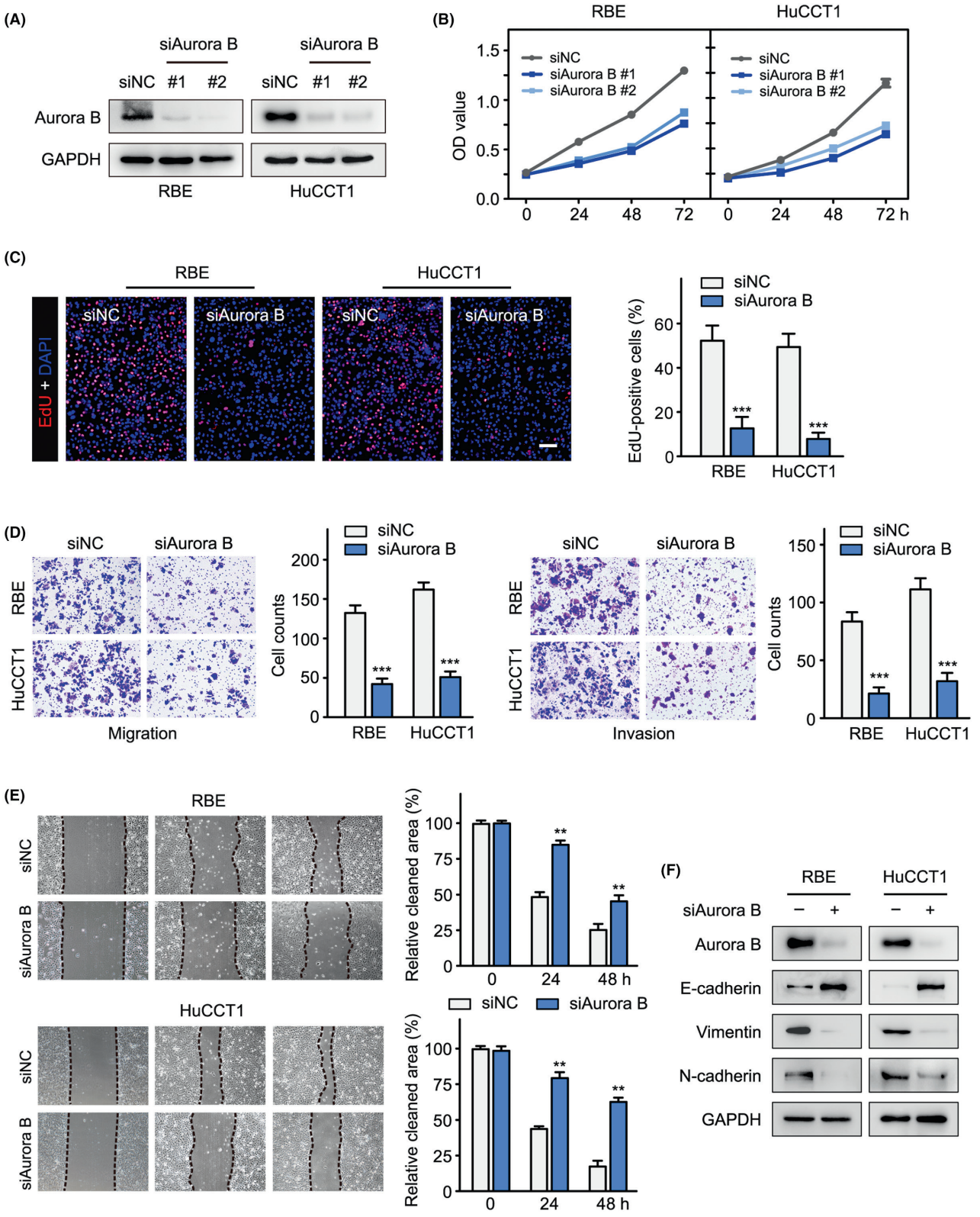

FIGURE 3 Effects of Aurora B on CCA proliferation and invasion. (A) Western blot analysis of Aurora B expression in RBE and HuCCT1 cells infected with Aurora B siRNA (400 nM) for 72 h. (B) The effect of Aurora B siRNA (400 nM) on cell viability was determined by CCK-8 assays. (C) RBE and HuCCT1 cells were treated with 400 nM siNC or siAurora B, and cell proliferation was detected by EdU incorporation assay. Scale bar: 100 μm, $^{***}p < 0.001$ versus siNC. (D) Representative images of the migration and invasion of RBE and HuCCT1 cells transfected with 400 nM siNC or siAurora B. The numbers of migrating cells per field were calculated. $^{***}p < 0.001$ versus siNC. (E) Representative images and data from wound healing migration assays performed with the indicated CCA cells transfected with 400 nM siNC or siAurora B. $^{**}p < 0.01$ versus siNC. (F) The expression of Aurora B, E-cadherin, vimentin, and N-cadherin was detected by Western blotting in the indicated cells transfected with 400 nM siNC or siAurora B for 72 h.

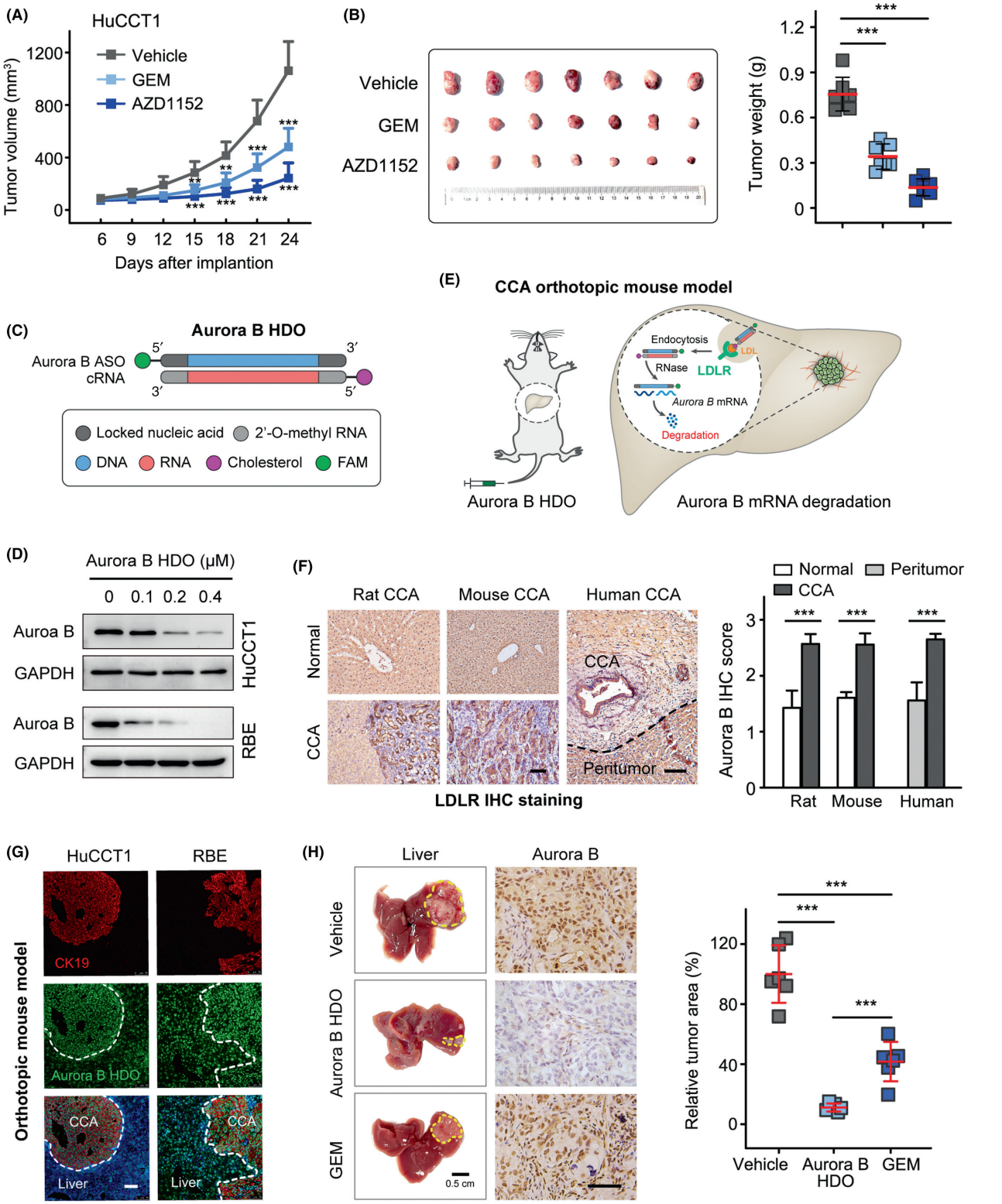

FIGURE 4 Effects of pharmacological inhibition of Aurora B on CCA progression. (A) Tumor volumes of HuCCT1 xenografts were measured and recorded every 3 days, and a growth curve was plotted. $^{**}p<0.01$, $^{***}p<0.001$ versus vehicle. (B) The left panel shows an image of subcutaneous HuCCT1 xenografts, and the right panel shows the final weights of the subcutaneous xenografts. $^{***}p<0.001$. (C) Schematic of the structure of Aurora B HDO. (D) The dose-dependent decrease in gene silencing of HuCCT1 cells and RBE cells by Aurora B HDO (72 h) was detected by Western blotting. (E) After tail vein injection of Aurora B HDO, cholesterol-labeled Aurora B HDO accumulates in LDLR-positive cells through LDLR-mediated endocytosis, resulting in a decrease in Aurora B mRNA expression. (F) Immunohistochemical staining of LDLR in normal liver or CCA tissues from rat and mouse spontaneous CCA models, as well as in a human CCA tissue. $n=5$. Scale bar = 100 μm, $^{***}p<0.001$. (G) Fluorescence image of mouse liver tissue in orthotopic HuCCT1 or RBE xenograft mouse model treated with Aurora B HDO. Scale bar = 100 μm. (H) Representative images of tumor morphology and immunohistochemical staining of Aurora B in an orthotopic HuCCT1 xenograft model treated with Aurora B HDO or gemcitabine and statistical analysis of tumor nodules. Scale bar = 100 μm, $^{***}p<0.001$.

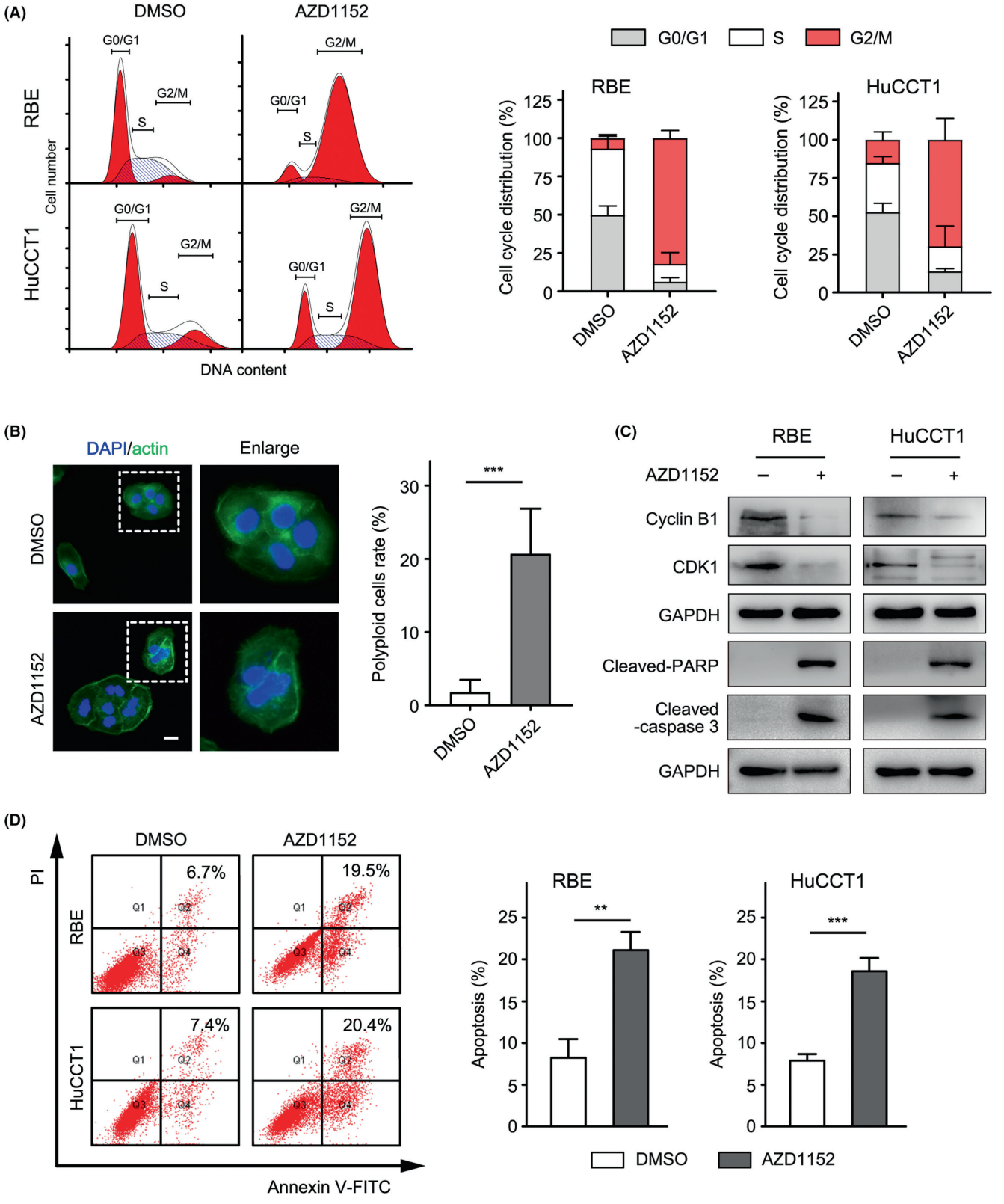

FIGURE 5 Aurora B inhibition induced G2/M cell cycle arrest and apoptosis in CCA cells. (A) Cell cycle analysis was performed in RBE and HuCCT1 cells treated with AZD1152 (20 μM) for 24 h. The bar graphs show the percentage of cell cycle distribution. (B) Fluorescence microscopy was performed to analyze the percentage of polyploidy cells in RBE cells treated with AZD1152 (20 μM) for 24 h. Scale bar, 10 μm, ***$p < 0.001$. (C) Cell cycle-related proteins and cell apoptosis-related proteins in RBE and HuCCT1 cells treated with AZD1152 (20 μM) for 24 h were measured by Western blotting. (D) RBE and HuCCT1 cells treated with AZD1152 (20 μM; 24 h) were stained with a combination of Annexin V-FITC and PI and analyzed by FACS. The bar graphs show the percentage of apoptotic cells. **$p < 0.01$, ***$p < 0.001$.

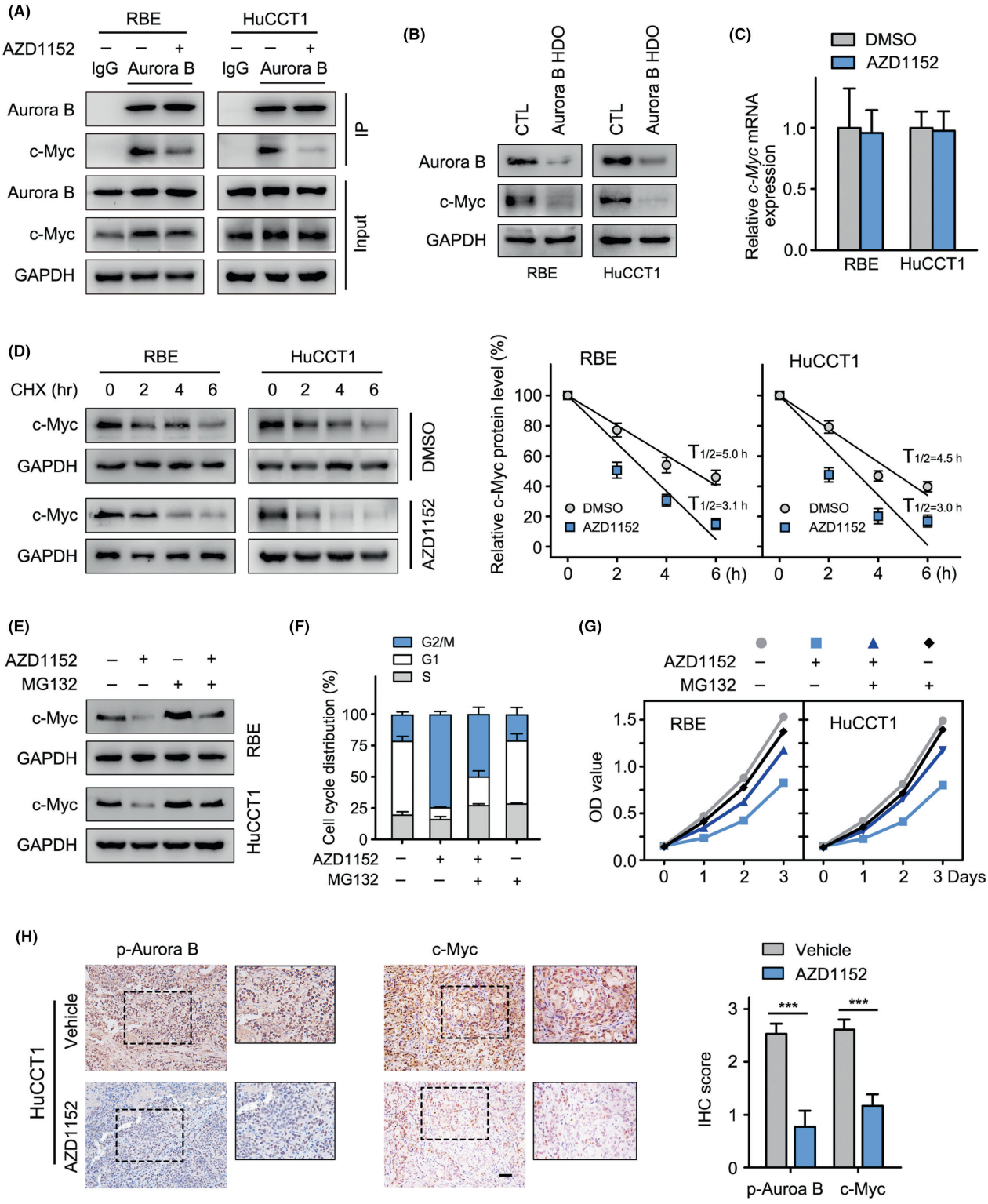

FIGURE 6 Aurora B sustains the stability of c-Myc in human CCA cells. (A) RBE and HuCCT1 cells treated with AZD1152 (20 μM) for 12 h were subjected to co-IP and immunoblotting to detect endogenous c-Myc and Aurora B interactions. (B) c-Myc protein levels were analyzed by Western blotting in RBE and HuCCT1 cells transfected with Aurora B HDO (400 nM) for 72 h. (C) qRT–PCR analysis of c-Myc mRNA levels in RBE and HuCCT1 cells upon AZD1152 treatment (20 μM) for 24 h. (D) RBE and HuCCT1 cells were treated with CHX (1 μM) for the indicated times in the presence or absence of AZD1152 (20 μM). c-Myc protein levels were analyzed by Western blotting. (E) and (F) RBE and HuCCT1 cells were treated with AZD1152 (20 μM) in the presence or absence of MG132 (10 μM) for 24 h, c-Myc protein levels were analyzed by Western blotting (E), and cell cycle distribution was analyzed by flow cytometry (F). (G) RBE and HuCCT1 cells were treated with AZD1152 (20 μM) in the presence or absence of MG132 (10 μM) for the indicated times, and cell viability was determined by CCK-8 assay. (H) Representative images of immunohistochemical staining of c-Myc and p-Aurora B in a subcutaneous HuCCT1 xenograft model treated with vehicle or AZD1152 (25 mg/kg); the right panel shows the analysis of IHC staining. $n = 7$. Scale bar: 50 μm, $^{***}p < 0.001$.

via protein degradation. We then examined whether the ubiquitin-proteasome system was involved in AZD1152-mediated downregulation of c-Myc. The data demonstrated that treatment with MG132 (a proteasome inhibitor) counteracted AZD1152-mediated c-Myc downregulation, indicating that proteasomal degradation of c-Myc was involved in the effect of Aurora B inhibition (Figure 6E). Of particular interest, rescue of c-Myc protein expression by MG132 reversed G2/M arrest (Figure 6F) and restored cell viability (Figure 6G). Moreover, the immunohistochemical staining revealed that expression of p-Aurora B decreased after AZD1152 treatment, leading to a reduction in c-Myc expression in subcutaneous CCA xenografts (Figure 6H). Altogether, these findings demonstrate that Aurora B stabilizes the expression of c-Myc, thus inducing cell cycle progression and promoting CCA cell proliferation.

3.7 | Sequential application of Aurora B inhibitor enhances the sensitivity of CCA cells to gemcitabine

It has been reported that the effectiveness of gemcitabine is limited by the frequent development of drug resistance in CCA.[39] AZD1152 enhances gemcitabine effectiveness in pancreatic cancer, suggesting that Aurora B is associated with gemcitabine resistance.[40] Therefore, we explored whether different treatment strategies of Aurora B inhibitors can increase the effectiveness of combined chemotherapy with gemcitabine. First, the sensitivity of four CCA cell lines to AZD1152 and gemcitabine was determined. These data showed that in contrast to RBE or Huh28 cells, HuCCT1 and TFK-1 cells were more sensitive to AZD1152 but resistant to gemcitabine (Figure 7A). Then, three different treatment strategies were implemented to evaluate the combination efficiency of gemcitabine with Aurora B inhibitors in HuCCT1 and TFK-1 cells (Figure 7B). In Group A, CCA cells were treated with gemcitabine alone for 72 h (Figure 7B, Group A). In the sequential therapy strategy, to establish the inhibitory effect of Aurora B, pretreatment with AZD1152 (Group B) or Aurora B HDO (Group C) was conducted for 24 h before addition of gemcitabine. Compared with gemcitabine treatment alone, sequential application of AZD1152 or Aurora B HDO synergistically improved the antiproliferative effects of gemcitabine in both TFK-1 and HuCCT1 cells (Figure 7C). To further verify the mode of cell death in gemcitabine-resistant HuCCT1 and TFK-1 cells, sequential treatment with AZD1152/Aurora B HDO and gemcitabine was performed, which markedly increased the percentage of apoptotic cells (Figure 7D). Cell cycle analysis also confirmed the varied sensitivity of HuCCT1 and TFK-1 cells to sequential application of AZD1152/Aurora B HDO and gemcitabine, as demonstrated by an increase in polyploid cells with DNA content >4 N due to Aurora B inhibitor pretreatment (Figure 7E). These data imply that polyploid CCA cells in G2/M phase may be more vulnerable to gemcitabine treatment. Clarifying c-Myc function in the combined therapy of AZD1152/Aurora B HDO and gemcitabine, our findings showed that c-Myc levels decreased as cells underwent the synergistic induction and suppression of apoptosis and proliferation (Figure 7F). These data imply that c-Myc can be used as a marker for gemcitabine-resistant CCA cells and that c-Myc downregulation can enhance the response of CCA cells to gemcitabine. Indeed, c-Myc contributes to chemotherapy resistance in several other cancers.[41–43] As expected, in vivo studies also validated these findings in a subcutaneous CCA xenograft model. Compared with gemcitabine treatment alone, the combination strategies showed better therapeutic effects (Figure 7G–I). The immunohistochemical staining showed that the combination therapies resulted in a reduction of p-Aurora B and c-Myc expression in CCA cells (Figure S2D). Altogether, these findings demonstrate that sequential application of Aurora B inhibitors can enhance the activity of gemcitabine in CCA cells.

4 | DISCUSSION

The present study discussed the expression, clinical significance, molecular mechanism, and targeted strategies of Aurora B in CCA. Aurora B is upregulated in CCA tissues compared to adjacent nontumor and normal bile duct tissues. Aurora B upregulation was positively correlated with malignant characteristics and poor prognosis in CCA patients. Inhibiting its kinase activity with AZD1152 or suppressing its expression using Aurora B HDO significantly inhibited the progression of CCA and enhanced its response to gemcitabine. The present data suggests that Aurora B kinase may serve as a potential molecular biomarker and therapeutic target in CCA.

In solid tumors, Aurora B is a well-established therapeutic target. Over the past decade, various Aurora kinase inhibitors have been designed and synthesized.[44,45] AZD1152 is an ATP-competitive inhibitor of Aurora B. As Aurora B is a mitotic checkpoint kinase responsible for proper chromosome segregation and mitosis, numerous studies suggest that AZD1152-induced tumor cell death is primarily achieved through the induction of cell cycle arrest.[46–49] However, the specific mechanisms by which cell cycle arrest induction are diverse. In hepatocellular carcinoma cells, AZD1152 inhibited the Aurora B-mediated phosphorylation of histone H3 at Ser10 and led to an accumulation of DNA content at 4 N/8 N, and cell cycle arrest.[47] In Burkitt's lymphoma and Hodgkin's lymphoma, AZD1152 suppressed survivin binding to the Aurora B kinase and inner centromere protein, ultimately leading to mitotic inhibition.[48] In acute promyelocytic leukemia cells, AZD1152 triggers the functionality of ATM/ATR kinases, inducing DNA damage, and promoting alterations in the cell cycle.[49] In the current study, AZD1152 induced CCA cell cycle arrest by suppressing Aurora B phosphorylation and reducing c-Myc stability. c-Myc degradation was a key factor in AZD1152-induced cell cycle arrest and blocking c-Myc degradation by MG132 can restore cell cycle progression. However, the precise regulatory mechanisms of Aurora B on c-Myc have not been extensively explored. Here we validated that this effect is related to Aurora B kinase activity, and the decrease in c-Myc expression occurs through proteasomal degradation rather than downregulation of mRNA levels.

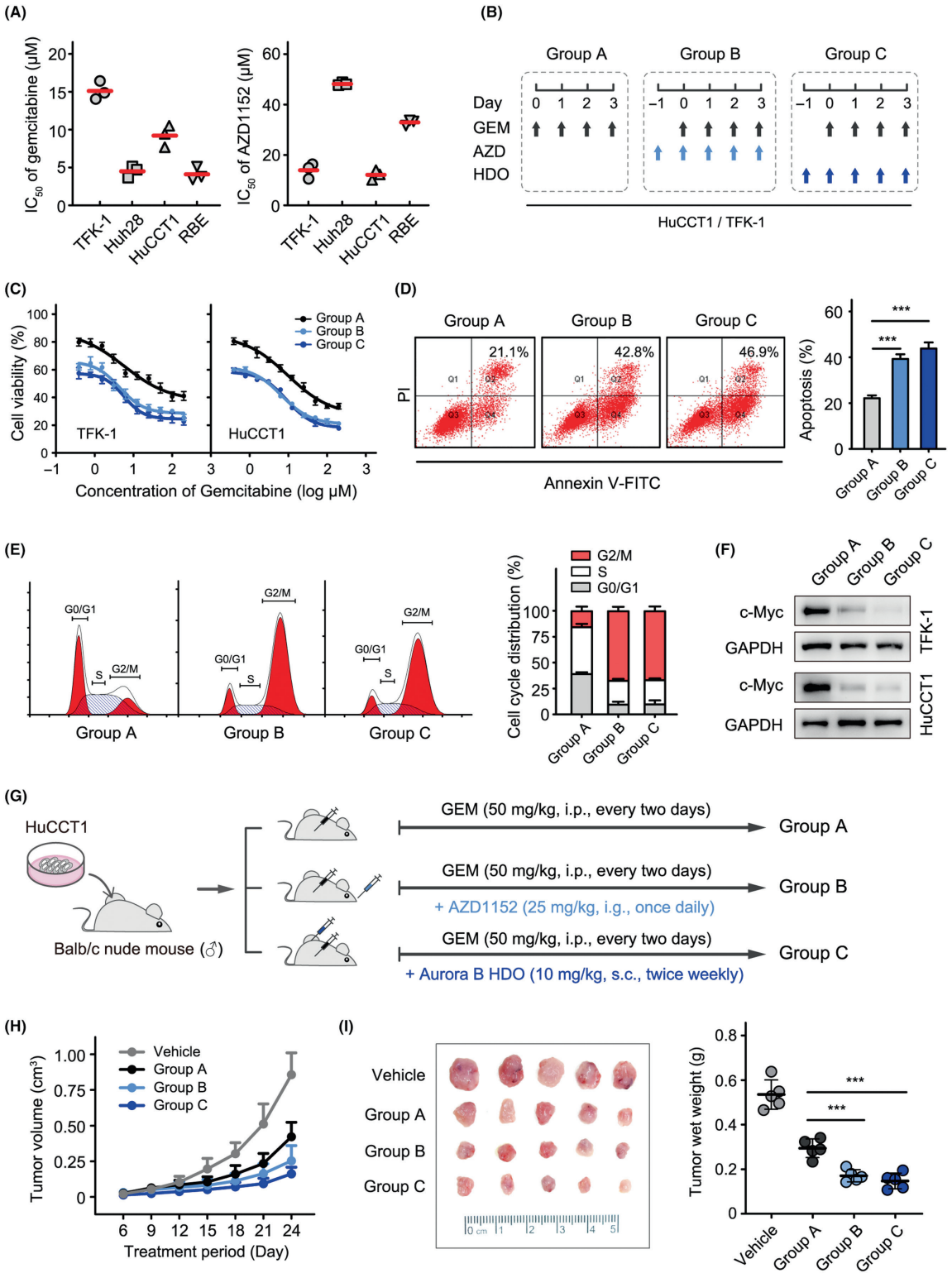

FIGURE 7 Sequential application of AZD1152 or Aurora B HDO enhanced the therapeutic efficiency of gemcitabine in CCA. (A) Cell sensitivity to gemcitabine and AZD1152 was determined by CCK-8 assay. (B) Three different drug combination strategies were designed to test the efficacy of combination therapy in vitro. AZD1152 (5 μM, nontoxic dose) and Aurora B HDO (200 nM, nontoxic dose). (C) The proliferation of TFK-1 cells or HuCCT1 cells under different combination strategies of gemcitabine with AZD1152/Aurora B HDO was determined by CCK-8 assay. (D) Apoptosis was investigated by flow cytometry in CCA cells (HuCCT1) after three different combination strategies. ***$p < 0.001$. (E) Cell cycle analysis was performed in TFK-1 and HuCCT1 cells with different combination strategies. The bar graphs show the percentage of cell cycle distribution. (F) c-Myc expression levels in CCA cells with different combination strategies were detected by Western blot analysis. (G) Tumor volumes of mice with different drug combination strategies in a subcutaneous CCA xenograft model. $n = 5$ for each group. (H) The left panel shows an image of subcutaneous HuCCT1 xenografts, and the right panel shows the final weights of the xenograft tumors. ***$p < 0.001$.

A previous study showed that Aurora B can directly bind to Myc and phosphorylate its Ser67 residue. Phosphorylated c-Myc (Ser67) failed to interact with GSK3β, which impedes the recognition by the E3 ubiquitin ligase FBXW7. This, in turn, enhanced Myc protein stability, further reinforcing its oncogenic functions.[38] At this point, our findings were consistent with such a mechanism. However, whether Aurora B is associated with Ser67 phosphorylation of c-Myc in CCA warrants further exploration. The proteasomal degradation of c-Myc is primarily regulated by phosphorylation at residues Ser67, Ser62, Thr58, and so on.[50] These phosphorylation pathways are typically influenced by cell signaling pathways and regulatory proteins, thereby modulating the stability and activity of c-Myc protein.[51] Does Aurora B, a typical serine/threonine protein kinase, directly regulate these phosphorylation sites on c-Myc? To address this, further investigations using quantitative phosphorylation proteomics can be employed to identify new phosphorylation sites on c-Myc that are directly regulated by Aurora B. Besides direct regulation, Aurora B can also regulate c-Myc protein stability through indirect mechanisms. For example, Aurora B induced phosphorylation of p53 and triggered its ubiquitination-proteasomal degradation, while p53 is a negative regulator of c-Myc expression.[52,53] Therefore, an increase in Aurora B activity can indirectly regulate c-Myc stability by promoting p53 degradation. ~whether Aurora B regulates c-Myc directly or indirectly in CCA still needs further study.

Aurora kinases are often associated with chemotherapy resistance in tumor cells. Studies have indicated that overexpression of Aurora B is linked to drug resistance in breast cancer, lung cancer, and head and neck squamous cell carcinoma.[54–56] This association is also evident in CCA. In this study, sequential treatment with Aurora B inhibitors improves the effectiveness of gemcitabine in CCA treatment, indicating that high expression of Aurora B is associated with gemcitabine resistance in CCA. Anti-tumor effects of gemcitabine depend on the inhibition of DNA synthesis, with the S-phase of cell division being the critical period of action.[57] Conversely, overexpression of Aurora B induces cells to enter the G2/M phase, increasing the frequency of cell mitosis, consequently reducing the duration during which drugs like gemcitabine can act. This diminishes the sensitivity of CCA to gemcitabine treatment. Furthermore, high expression of Aurora B may disrupt the mitotic checkpoint and chromosome segregation, potentially leading to the accumulation of gene mutations that contribute to drug resistance. In summary, abnormal Aurora B expression is closely linked to drug resistance in tumors including CCA. However, further studies are needed to elucidate its mechanisms and potential clinical applications in detail.

This study highlights Aurora B as a potential therapeutic target for CCA. Recently, nucleic acid drugs have gained success in clinical applications due to their specificity and safety profile.[58,59] HDO, a new oligonucleotide, consists of ASOs paired with complementary RNA strands in a double-stranded structure, which enhances its stability in the plasma.[60] By conjugating with cholesterol, HDO can accumulate in the liver, due to liver cells being rich in low-density lipoprotein receptors (LDLRs). Given the anatomical proximity of CCA and liver, Cho-HDO was employed in this study. We used human CCA specimens and animal CCA models to establish that CCA also highly expresses LDLRs and verified the enrichment of Cho-HDO in CCA and its therapeutic effect. Therefore, Aurora B HDO shows advantages over oral AZD1152. It only targets DNA or RNA with complementary sequences, avoiding affecting non-target molecules. It is enriched in tumor lesions while the risk of side effects is reduced as much as possible. However, immunogenicity is a common side effect of nucleic acid drugs.[59] Therefore, the hepatic immune response and other side effects of Aurora B HDO still need further evaluation.

5 | CONCLUSIONS

This research presents the molecular basis for inhibition of Aurora B and suggests that targeting Aurora B could be an effective anticancer strategy against CCA. AZD1152 or Aurora B HDO administration before gemcitabine was proven to be the optimal sequence to maximize the antitumor effects of these chemotherapeutic drugs in CCA. The findings provide new insights into the implementation of sequential treatment in CCA therapy and support the therapeutic effects of targeting Aurora B.

AUTHOR CONTRIBUTIONS

Ke Liu and Fei Huang performed the experiments, organized the data, and wrote the manuscript. Xuxuan Zhou performed the experiments, organized the data, and revised the manuscript. Lihao Liu, Zijian Xu, Chongqing Gao and KeKe Zhang helped to perform the animal studies. Jian Hong helped to design the study and revise the manuscript. Nan Yao and Guohua Cheng designed the study, guided the research, and finalized the manuscript.

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CONFLICT OF INTEREST STATEMENT

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.

ETHICS STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki. The experimental protocol involving human

tissues was approved by the Research and Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (approval no. [2021]678). The experimental protocol involving animal study was approved by the Institutional Animal Ethical Committee, Experimental Animal Center of Jinan University (approval no. 20220222–40).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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