Original Article
Targeting sphingosine kinase 2 suppresses cell growth and synergizes with BCL2/BCL-XL inhibitors through NOXA-mediated MCL1 degradation in cholangiocarcinoma

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Abstract: Sphingosine kinase 2 (SPHK2) is a key factor within sphingolipid metabolism, responsible for the conversion of pro-apoptotic sphingosine to the pro-survival sphingosine-1-phosphate. We have previously shown that ABC294640, a first-in-class SPHK2 inhibitor, inhibits growth of cholangiocarcinoma cells. In a Phase I study of ABC294640 in tumors, the best response was achieved in a cholangiocarcinoma patient. These data suggest SPHK2 as a novel therapeutic target of cholangiocarcinoma. However, the antitumor mechanism of ABC294640 in cholangiocarcinoma remains not clear. In the current study, we found that ABC294640 upregulated expression of pro-apoptotic NOXA. In cholangiocarcinoma patients, high NOXA mRNA expression was associated with better overall survival. Also, SPHK2 mRNA expression was negatively correlated with NOXA mRNA expression. NOXA is known to degrade MCL1, an anti-apoptotic BCL2 protein. We showed that ABC294640 directed MCL1 for proteasome degradation. Knockdown of NOXA prevented ABC294640-induced MCL1 degradation and apoptosis. In addition, ABC294640 had a synergistic effect with BCL2/BCL-XL inhibitors ABT-263 and Obatoclax in inhibiting cell growth. Combined treatment with ABC294640 and BCL2/BCL-XL inhibitors induced potent apoptosis. Silencing of MCL1 also potentiated ABT-263-induced cytotoxicity. Furthermore, we found that both SPHK2 and MCL1 protein expression were significantly higher in cholangiocarcinoma than that in nontumoral bile ducts. SPHK2 expression correlated significantly with MCL1 expression. Our study reveals that ABC294640 inhibits cholangiocarcinoma cell growth and sensitizes the antitumor effect of BCL2/BCL-XL inhibitors through NOXA-mediated MCL1 degradation. Combinations of ABC294640 with BCL2/BCL-XL inhibitors may provide novel strategies for the treatment of cholangiocarcinoma.

Keywords: Cholangiocarcinoma, ABC294640, NOXA, MCL1, ABT-263, obatoclax

Introduction

Cholangiocarcinoma is a heterogeneous disease and is often characterized by its aggressive clinical course and poor prognosis. Most of cholangiocarcinoma patients are diagnosed at advanced stages and are not eligible for potential curative therapies (surgical resection or liver transplantation) [1]. Current standard of care for patients at advanced stages is chemo-therapy, with limited efficacy. Therefore, there is an urgent and unmet need to develop more effective and less toxic treatments with cholangiocarcinoma.

Sphingosine kinase 2 (SPHK2) is a ubiquitously expressed lipid kinase that catalyze the conversion of the pro-apoptotic sphingosine to the pro-survival sphingosine-1-phosphate (S1P) [2]. SPHK2 has recently been implicated in contrib-

uting to neoplastic transformation, tumorigenesis and cancer progression, representing a novel target for cancer therapeutics [3-5]. Recent finding suggests that S1P, probably generated from SPHK2, may contribute to cholangiocarcinoma proliferation and migration [6].

ABC294640 is a first-in-class, orally-administered SPHK2 inhibitor with highly selectivity and low potential off-target inhibition of protein kinases [7]. ABC294640 has shown a good anti-tumor activity in a variety of cancers [4, 8-15]. It has a good oral bioavailability and safety profile in preclinical studies [7]. Based on these strong preclinical profiles, a first-in-human phase I trial with ABC294640 was taken to determine the drug's safety in advanced solid tumors [16]. Of note, one metastatic cholangiocarcinoma patient achieved the best response (sustained partial response, Overall Survival = 20.3 months) [16]. The other two advanced cholangiocarcinoma patients receiving ABC294640 had stable disease (Overall Survival = 17.6 and 16.3 months). We have previous demonstrated that SPHK2 is overexpressed in human cholangiocarcinoma cell lines compared to normal cholangiocytes [17]. In cholangiocarcinoma cells, ABC294640 inhibits proliferation and induces apoptosis [17]. Collectively, both our in vitro study and Phase I clinical study suggests SPHK2 as a potential novel target for the treatment of cholangiocarcinoma. The phase II study of ABC-294640 in the treatment of patients with advanced cholangiocarcinoma is ongoing.

Despite the important roles of SPHK2 in cholangiocarcinoma biology, how SPHK2 regulate cholangiocarcinoma cell proliferation and apoptosis remains poorly understood. Here, we showed that SPHK2 specific inhibitor ABC294640 increased transcription of pro-apoptotic NOXA and degradation of pro-survival BCL2 family molecule MCL1 in four human cholangiocarcinoma cell lines (RBE, HCCC9810, HuH28 and HuCCT1). We demonstrated that NOXA and MCL1 played important roles in the regulation of cholangiocarcinoma cells sensitivity to ABC294640 treatment. Furthermore, ABC294640 synergized with BCL2/BCL-XL inhibitors ABT-263 and Obatoclax in inducing cholangiocarcinoma cell death. Our results provide a rationale for clinical drug studies for this combination therapy in cholangiocarcinoma.

Materials and methods

Materials

ABC294640 was purchased from Selleck (Houston, TX, USA). K145, ABT-263 (Navitoclax) and Obatoclax were purchased from Medchemexpress (Monmouth Junction, NJ, USA). Anti-β-actin primary antibody was purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against human cleaved PARP (#5625), NOXA (#14766), BCL-XL (#2764) and MCL1 (#5453) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against human BCL2 (SC-7382) was from Santa Cruz (Santa Cruz, CA, USA). Immobilon Western Chemiluminescent HRP detection kit was from Millipore (Burlington, MA, USA). The Bromodeoxyuridine (BrdU) ELISA kit was from Roche (Basel, Switzerland). Cell counting kit-8 (CCK-8) was from Dojindo Laboratories (Kyushu, Japan). Caspase-Glo 3/7 assay kit was from Promega (Madison, WI, USA). cDNA Reverse Transcription Kit and SYBR® Green qPCR detection Kit were from TaKaRA (Tokyo, Japan). RNeasy Plus Mini kit was from QIAGEN (Duesseldorf, Germany). Annexin V-FITC Apoptosis Detection kit was from BD Pharmingen (Franklin Lakes, NJ, USA). Lipofectamine RNAiMAX was from Invitrogen (Carlsbad, CA, USA). Cell culture medium was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Biological Industries (Kibbutz Beit Haemek, Israel). ABC-294640 was dissolved in Dimethyl sulfoxide (DMSO) to make a stock solution of 50 mM. K145, ABT-263 and Obatoclax were dissolved in DMSO to make stock solutions of 10 mM.

Cell culture

Four human cholangiocarcinoma cell lines RBE, HCCC9810, HuH28 and HuCCT1 were used. HuH28 and HuCCT1 were provided by Lewis R.Roberts (Mayo Clinic, MN, USA), which were originally obtained from the Japanese Collection of Research Bioresources. RBE and HCCC-9810 were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cell lines were authenticated using short tandem repeat profiling. All cell lines used were cultured in RPMI 1640 with 10% FBS and maintained at 37°C in the presence of 5% CO_2.

BrdU cell proliferation ELISA assay

Cholangiocarcinoma cells were plated in 96-well plates at 3000 cells/well in triplicate. After

Table 1. The sequences of primers for real-time RT-PCR

Gene	Primer sequence	
NOXA (PMAIP1)	Forward	CTGGAAGTCGAGTGTGCTACT
	Reverse	TCAGGTTCCTGAGCAGAAGAG
MCL1	Forward	CATTTCTTTTGGTGCCTTTGTG
	Reverse	CCAGTCCCGTTTTGTCCTTAC
BCL2	Forward	CATGTGTGTGGAGAGCGTCAA
	Reverse	GCCGGTTCAGGTACTCAGTCA
BCL-XL	Forward	GACAAGGAGATGCAGGTATTGG
	Reverse	TCCCGTAGAGATCCACAAAAGT
BIM	Forward	TAAGTTCTGAGTGTGACCGAGA
	Reverse	GCTCTGTCTGTAGGGAGGTAGG
BAD	Forward	CCCAGAGTTTGAGCCGAGTG
	Reverse	CCCATCCCTTCGTCGTCCT
BAX	Forward	CACCAGCTCTGAGCAGATCATGAAG
	Reverse	GCGGCAATCATCCTCTGCAG
BAK	Forward	CCCAGGACACAGAGGAGGTTT
	Reverse	GCCTCCTGTTCCTGCTGATG
BID	Forward	GGAGGAGGACCGGAACAGG
	Reverse	GAAAGACATCACGGAGCAAGGAC
BIK	Forward	TCTGCAATTGTCACCGGTTA
	Reverse	TTGAGCACACCTGCTCCTC
18S	Forward	TTGGAGGGCAAGTCTGGTG
	Reverse	CCGCTCCCAAGATCCAACTA

24 h, drugs were added and cells were incubated for the indicated time. The BrdU ELISA assay was performed according to the manufacturer's instructions.

Cell viability assay

Cell viability was detected by CCK-8 assay. Cells were seeded into 96-well plates at 2000-3000 cells/well in triplicate, cultured overnight then treated with drugs for indicated time. The CCK-8 assay was performed as previously described [18]. For analysis of synergy between ABC294640 with ABT-263 and Obatoclax, cells were exposed to single drugs or different drug combinations for 72 h and cell viability was assessed by the CCK-8 assay. The combination index (CI) was determined using CompuSyn software, based on the median-effect model of Chou-Talalay [19]. A CI < 1 indicates synergism.

Annexin V-FITC apoptosis assay

Cells were seeded in 6-well plates at 10^5 cells/well and treated with varying concentrations of

ABC294640 alone or in combination with ABT-263 and Obatoclax for 72 h. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection kit and performed according to the manufacturer's instructions. Data were analyzed using FlowJo software.

Caspase 3/7 activity assay

Caspase 3/7 activity was analyzed using the Caspase-Glo 3/7 assay kit according to the manufacturer's instructions. 3000 cells were seeded into 96-well white opaque plates and a corresponding optically clear 96-well plate and then allowed to adhere overnight. The next day, cells were treated with varying concentrations of indicated drugs for 48 h. At the end of the incubation time, Caspase-Glo reagent was added to each well. Plates were gently mixed and incubated for 1 h at room temperature. The luminescence was then measured in a GloMax Luminometer (Promega, Madison, WI, USA). The corresponding 96-well clear plate was used to measure the relative number of viable cells with the CCK-8 assay. Caspase 3/7 activity was normalized to viable cell number.

Western immunoblotting

Equivalent amounts of protein were separated on a 4-20% Tris-HCl gel and transferred to PVDF membranes. Membranes were probed with the appropriate primary antibodies. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and signals were visualized using the HRP detection kit. β-actin was used as a loading control. Quantitation of the signal was performed using Image J software.

Real-time qPCR

MRNA was extracted using the RNeasy Plus Mini kit and cDNA was synthesized from 500 ng mRNA using High Capacity cDNA Reverse Transcription Kits according to the manufacturer's instructions. Quantitative real-time PCR was done with the Real-time PCR System (Roche, Basel, Switzerland) using the SYBR qPCR detection Kit. 18S was used as the internal control. The primers used were listed in **Table 1**.

RNA interfering

SiRNA targeting human NOXA (5'-GGGUAUCU-GUAUAGAUAAUTT-3' and 5'-AUUAUCUAUACAG-AUACCCTT-3') and MCL1 (5'-GAUUAUCUCUCG-GUACCUUTT-3' and 5'-AAGGUACCGAGAGAUA-AUCTT-3') were synthesized by GenePharma (Shanghai, China). For gene knockdown experiments, cells were plated in 6-well plates 24 h before transfection and were then transfected with 50 nM of siRNA using Lipofectamine RNAiMAX, as per the manufacturer's protocol. Cells were used at 48 h after transfection for further experiments.

Immunohistochemistry (IHC) assay

The cholangiocarcinoma tissue microarray slides (HBiDC122Su01) were obtained from SHANGHAI OUTDO BIOTECH (Shanghai, China). The microarray was built by SHANGHAI OUTDO BIOTECH using tissues from National Human Genetic Resources Sharing Service Platform (2005DKA21300). The study methodologies conformed to the standards set by the Declaration of Helsinki. IHC was performed to determine the protein expression of SPHK2 and MCL1 in cholangiocarcinoma and nontumoral surrounding intrahepatic bile ducts using the primary antibodies of SPHK2 from Abgent (AP7238b, San Diego, CA, USA) and MCL1 from Abcam (ab32087, Cambridge, MA, USA). Staining intensity was graded as follows: 0 (negative), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The percentage of staining was graded as follows: 0 (no positive cells), 1 (< 25% positive cells), 2 (25%-50% positive cells), 3 (50%-75% positive cells) and 4 (≥ 75% positive cells). The total score was calculated by combining the two parameters. Immunohistochemical staining was analyzed by two pathologists in a blinded manner.

Dataset analysis

Publicly available cholangiocarcinoma dataset GSE26566 was downloaded and used to analyze the correlation of SPHK2 and NOXA [20]. Moreover, publicly available data generated by The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/) was used to perform survival-outcome analyses [21].

Statistical analysis

All statistical tests were conducted with GraphPad Prism 6.0. The half maximal inhibitory concentration (IC_{50}) was calculated using non-linear regression analysis in Prism 6.0. Parametric Student's t test or nonparametric Mann-Whitney test were used to compare two groups. In experiments involving more than two groups, one-way ANOVA with a Turkey *post hoc* test was used. Results were considered statistically significant at $P < 0.05$.

Results

ABC294640 inhibits proliferation and induces apoptosis of RBE and HCCC9810 cells

Previous data from our team showed that ABC-294640 decreases the proliferation of six cholangiocarcinoma cell lines (HuH28, HuCCT1, WITT, EGI-1, OZ and LIV27) [17]. In the current study, we evaluated its effect on two additional cholangiocarcinoma cell lines RBE and HCCC-9810. Cholangiocarcinoma cells were exposed to increasing concentrations of ABC294640 for 72 h and cell proliferation was evaluated by BrdU ELISA assay. ABC294640 dose-dependently inhibited RBE and HCCC9810 cell proliferation with IC_{50} 33.03 μM and 42.49 μM respectively (**Figure 1A**). To characterize ABC294640-induced cytotoxicity, apoptotic cell death was assessed by Annexin V/PI double staining. Decrease in cell viability and increase in apoptosis were observed in both RBE and HCCC9810 cells after 50 μM ABC294640 treatment for 72 h (**Figure 1B** and **1C**), consistent with our previous study using other cholangiocarcinoma cell lines. Collectively, these data further prove that SPHK2 may play a role in the regulation of cholangiocarcinoma proliferation and apoptosis.

ABC294640 induces pro-apoptotic NOXA expression

The BCL2 protein family, which includes both pro-apoptotic and anti-apoptotic proteins, is a major regulator of cell apoptosis [22]. To investigate the underlying molecular mechanism by which SPHK2 regulates cholangiocarcinoma cell survival and apoptosis, we first evaluated the expression of several common genes in the BCL2 family in RBE and HCCC9810 cells, including NOXA, BAX, BAK, BID, BIM, BAD, BIK, MCL1, BCL2 and BCL-XL, using real-time qPCR. We observed significant induction of NOXA (PM-AIP1) mRNA levels when cells were treated by 50 μM ABC294640 for 24 h in both RBE and HCCC9810 cells (**Figure 1D**). Also, ABC294640
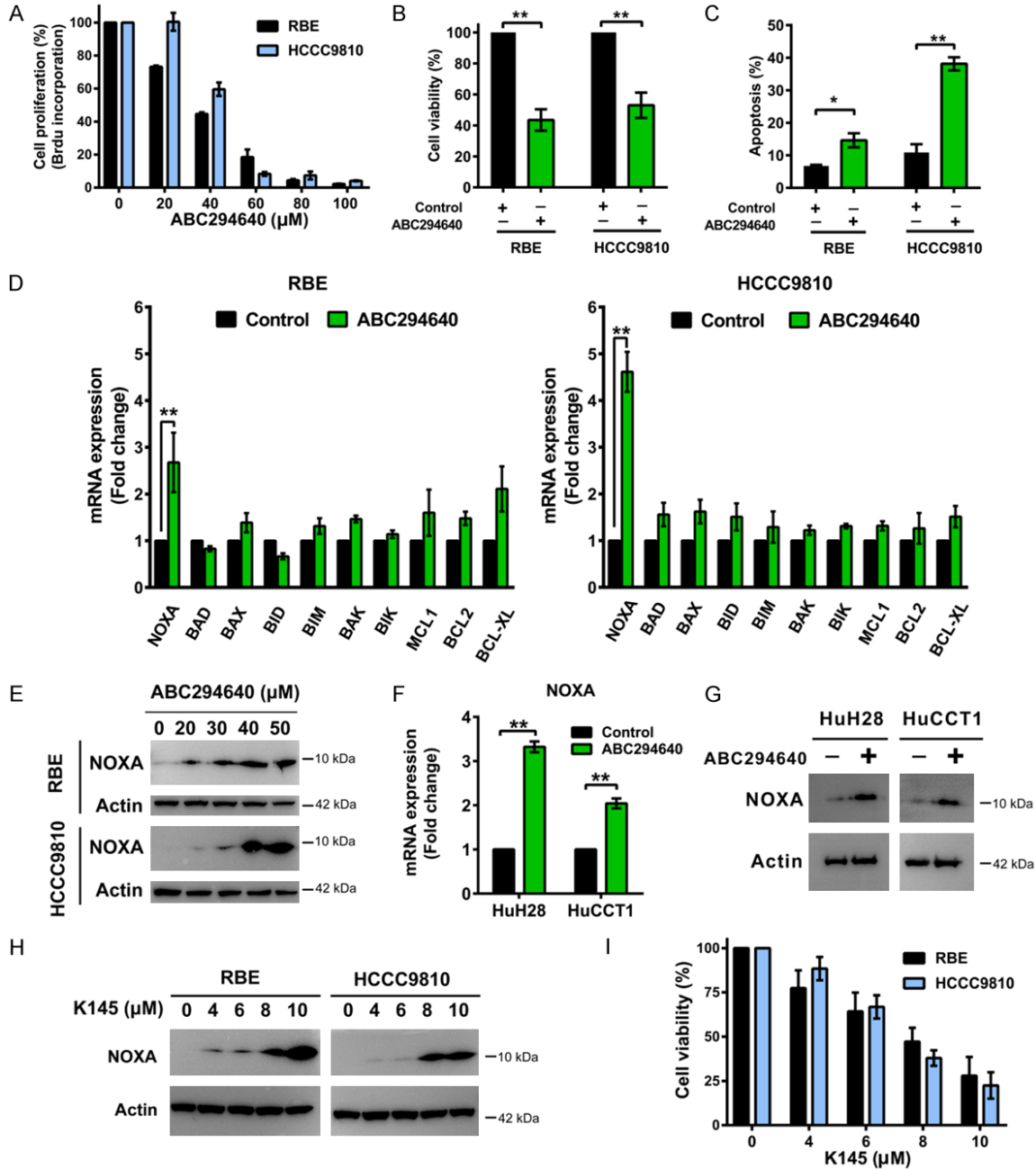
Figure 1. SPHK2 inhibition suppresses cholangiocarcinoma cell growth, induces apoptosis and upregulates NOXA expression. A. RBE and HCCC9810 cells were treated with ABC294640 for 72 h and cell proliferation was quantified by BrdU ELISA assay. B. Cells were treated with ABC294640 at 50 μM for 72 h and cell viability was determined by CCK-8 assay. C. Cells were treated with ABC294640 at 50 μM for 72 h and cell apoptosis was then measured by Annexin V-FITC/PI labeling followed by flow cytometry. D. Real-time qPCR analysis of BCL2 family mRNA level in RBE and HCCC9810 cells treated with 50 μM ABC294640 or no drug control for 24 h. E. Western immunoblotting analysis of NOXA protein levels in RBE and HCCC9810 cells treated with different concentrations of ABC294640 for 24 h. Data shown represents 3 independent experiments. F. Real-time qPCR analysis of NOXA mRNA level in HuH28 and HuCCT1 cells treated with 50 μM ABC294640 for 24 h. G. Western immunoblotting analysis of NOXA protein levels in HuH28 and HuCCT1 cells treated with 50 μM ABC294640 or no drug control for 24 h. Data shown represents 3 independent experiments. H. Western immunoblotting analysis of NOXA protein levels in RBE and HCCC9810 cells treated with different concentrations of K145 for 24 h. Data shown represents 2 independent experiments. I. RBE and HCCC9810 cells were treated with different concentrations of K145 for 72 h and cell viability were determined by CCK-8 assay. Quantitative analysis from 3 independent experiments (Student's t test; data are shown as mean ± SEM; *P < 0.05, **P < 0.01) are shown.

dose-dependently increased the protein level of NOXA in these two cell lines (**Figure 1E**). Up-regulation of NOXA mRNA and protein level by ABC294640 was also observed in the other two cholangiocarcinoma cell lines (HuH28 and HuCCT1) (**Figure 1F** and **1G**). K145 is another recently reported SPHK2 specific inhibitor. It does not inhibit SPHK1 with a concentration up to 10 μM [23]. Likewise, treatment of RBE and HCCC9810 cells with K145 showed a dose-dependent increase in NOXA expression (**Figure 1H**), confirming this NOXA inducing mechanism is mediated through SPHK2. Consistent with its effects on NOXA, K145 blocked cell survival in a dose-dependent manner (**Figure 1I**). Our results showed that SPHK2 inhibition upregulated pro-apoptotic NOXA expression in cholangiocarcinoma cells.

NOXA plays important role in regulation of cholangiocarcinoma cell apoptosis to SPHK2 inhibition

To determine whether NOXA is a critical component in defining the therapeutic efficacy of ABC294640, we functionally silenced NOXA mRNA expression and assessed the response of RBE and HCCC9810 cells to treatment. Silencing of NOXA by RNAi greatly reduced the NOXA mRNA and protein expression with cholangiocarcinoma cells exposed to ABC294640 (**Figure 2A** and **2B**). Further analysis showed that loss of NOXA expression effectively protected cholangiocarcinoma cells from cell death induced by ABC294640 in both RBE and HCCC9810 cells (**Figure 2C-E**). We then determined the clinical relevance of NOXA expression levels to cholangiocarcinoma patient outcome through analyzing the TCGA dataset of cholangiocarcinoma patient samples [21]. Kaplan-Meier survival analysis revealed that patients with high tumor NOXA mRNA expression levels was associated with longer overall survival (P < 0.05) (**Figure 2F**). In addition, SPHK2 mRNA expression is negatively correlated with NOXA mRNA expression in the cholangiocarcinoma dataset GSE-26566 (P < 0.01) [20] (**Figure 2G**). Together, these data suggest a role for NOXA in enabling cytotoxicity after SPHK2 inhibition in cholangiocarcinoma cells.

*Pro-apoptotic effects of SPHK2 inhibition are mediated by MCL1 degradation*

Cholangiocarcinoma cells frequently overexpress the anti-apoptotic BCL2 family member MCL1 to inhibit apoptosis and promote cell survival [24]. Because previous study shows that NOXA can promote intrinsic apoptosis through proteasomal degradation of the anti-apoptotic protein MCL1 [25, 26], we examined the changes in protein levels of MCL1 and along with two other pro-survival BCL2 family members BCL2 and BCL-XL after ABC294640 treatment. After 24 h treatment, ABC294640 dose-dependently reduced protein levels of MCL1 without decreasing the expression of BCL2 and BCL-XL (**Figure 3A** and **3B**). Notably, this loss of MCL1 protein was not associated with the reduction of MCL1 mRNA expression (**Figure 1D**), suggestive of post-transcriptional regulation of MCL1 expression. Instead, we observed that MCL1 mRNA was slightly elevated by ABC294640, which may compensate for its protein loss. We then tested if ABC294640 treatment promoted MCL1 degradation in a proteasome-dependent manner. RBE and HCCC9810 cells were treated with ABC294640 alone, proteasome inhibitor MG132 alone or ABC294640 in combination of MG132 for 12 h. Indeed, MG-132 protected MCL1 from degradation induced by ABC294640 treatment (**Figure 3C**). Similar effects of ABC294640 on MCL1 expression were observed in two other cholangiocarcinoma cell lines (HuH28 and HuCCT1) tested (**Figure 3D** and **3E**). Following knock down of NOXA, MCL1 degradation was in part rescued (**Figure 3F** and **3G**). These data suggested that ABC294640 decreased pro-survival MCL1 expression probably through NOXA mediated proteasome degradation.

ABC294640 synergizes with BCL2/BCL-XL inhibitors ABT-263 and Obatoclax in inducing cholangiocarcinoma cell death

The BH3-mimetic ABT-263, which inhibits the other pro-survival BCL2 family proteins (BCL2, BCL-XL and BCL-W), but not MCL1, shows promise for treating tumors which have low dependency on MCL1 for cell survival [27]. Cholangiocarcinoma cells appear resistant to ABT-263 due to elevated expression of MCL1 [28]. Therefore, we examined whether targeting MCL1 levels via SPHK2 inhibition could sensitize cholangiocarcinoma cells to ABT-263. Indeed, we found that sub-cytotoxic combinations of ABC-294640 and ABT-263 induced synergistic cell death in all three cholangiocarcinoma cells (RBE, HCCC9810 and HuH28) tested (**Figure 4A**, CI < 1). Then, we extended our investigations to another BH3-mimetic Obatoclax. Oba-
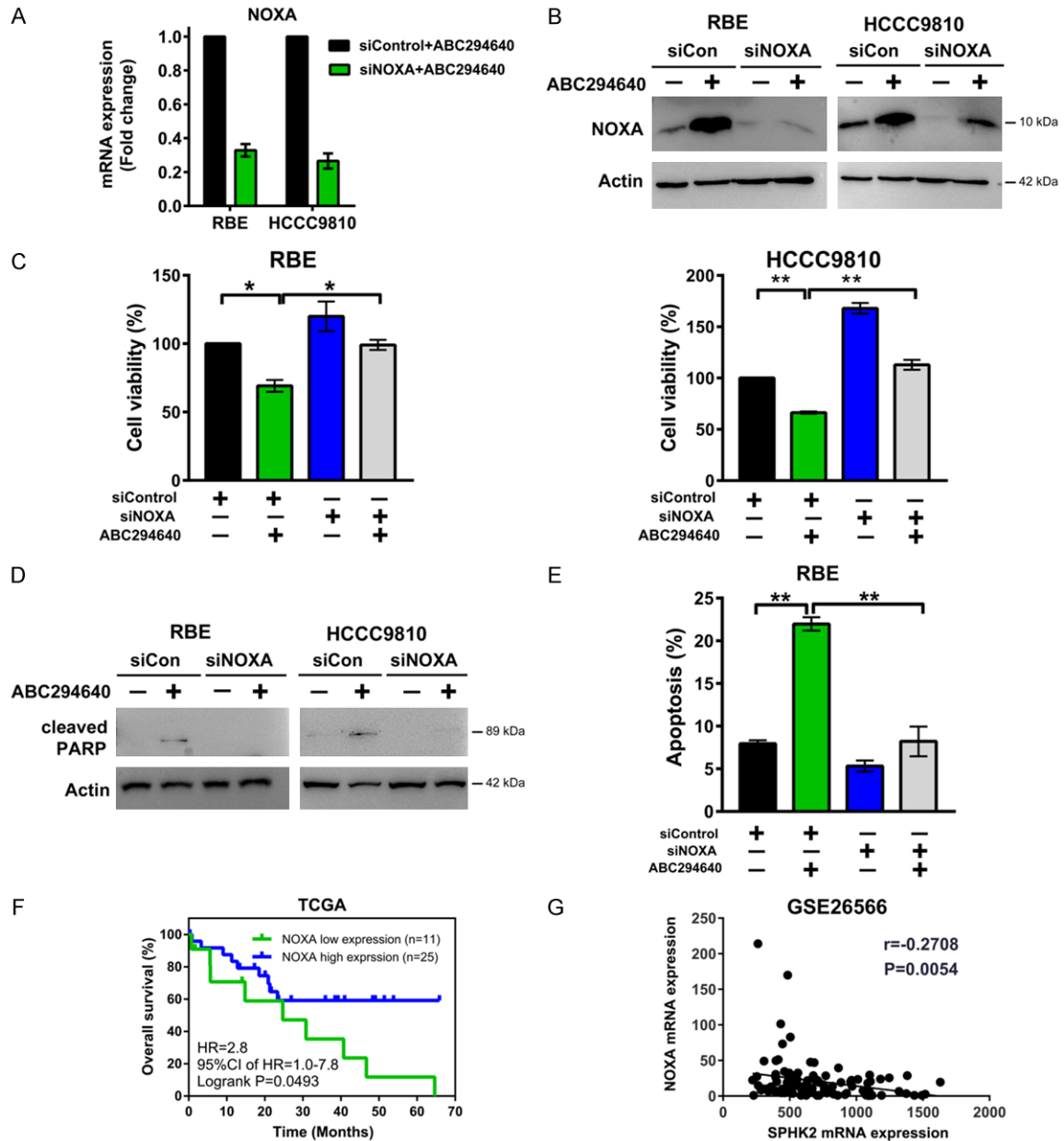
Figure 2. SPHK2 regulates cholangiocarcinoma cell apoptosis via NOXA. A. Cells were transfected with 50 nM Control siRNA (siCon) or NOXA siRNA (siNOXA) for 48 h and then treated with 50 µM ABC294640 for 48 h. NOXA mRNA level was analyzed by Real-time qPCR. B. Cells were transfected with 50 nM Control siRNA (siCon) or NOXA siRNA (siNOXA) for 48 h and then treated with 50 µM ABC294640 for 24 h. NOXA protein level was analyzed by Western immunoblotting. C-E. Cells were transfected with 50 nM Control siRNA (siCon) or NOXA siRNA (siNOXA) for 48 h and then treated with 50 µM ABC294640 for 72 h. Cell viability was determined by CCK-8 assay. Apoptosis was analyzed by PARP cleavage through Western immunoblotting in both cell lines and by Annexin V-FITC/PI labeling followed by flow cytometry in RBE cells. F. Kaplan-Meier curves showing the overall survival rate of 36 patients in the TCGA dataset according to the expression status of NOXA. G. Correlation between SPHK2 mRNA expression and NOXA mRNA expression in a cholangiocarcinoma dataset (GSE26566). The r and *P* values were determined by Pearson correlation analysis. Quantitative analysis from 3 independent experiments (one-way ANOVA with a Turkey post hoc test; data are shown as mean ± SEM; *P < 0.05, **P < 0.01) are shown.

toclax differs from ABT-263 in that in addition to targeting BCL2/BCL-XL/BCL-W, it also targets MCL1 and Bfl-1. Similar to ABT-263, obvi-ous synergism was observed between Obato-clax and ABC294640 (**Figure 4B**, CI < 1). Apop-tosis induction by ABT-263 and Obatoclax was
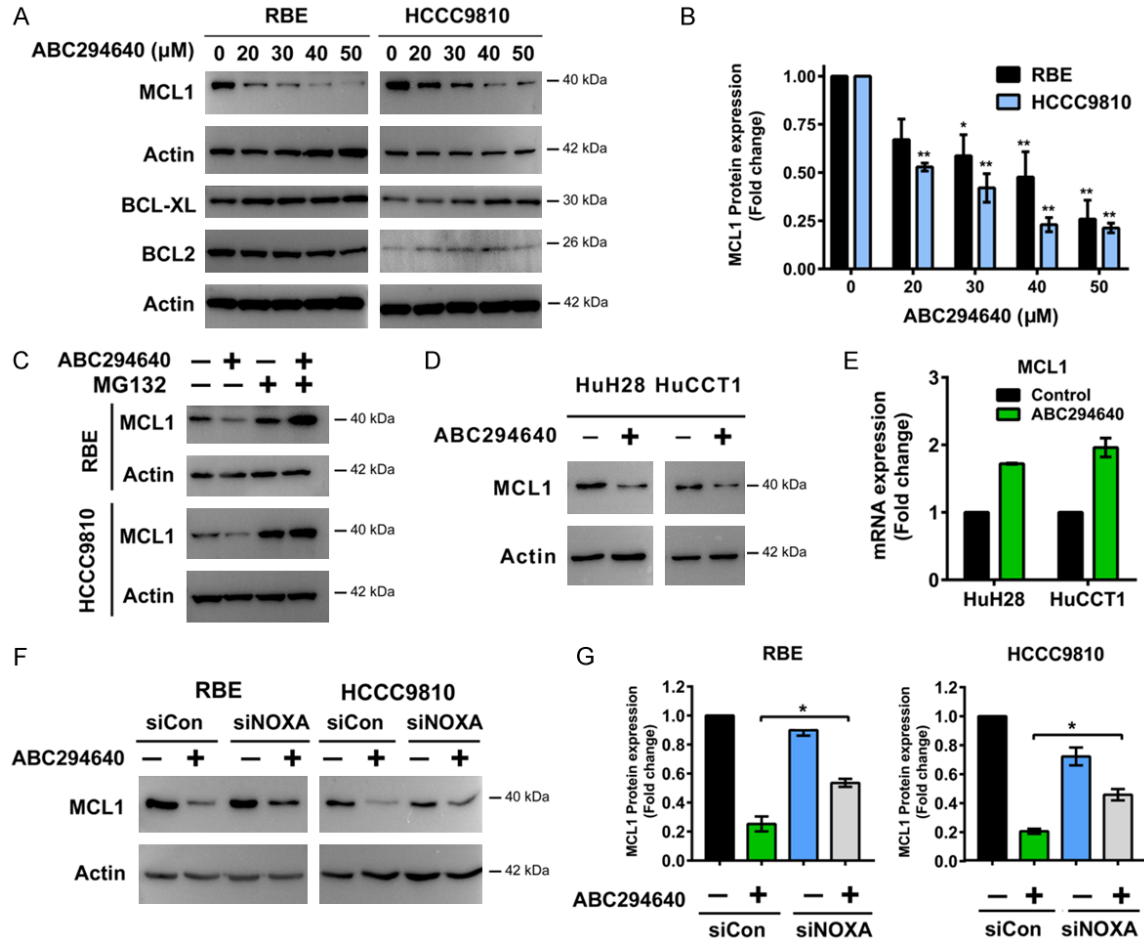
Figure 3. ABC294640 induces degradation of pro-survival protein MCL1. A, B. Western immunoblotting analysis of MCL1, BCL-XL and BCL2 protein levels in RBE and HCCC9810 cells treated with different concentrations of ABC294640 for 24 h. MCL1 protein level following ABC294640 treatment for 24 h was quantified using Image J. Quantitative analysis from three independent experiments (one-way ANOVA with a Turkey post hoc test; data are shown as mean ± SEM; *P < 0.05, **P < 0.01) are shown. C. RBE and HCCC9810 cells treated with no drug control buffer or Proteasome inhibitor MG132 (200 nM) for 2 h, followed by treatment with 50 μM of ABC294640 for additional 12 h. Whole cell lysate was prepared and analyzed for MCL1 expression by Western immunoblotting. Result shown represents 2-3 independent experiments. D. Western immunoblotting analysis of MCL1 protein levels in HuH28 and HuCCT1 cells treated with 50 μM of ABC294640 for 24 h. Result shown represents 3 independent experiments. E. Real-time qPCR analysis of MCL1 mRNA levels in HuH28 and HuCCT1 cells treated with 50 μM of ABC294640 for 24 h. Data are shown as mean ± SEM from 3 independent results. F. Cells were transfected with 50 nM Control siRNA (siCon) or NOXA siRNA (siNOXA) for 48 h and then treated with 50 μM ABC294640 for 24 h. MCL1 protein level was analyzed by Western immunoblotting. Result shown represents 2-3 independent experiments. G. MCL1 protein level of previous experiment was quantified using Image J. Quantitative analysis from 2-3 independent experiments (one-way ANOVA with a Turkey post hoc test; data are shown as mean ± SEM; *P < 0.05) are shown.

also markedly enhanced by ABC294640 in all three cholangiocarcinoma cells assessed by Annexin V/PI staining assay (**Figure 5A** and **5B**). Additionally, combined inhibition of SPHK2 and BCL2/BCL-XL induced a strong increase in Caspase 3/7 activity (**Figure 5C**). Taken together, ABC294640 acted synergistically with ABT-263 and Obatoclax in reducing cell viability and inducing apoptosis in cholangiocarcinoma cells.

Silencing of MCL1 enhances the antitumor effects of ABT-263

We knocked down MCL1 by specific siRNA and observed that silencing MCL1 potently sensitized RBE and HCCC9810 cells to the cytotoxic effects of ABT-263 (**Figure 6A** and **6B**), proving that MCL1 levels are key regulators in ABT-263 mediated apoptosis and ABC294640 may sen-
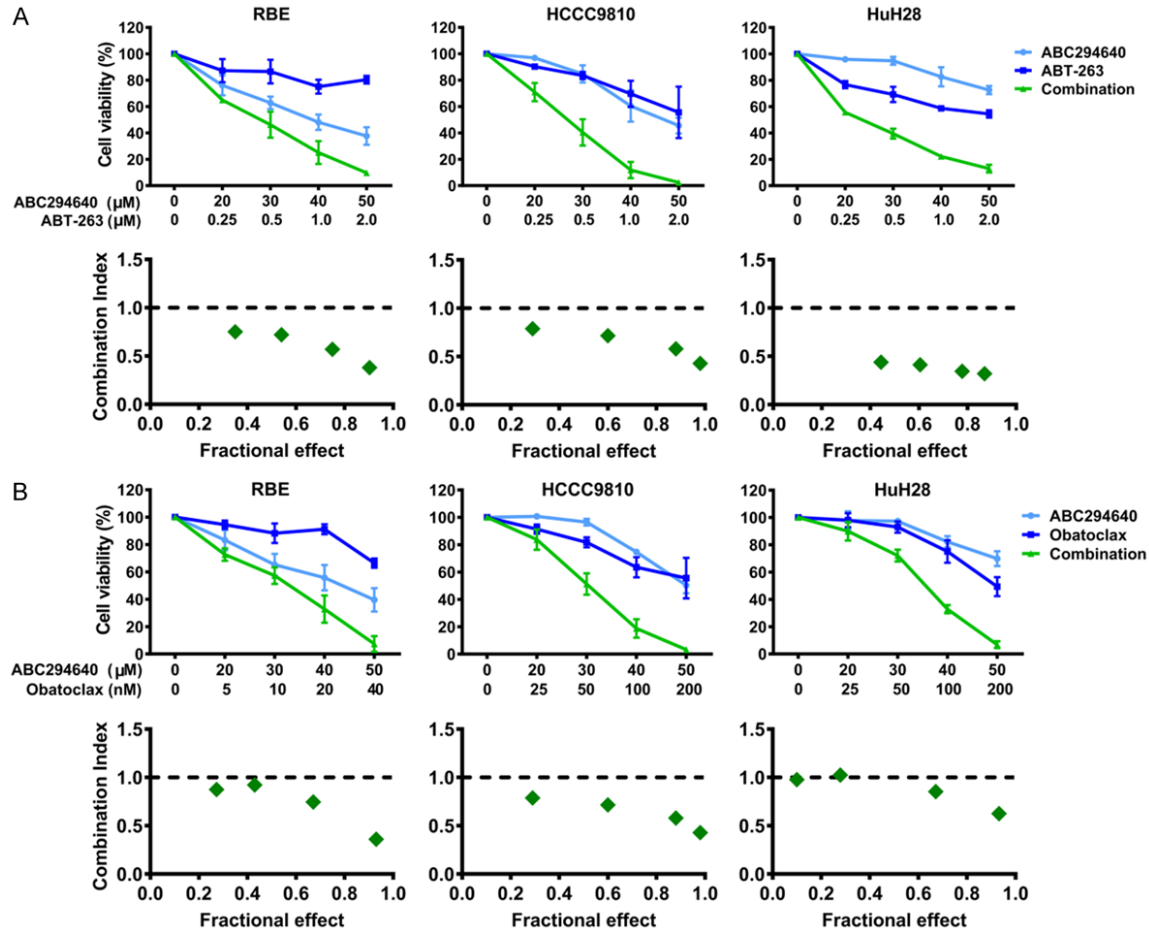
Figure 4. ABC294640 acts synergistically with BH3-mimetics in inhibiting cholangiocarcinoma cell growth. A. RBE, HCCC9810 and HuH28 cells were treated with various concentrations of ABC294640 or ABT-263 alone or their combination for 72 h, then the cell viability was analyzed by CCK-8 assay. The results are presented as mean ± SEM from 3-4 independent experiments. The combination index (CI) was determined using the Chou-Talalay Method. CI < 1 indicates that the interaction between ABC294640 and ABT-263 was synergistic. B. RBE, HCCC9810 and HuH28 cells were treated with various concentrations of ABC294640 or Obatoclax alone or their combination for 72 h, then the cell viability was analyzed by CCK-8 assay. The results are presented as mean ± SEM from 3-4 independent experiments. CI was determined using the Chou-Talalay Method. CI < 1 indicates that the interaction between ABC294640 and Obatoclax was synergistic.

sitize cholangiocarcinoma cells to BCL2/BCL-XL inhibitors mainly through inhibition of MCL1.

SPHK2 and MCL1 are overexpressed in cholangiocarcinoma and has a positive correlation

We have done immunohistochemistry analysis of SPHK2 and MCL1 in cholangiocarcinoma tissue microarray slides. We found that both SPHK2 and MCL1 protein expression were significantly higher in cholangiocarcinoma than that in nontumoral bile ducts (**Figure 7A-D**). Additionally, SPHK2 expression had a significant correlation with MCL1 expression in the cholangiocarcinoma tissue microarray (r = 0.4218, P < 0.0001) (**Figure 7E**).

Discussion

S1P has been proposed to contribute to tumor proliferation, migration and angiogenesis in a number of cancers including cholangiocarcinoma. As S1P is converted by SPHK1 and SPHK2, both two isoforms can be therapeutic target in cholangiocarcinoma. SK1-I is the specific inhibitor of SPHK1 [29]. Chen et al demonstrated that SK1-I has potent antiproliferative activity in cholangiocarcinoma in vitro and in vivo [30]. The findings of this study and our study suggest that both SPHK1 and SPHK2 have important roles in inducing cholangiocarcinoma cell survival. Liu at al analyzed mRNA levels of SPHK1 and SPHK2 in rat and human cholangiocarci-
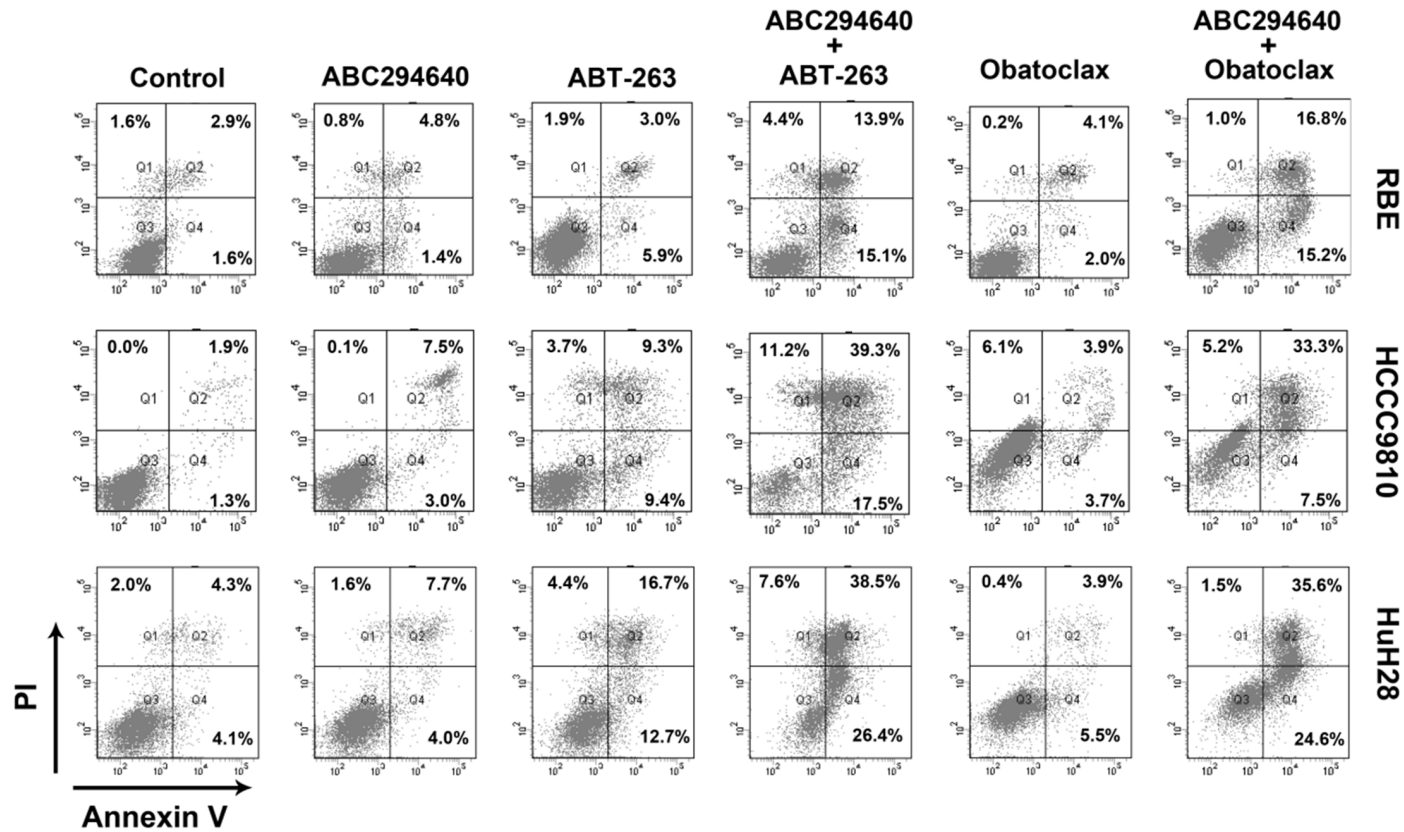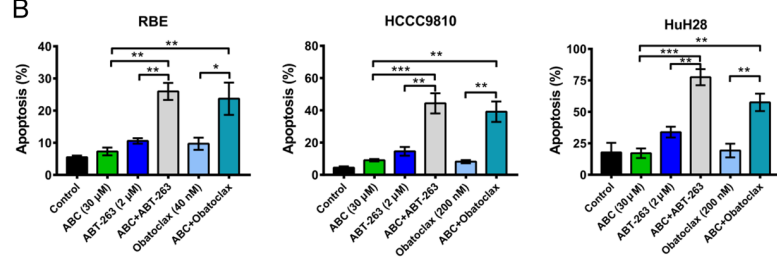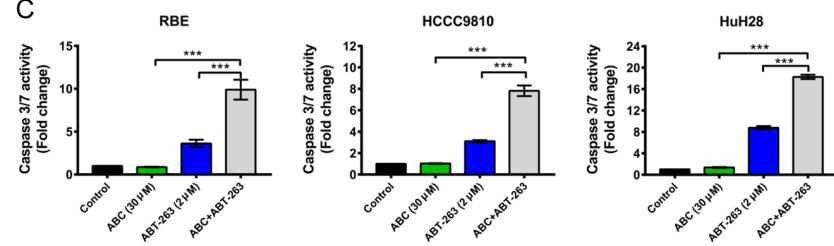
Figure 5. ABC294640 potentiates apoptosis induced by BH3-mimetics in cholangiocarcinoma cells. A, B. RBE, HCCC9810 and HuH28 cells were treated with ABC294640 (ABC) alone or in combination with ABT-263 or Obatoclax at indicated concentrations for 72 h. Cell apoptosis was measured by Annexin V-FITC/PI staining followed by flow cytometry. C. RBE, HCCC9810 and HuH28 cells were treated with ABC294640 alone or in combination with ABT-263 at indicated concentration for 48 h. Cell apoptosis was determined by the Caspase 3/7 activity assay. Quantitative analysis from 3 independent experiments (one-way ANOVA with a Turkey post hoc test; data are shown as mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) are shown.
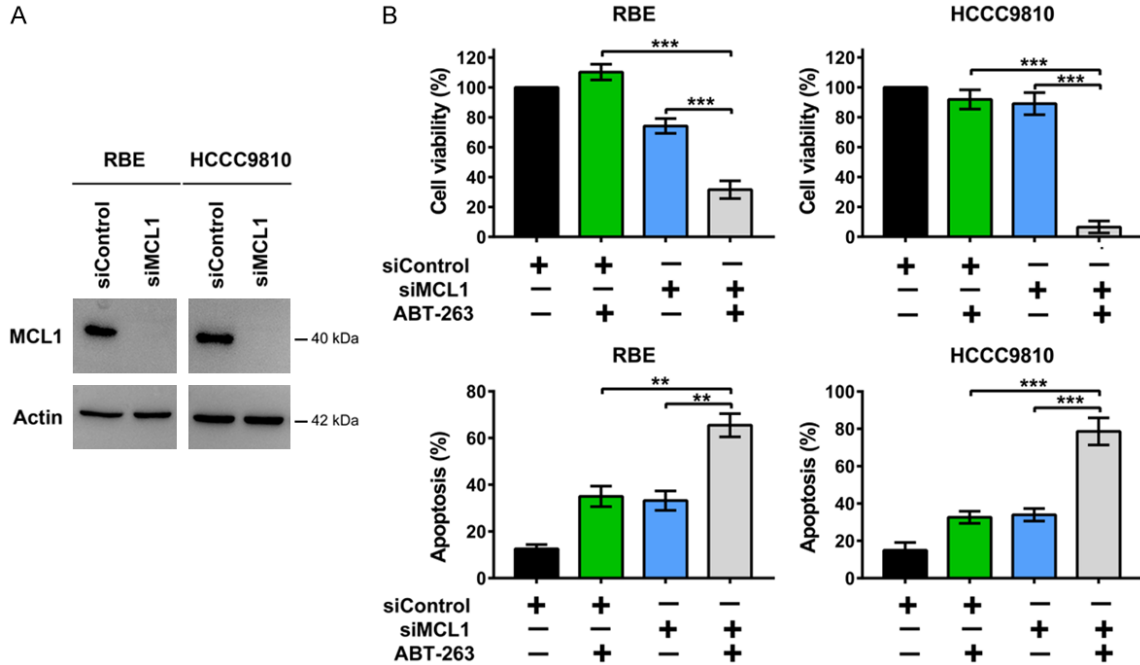
Figure 6. Silencing of MCL1 enhances the antitumor effects of ABT-263. A. Western immunoblotting analysis of MCL1 protein levels in RBE and HCCC9810 cells transfected with siRNA for 48 h. Data shown represents 2 independent experiments. B. RBE and HCCC9810 cells were transfected with control siRNA or MCL1 siRNA for 48 h and then treated with ABT-263 at indicated concentrations for 48 h. Cell viability was determined by CCK-8 assay. Cell apoptosis was measured by Annexin V-FITC/PI staining followed by flow cytometry. Quantitative analysis from 3 independent experiments (one-way ANOVA with a Turkey post hoc test; data are shown as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001) are shown.

noma cell lines and found that SPHK2 mRNA expression being predominant over that of SPHK1 in the cholangiocarcinoma cells, suggesting SPHK2 may play a relatively more important role than SPHK1 in cholangiocarcinoma [6]. However, SPHK1 inhibitors will provide additional opportunities to discover effective therapies in cholangiocarcinoma.

We have previously shown that SPHK2 is overexpressed in cholangiocarcinoma cell lines and its inhibition suppresses cholangiocarcinoma cell proliferation and survival [17]. In this study, we provide new mechanistic insights regarding how SPHK2 regulate cholangiocarcinoma cell survival. These data indicate that (1) SPHK2 negatively regulates pro-apoptotic NOXA mRNA expression; (2) SPHK2 promotes MCL1 stabilization probably through NOXA inhibition, providing resistance to cell death induced by BH3-mimetics ABT-263 and Obatoclax. These findings are summarized and illustrated in **Figure 7F** and discussed in greater detail below.

ABC294640 is a competitive inhibitor of SPHK2 with respect to sphingosine, which has minimal off-target effects. In vitro, it does not inhibit SPHK1 at concentration up to at least 100 µM [7]. ABC294640 depletes S1P, elevates ceramide and suppresses ERK and AKT proliferative signaling in tumor cells [10, 31]. Recently, ABC294640 has also been shown to down-regulate oncogenic c-Myc expression in a variety of tumor cell lines through both transcriptional and post-transcriptional mechanisms [4, 9, 10, 14].

Here, we identified selective induction of pro-apoptotic NOXA mRNA expression after SPHK2 inhibition in cholangiocarcinoma cell lines. NOXA knockdown afforded significant protection from ABC294640-induced apoptosis, demonstrating the critical role of NOXA in the cytotoxicity of ABC294640. Furthermore, high NOXA mRNA expression within cholangiocarcinoma tumors correlates with longer overall survival in the TCGA dataset. An inverse correlation was also observed between SPHK2 and NOXA mRNA expression in cholangiocarcinoma patients. These data suggest NOXA may be a critical downstream target of SPHK2.
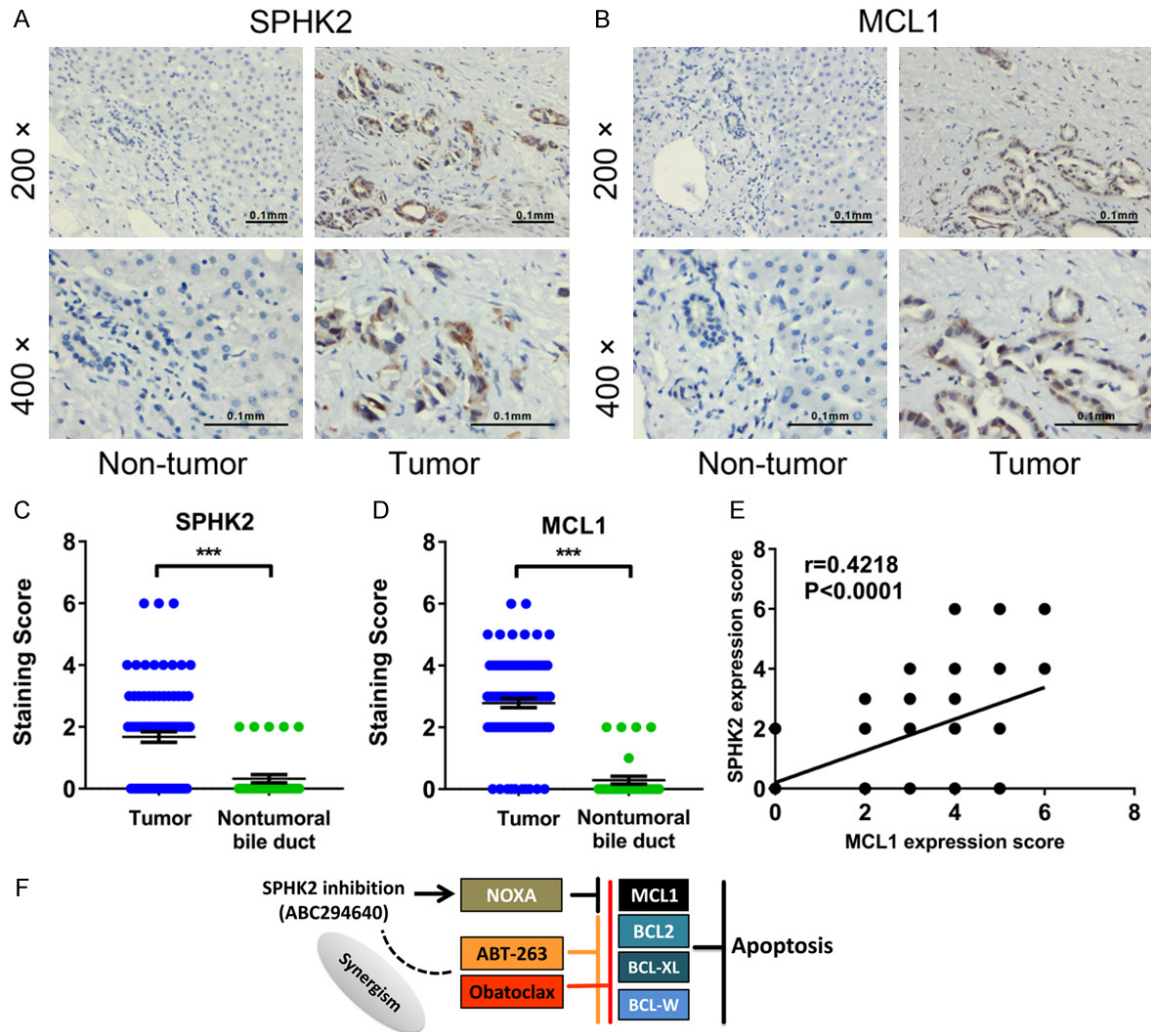
Figure 7. SPHK2 and MCL1 are overexpressed in cholangiocarcinoma and has a positive correlation. A, B. Immunohistochemical staining with anti-SPHK2 and anti-MCL1 antibodies were performed on 89 human cholangiocarcinoma tissues and 31 adjacent non-tumor tissues. Representative images were shown (200× and 400×). C, D. Semi-quantitative analysis of immunohistochemical staining showed that SPHK2 and MCL1 protein expression were upregulated in cholangiocarcinoma in the tissue microarray. Mann-Whitney test were used to compare two groups. ***P < 0.001. E. The expression level of SPHK2 showed a significant positive correlation with MCL1 expression in the tissue microarray. The r and *P* values were determined by Spearman correlation analysis. F. Schematic representation of the mechanisms of action of ABC294640 in cholangiocarcinoma cells.

Pro-survival BCL2 family protein MCL1 is the well-known target of NOXA. Previous studies have demonstrated that when NOXA is expressed, it is localized at the mitochondria via its mitochondrial targeting domain [25]. Then MCL1 is recruited from the cytosol to the mitochondria by binding to the BH3 domain of NOXA, which initiates MCL1 phosphorylation and subsequent ubiquitination triggering proteasome-mediated degradation [25, 32]. Consistent with it, we subsequently found that SPHK2 inhibition in cholangiocarcinoma cells

resulted in loss of MCL1 through proteasome-dependent degradation, whereas BCL2 and BCL-XL expression were unaffected. Many studies have shown that MCL1 has a critical role in a variety of hematological malignancies and solid tumors including cholangiocarcinoma [33, 34]. In particular, MCL1 has been implicated in drug resistance in cholangiocarcinoma [24, 35]. In addition, MCL1 is frequently amplified in cholangiocarcinoma [36, 37]. We and others previously have shown that MCL1 is critical for cholangiocarcinoma survival [18, 24]. In the

current study, we found that MCL1 was overexpressed in cholangiocarcinoma. Thus, our new finding is of particular interest.

Targeting anti-apoptotic proteins BCL2, BCL-XL and BCL-W with BH3-mimetics such as ABT-263 represents one of the most promising antitumor drugs [27]. However, ABT-263 has shown limited success against cancers with high MCL1 expression [28, 38]. Obatoclax, another BH3-mimetic, which inhibits MCL1 in addition to BCL2/BCL-XL/BCL-W, has been limited by its neurologic toxicity [27]. Therefore, many groups have developed combinational therapies whereby BCL2/BCL-XL inhibitors were combined with inhibitors of MCL1. Targeting these cooperative survival signaling pathways may overcome drug resistance, improve therapeutic efficacy and reduce side effects [25, 39-42]. Previous study using short hairpin RNA-targeted knockdown of MCL1 sensitized KMCH cholangiocarcinoma cells to ABT-263 mediated apoptosis [28]. Therefore, we further explored whether targeting SPHK2 would be similarly effective in restoring cholangiocarcinoma cell susceptibility to BCL2/BCL-XL inhibitors. Indeed, ABC294640 synergized with BH3-mimetics ABT-263 and Obatoclax in inhibiting cell survival and inducing cell apoptosis in three cholangiocarcinoma cell lines. As a first-in-class SPHK2 inhibitor, ABC294640 shows promise against cholangiocarcinoma in both in vitro experiments and first-in-human clinical trial. As we demonstrated that targeting SPHK2 with ABC294640 and BCL2/BCL-XL inhibitors induced synergistic lethality in cholangiocarcinoma, these rational combinational therapies may prove more efficacious for the treatment of cholangiocarcinoma.

S63845 is a specific MCL1 inhibitor [34]. S63845 has been shown to have combination effect with BCL-2 inhibitor ABT-199 or BCL-2/BCL-XL inhibitor ABT-263 in different cancer models [43, 44]. Thus, S63845 may also have synergistic effect with BCL2/BCL-XL inhibitors in cholangiocarcinoma. However, ABC294640 also provide an apoptotic stimulus through inhibition AKT, STAT3 or other survival signaling pathways. Therefore, ABC294640 may be superior to MCL1 inhibitors in the combination therapy. In any case, the availability of MCL1 inhibitors will provide new opportunities to discover efficacious combinations that may be effective in cholangiocarcinoma.

Our results suggest that SPHK2 may regulate cholangiocarcinoma cell survival through inhibition of NOXA expression and MCL1 proteasomal degradation. Our data also provide preliminary insight into the possible use of SPHK2 inhibitor ABC294640 in combination with BCL2/BCL-XL inhibitors such as ABT-263 and Obatoclax to improve the treatment of cholangiocarcinoma.

Disclosure of conflict of interest

None.

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