Original Article

DAPT suppresses proliferation and migration of hepatocellular carcinoma by regulating the extracellular matrix and inhibiting the Hes1/PTEN/AKT/mTOR signaling pathway

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Background: The aim of the present study was to investigate the antitumor properties of N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) against hepatocellular carcinoma (HCC), as well as the underlying mechanism.

Methods: Immunohistochemistry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay were used to determine the expression of Notch1 in HCC tissues. The expression of Notch1 in 3 HCC cell lines was evaluated by qRT-PCR and Western blot. The proliferation ability of cells was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays. Flow cytometry and Transwell assay were used to check the apoptosis and migration of HepG2 cells, respectively. Western blot was used to determine the expression level of Notch1, Hes1, Phosphatase and tensin homolog (PTEN), protein kinase B1 (AKT1), phosphorylated AKT1, mammalian target of rapamycin (mTOR), phosphorylated mTOR, intracellular adhesion molecule-1, vascular cell adhesion protein 1, matrix metalloproteinase (MMP)-2, MMP-9, and focal adhesion kinase in cells and tumor tissues. A HepG2 xenograft experiment was conducted to evaluate the *in vivo* antitumor properties of DAPT.

Results: Notch1 was found to be significantly upregulated in both HCC tissues and cell lines. DAPT significantly inhibited the proliferation and migration of HepG2 cells in a dose-dependent manner, accompanied by the suppression of Notch1/Hes1 signaling, inactivation of AKT/mTOR signaling, downregulation of MMPs, and decreased expression of adhesion molecules. The activation of Notch1/Hes1 or AKT/mTOR signaling removed the inhibitory effect of DAPT on the proliferation and migration of HepG2 cells, as well as the inhibitory properties of DAPT on the expression of MMPs and adhesion molecules. The antitumor properties and regulatory effect of DAPT against the extracellular matrix (ECM) and Hes1/PTEN/AKT/mTOR signaling were verified by the HepG2 xenograft experiments.

Conclusions: DAPT could suppress the proliferation and migration of HCC by regulating the ECM and inhibiting the Hes1/PTEN/AKT/mTOR signaling pathway.

Keywords: Notch1; hepatocellular carcinoma; protein kinase B; mammalian target of rapamycin; N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors (1). The overall survival rate of HCC patients is relatively low. In addition, in China, the survival rate during the early stage of HCC has been reported to be around 60%, while the 5-year survival rate is approximately 33% (2). Global oncology epidemiological investigations indicate that approximately 0.7 million patients are diagnosed with HCC annually, and 0.65 million patients die from HCC every year, with half from China (3). Health statistics from China's Ministry of Health indicate that HCC has a severe impact on public health owing to its secondary death rate in all types of malignant tumors (4). Therefore, it is important to investigate the pathological mechanism of HCC and develop effective therapies for its treatment.

Several molecular signaling pathways have been reported to be involved in the development and progression of HCC, including the mitogen-activated protein kinase (MAPK), Phosphatase and tensin homolog (PTEN)/mammalian target of rapamycin (mTOR)/protein kinase B (AKT), and Notch signaling pathways (5). The Notch signaling pathway is an important signaling cassette that exists in multiple species, and consists of the membrane ligand, receptor, and downstream molecules (6). The abnormal activation of the Notch signaling pathway has been reported to be closely related to the pathogenesis of HCC, in which Notch1 is a vital regulator that is claimed to be involved in epithelial–mesenchymal transition, embryo development, cellular proliferation and differentiation, and the repair of impaired organs, as well as the pathogenesis of malignant tumors (7). In HCC tissues, Notch1 is found to be significantly upregulated (8). Further functional investigations indicate that the expression of cell circle-related proteins, such as cyclin A, E2, and D1, can be significantly suppressed by Notch1 (9). Increasing evidence has suggested that Notch1 facilitates the development of HCC. Zhang *et al.* reported that Notch1, 3, and 4 were found to be highly expressed in HCC tissues, which played an important role in the prognosis of HCC (10). Villanueva *et al.* reported that the Notch signaling is activated in human HCC and contributes to tumor formation in animal models (11). In addition, the suppression of the Notch1 signaling pathway has been reported to be involved in the mechanism of multiple anti-HCC molecules, such as ICBP90, LINC00261, and salidroside (12-14). Therefore, blocking the Notch1 signaling pathway might be an effective method for the treatment of HCC. N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) is an inhibitor of Notch1 that is widely used for the verification of the involvement of the Notch1 signaling pathway as a blocker (15,16). DAPT is an inhibitor of γ-Secretase and inhibits the segmentation processing of S3 domain in Notch, which release the Notch intracellular domain to further activate the transcription of downstream genes. DAPT has been widely used for the inhibition of Notch pathway (doi: 10.1002/jnr.21368). Although the antitumor property of DAPT in hepatocellular carcinoma has already been reported (30309512), the underlying mechanism have not been illustrated clearly. Therefore, in the present study, we explored the potential therapeutic properties of DAPT against HCC by investigating regulation of DAPT on Hes1/PTEN/AKT/mTOR signaling pathway. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/jgo-21-235).

Methods

Tissues, cells, animals, and treatments

Ten pairs of HCC tissues and para-carcinoma tissues were obtained from Ningbo Medical Center Lihuili Hospital (Ningbo, China), All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee Board of Ningbo Medical Center Lihuili Hospital (NO: KY2020PJ190). Because of the retrospective nature of the study, the requirement for informed consent was waived. HCC cell lines, including HepG2, MHCC97H, and HCCLM3, as well as the normal hepatocyte L-02, were purchased from American Type Culture Collection (Manassas, VA, USA), which were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum and 0.1% penicillin and streptomycin at 37 ℃. Fifteen BABL/c nude mice (T cell defect, 6–8 weeks old, 18–23 g) were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China) for the *in vivo* xenograft experiment, the experimental unit of which was a single animal. The animals had access to food and water during the experiments, Experiments were performed under a project license (NO: 2019-381) granted by the Animal Ethics committee of Ningbo University School of Medicine, in compliance with institutional

Table 1 Sequences of primers for Notch1 and GAPDH

Primer name	Primer sequence (5'-3')	Primer length (bp)
Notch1 forward	GAGGCGTGGCAGACTATG	18
Notch1 reverse	CTTGTACTCCGTCAGCGTG	19
GAPDH forward	CAATGACCCCTTCATTGACC	20
GAPDH reverse	GAGAAGCTTCCCGTTCTCAG	20

guidelines for the care and use of animals. The chemical molecules (DAPT and MHY-1485) were purchased from MedChemExpress (Monmouth Junction, NJ, USA).

Immunohistochemical (ICH) assay

ICH assay was performed on tissues. The tissues were fixed with 10% neutral buffered formalin solution (Bio-Optica, Milan, Italy), and were then embedded with paraffin to obtain 4-μm-thick sections, which were airdried overnight and fixed in acetone for 10 min. After airdrying for another 20 min, the sections were incubated with 0.2% galantine (Sigma, San Francisco, CA, USA) and 0.2% Triton X-100 in phosphate-buffered saline (PBS), and further blocked with an antibody diluent (Dako, Copenhagen, Denmark) for 1 h at room temperature. Subsequently, the slides were deparaffinized and stained with primary antibody against Notch1 (1:100; Cell Signaling Technology, Boston, MA, USA) at 4℃ overnight. After washing using PBS buffer, secondary antibodies (1:100; Cell Signaling Technology, USA) were added, and the sections were incubated for 1 h with Hoechst 33342 (Sigma, USA) at room temperature. After incubation, the sections were analyzed with LSM710 confocal microscopes (Zeiss, Oberkochen, Germany).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the cells and tissues, and the RNAs were further transcribing into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), according to manufacturer's instructions. Subsequently, cDNA was applied for the quantitative real-time PCR in a 20-μL PCR reaction system containing 10 μL 2× Power SYBR Green Master Mix (Invitrogen, USA). GAPDH was used to normalize the relative expression of target genes, which was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in *Table 1*.

Western blot assay

Cells or tumor tissues were lysed using cellular lysis buffer (Thermo Scientific, USA) to extract total proteins, which were loaded and separated using sodium dodecylsulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a polyvinylidene difluoride membrane, followed by incubation with 5% bicinchoninic acid solution to remove non-specific binding proteins. Subsequently, the membrane was incubated with a primary antibody against Notch1 (1:1,000; Cell Signaling Technology, USA), Hes1 (1:1,000; Cell Signaling Technology, USA), PTEN (1:1,000; Cell Signaling Technology, USA), phosphorylated AKT1 (p-AKT1; 1:1,000; Cell Signaling Technology, USA), AKT1 (1:1,000; Cell Signaling Technology, USA), AKT1 (1:1,000; Cell Signaling Technology, USA), phosphorylated mTOR (p-mTOR; 1:1,000; Cell Signaling Technology, USA), mTOR (1:1,000; Cell Signaling Technology, USA), intracellular adhesion molecule-1 (ICAM-1; 1:1,000; Cell Signaling Technology, USA), vascular cell adhesion protein 1 (VCAM-1; 1:1,000; Cell Signaling Technology, USA), matrix metalloproteinase (MMP)-2 (1:1,000; Cell Signaling Technology, USA), MMP-9 (1:1,000; Cell Signaling Technology, USA), focal adhesion kinase (FAK; 1:1,000; Cell Signaling Technology, USA) or GAPDH (1:1,000; Cell Signaling Technology, USA), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:1,000; Cell Signaling Technology, USA) at room temperature for 1–2 h, all antibodies used are shown in *Table 2*. Finally, the blots were incubated with enhanced chemiluminescence reagents (Thermo Scientific, USA) and visualized using an Image J software (National Institutes of

Table 2 Antibodies used in the Western blot assay.

Antibodies	Manufacturer	Dilution ratio
Anti-Notch1	Cell Signaling Technology	1:1,000
Anti-Hes1	Cell Signaling Technology	1:1,000
Anti-PTEN	Cell Signaling Technology	1:1,000
Anti-p-AKT1	Cell Signaling Technology	1:1,000
Anti-AKT1	Cell Signaling Technology	1:1,000
Anti-p-mTOR	Cell Signaling Technology	1:1,000
Anti-mTOR	Cell Signaling Technology	1:1,000
Anti-ICAM-1	Cell Signaling Technology	1:1,000
Anti-VCAM-1	Cell Signaling Technology	1:1,000
Anti-MMP-2	Cell Signaling Technology	1:1,000
Anti-MMP-9	Cell Signaling Technology	1:1,000
Anti-FAK	Cell Signaling Technology	1:1,000
Anti-GAPDH	Cell Signaling Technology	1:1,000

Health, Bethesda, Maryland, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was used to evaluate the proliferation ability of HCC cells. In brief, 10 μL of 5 mg/mL MTT solution was added to the medium (Thermo Scientific, USA), followed by incubation for 4 h. Subsequently, approximately 200 μL of formazan in dimethylsulfoxide (Genview, Beijing, China) was added to the medium. Lastly, absorbance (optical density value) at 490 nm was read using a microplate reader (BMG LABTECH, Offenburg, Germany).

Colony formation assay

Cells at the exponential phase were seeded onto 6-well plates at a density of 500 cells/well with 3 replicates. After incubating for 10–14 days, the cells were fixed with 4% paraformaldehyde (Solarbio Science & Technology, Beijing, China) for 15 min and stained with Giemsa (Solarbio Science & Technology, Beijing, China) for 30 min at room temperature. After washing the cells several times, the number of cell colonies was calculated.

Flow cytometry for the apoptosis analysis

The treated cells were digested and resuspended using 400 μL of 1× binding buffer, and approximately 100 μL, cell suspension was added to a 5-mL flow tube, followed by 5 μL Annexin V/Alexa Fluor647 (Beijing 4A Biotech, Beijing, China). After incubation at room temperature for 5 min in the dark, the samples were incubated with 10 μL propidium iodide and 400 μL PBS. Finally, the Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer was used to detect and analyze the fluorescence intensity of the cells.

Transwell migration assay

The upper chamber of the well plate was planted with 1×10^5 cells per well and filled with 100 μL of serum-free medium. Subsequently, approximately 600 μL of complete medium was added to the lower chamber, followed by incubation for 24 h at 37 ℃. The residual cells in the upper chamber were removed and the cells in the lower chamber were the migrated cells, which were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. Finally, the migrated cells were photographed using an inverted

fluorescence microscope (Magnification: 200×, Olympus, Tokyo, Japan).

Transfection

The pcDNA3.1-Notch1 and pcDNA3.1-Hes1 were designed and established by Genscript (Nanjing, China), and were transfected into HepG2 cells, along with the transfection reagent (Lipofectamine 3000; Thermo Fisher, Waltham, Massachusetts, USA) to obtain the Notch1- and Hes1-overexpressed HepG2 cells.

Xenograft experiments

For the *in vivo* xenograft experiment, we used female nude mice , the cells were randomly divided into 5 groups, using a computer-generated random numbers table as follows: control, DAPT+pcDNA3.1-negative control (NC), DAPT+pcDNA3.1-Notch1, DAPT+pcDNA3.1-Hes1, and DAPT+MHY-1485. No confounders were involved. The nude mice in the control group were planted with naive HepG2 cells, followed by being administered subcutaneously with normal saline for 3 weeks. The animals in the DAPT+pcDNA3.1-NC group were transplanted with pcDNA3.1-NC-transfected HepG2 cells and treated subcutaneously with 10 mg/kg/day DAPT for 3 weeks (17). HepG2 cells transfected with pcDNA3.1-Notch1 and DAPT+pcDNA3.1-Hes1, were planted into the nude mice, followed by being administered subcutaneously with 10 mg/kg/day DAPT for 3 weeks, respectively. In the DAPT+MHY-1485 group, HepG2 cells were pretreated with 10 μM mTOR activator MHY-1485 for 6 h and then transplanted into the nude mice (18), which were further administered subcutaneously with 10 mg/kg/day DAPT for 3 weeks. For each group, 3 animals were utilized. All the treatments were performed until the volume of the tumor reached 100 mm^3. Tumor volume was measured and calculated according to the following formula: $V = L \times W2/2$, where V represents volume (mm^3), L represents the greatest diameter (mm), and W represents the lowest diameter (mm). The animals were killed at the end of the experiment, and the tumors were pictured and weighed. The Sample size was determined based on the number of experimental groups and different time points of analysis. For all allocation, only designer was aware of the group allocation at the different stages of the experiment. No criteria used for including and excluding animals as there were no exclusions.

Statistical analysis

All experiments were repeated independently 3 times, and data are shown as mean ± standard deviations. Statistical analyses were performed using SPSS version 24.0 (IBM, Armonk, NY, USA), and significant differences were analyzed by one-way analysis of variance coupled with post-hoc comparisons. Student's t-test was used to analyze the difference between groups. Significance was defined as $P<0.05$.

Results

High expression of Notch1 in HCC tissues and cell lines

To investigate the function of Notch1 in the development and progression of HCC, we analyzed the expression of Notch1 in both HCC tissues from patients and HCC cell lines. As shown in *Figure 1A*,*B*, ICH and qRT-PCR assay revealed that, compared with para-carcinoma tissues, Notch1 was found to be significantly upregulated in HCC tissues from patients ($P<0.01$). Subsequently, we further detected the relative expression level of Notch1 in HCC cell lines and normal hepatocytes. As shown in *Figure 1C* and D, the expression level of Notch1 was significantly elevated in HCC cell lines (all $P<0.05$). In addition, in the 3 HCC cell lines, the highest expression of Notch1 was found in HepG2 cells. Therefore, HepG2 cells were used in our subsequent experiments to investigate the function of Notch1 in HCC.

Suppression of proliferation and migration ability of HepG2 cells by DAPT

To explore the function of DAPT, an inhibitor of Notch1, on the development of HCC, HepG2 cells were treated with different dosages of DAPT (25, 50, and 100 μM, n=3), with HepG2 cells incubated with blank medium as the control group. As shown in *Figure 2A*, the cell viability of HepG2 cells was significantly inhibited by the introduction of DAPT in a dose-dependent manner (all $P<0.05$). Compared with the control, in the colony formation assay, the colony number was found to be significantly suppressed

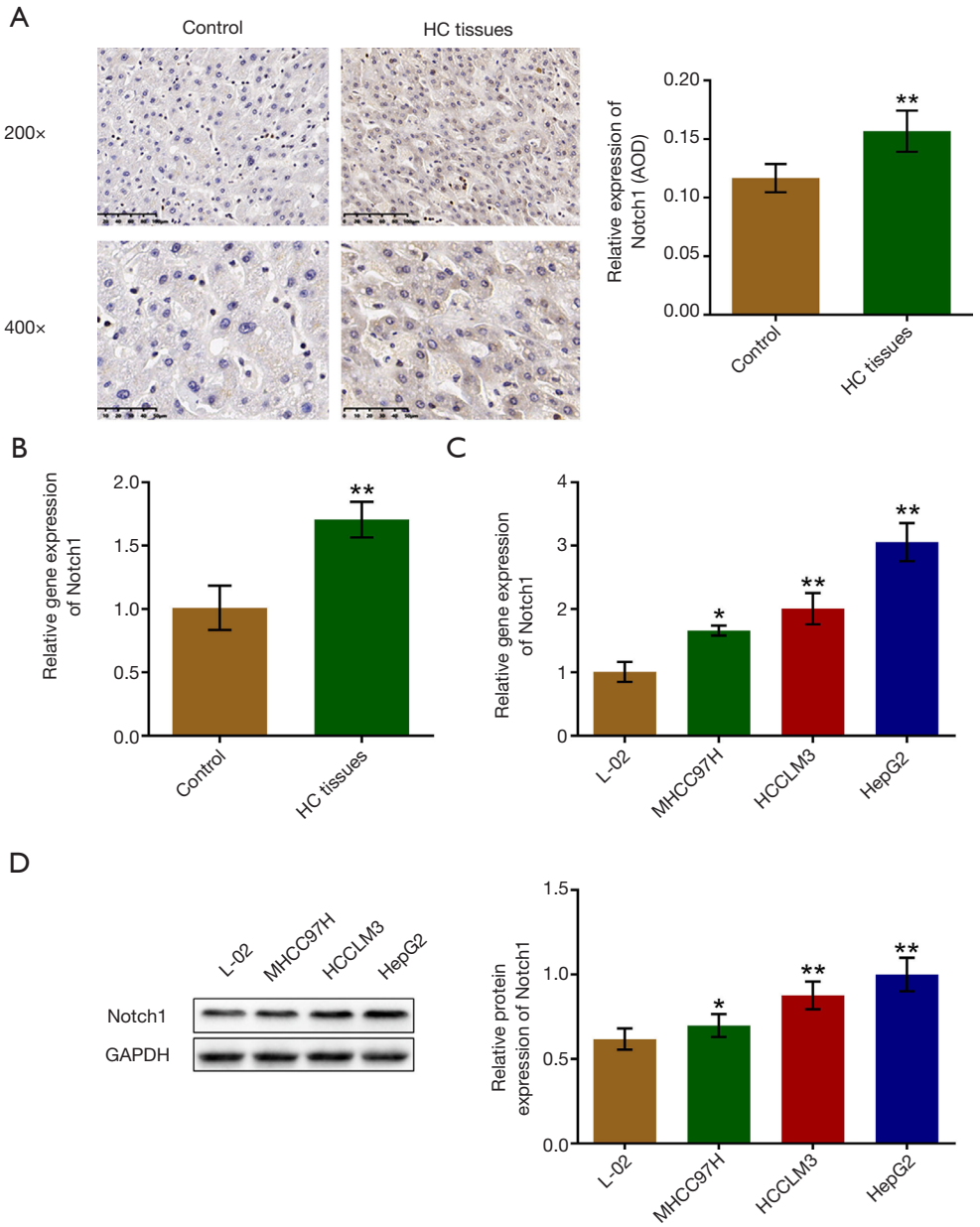

Figure 1 Notch1 was up-regulated in both hepatocellular carcinoma (HCC) tissues and cell lines. (A) Expression of Notch1 in HCC tissues was determined by immunohistochemical assay, Scale bar (200×): 20 μm. (B,C) Expression of Notch1 in HCC tissues and cell lines was detected by quantitative reverse transcription polymerase chain reaction assay. (D) Expression of Notch1 in HCC cell lines was evaluated by Western blot assay. *P<0.05 *vs.* control; **P<0.01 *vs.* control.

in the 50 and 100 μM DAPT groups (P<0.01) (*Figure 2B*). As shown in *Figure 2C*, compared with the control, the apoptotic rate of HepG2 cells increased from 5.45% to 15.71% and 18.29% in the 50 and 100 μM DAPT groups, respectively (P<0.01). Finally, in the Transwell assay, the number of migrated cells was significantly decreased by the treatment of DAPT in a dose-dependent manner (P<0.05) (*Figure 2D*). These data indicated that the proliferation and migration ability of HepG2 cells were significantly inhibited by DAPT.

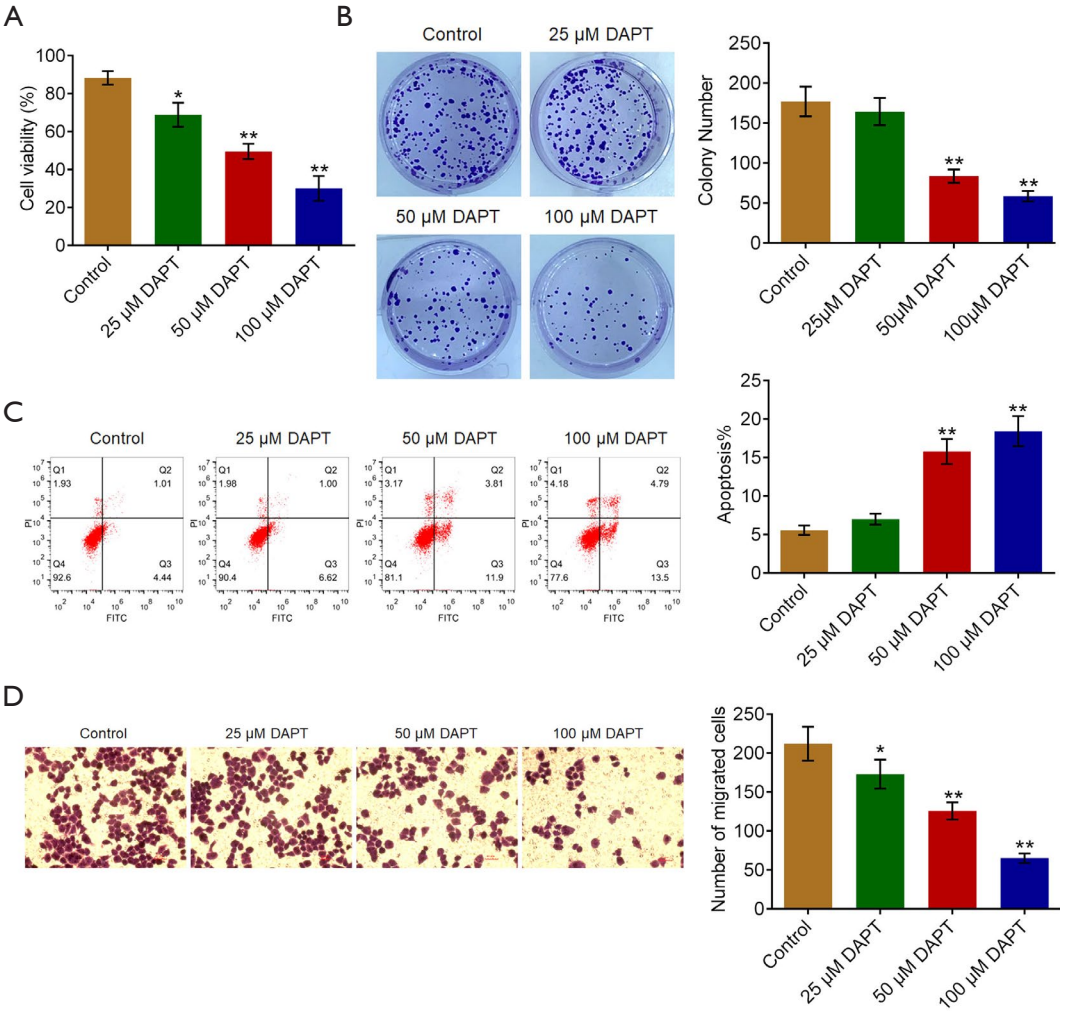

Figure 2 Proliferation and migration of HepG2 cells were suppressed, and the apoptotic rate of HepG2 cells was elevated by N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester in a dose-dependent manner. (A) Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; (B) proliferation ability was determined by colony formation assay; (C) apoptosis was detected by flow cytometry; (D) migration ability of HepG2 cells was checked by Transwell assay, Scale bar: 40 μm. *P<0.05 *vs.* control; **P<0.01 *vs.* control.

Effects of DAPT on the Hes1/PTEN/AKT/mTOR signaling pathway, extracellular matrix (ECM), and adhesion molecules

We further investigated the effects of DAPT on the downstream signaling pathway of Notch1 and the protection of the ECM. As shown in *Figure 3*, the expression of Notch1 and Hes1 was significantly inhibited, and the expression of PTEN was significantly elevated by the introduction of DAPT in a dose-dependent manner (all P<0.05), indicating negative regulation of DAPT on the Notch1/Hes1/PTEN signaling pathway. Subsequently, we found that p-AKT1 and p-mTOR were significantly downregulated by the treatment of DAPT in a dose-dependent manner (all P<0.05), indicating an inhibitory effect of DAPT on the AKT/mTOR signaling pathway. In addition, the expression of FAK, ICAM-1, and VCAM-1, was found to be significantly suppressed by DAPT (P<0.01). Finally, MMP-2 and MMP-9 were significantly downregulated by the treatment of DAPT in a dose-dependent manner (P<0.01), indicating protective

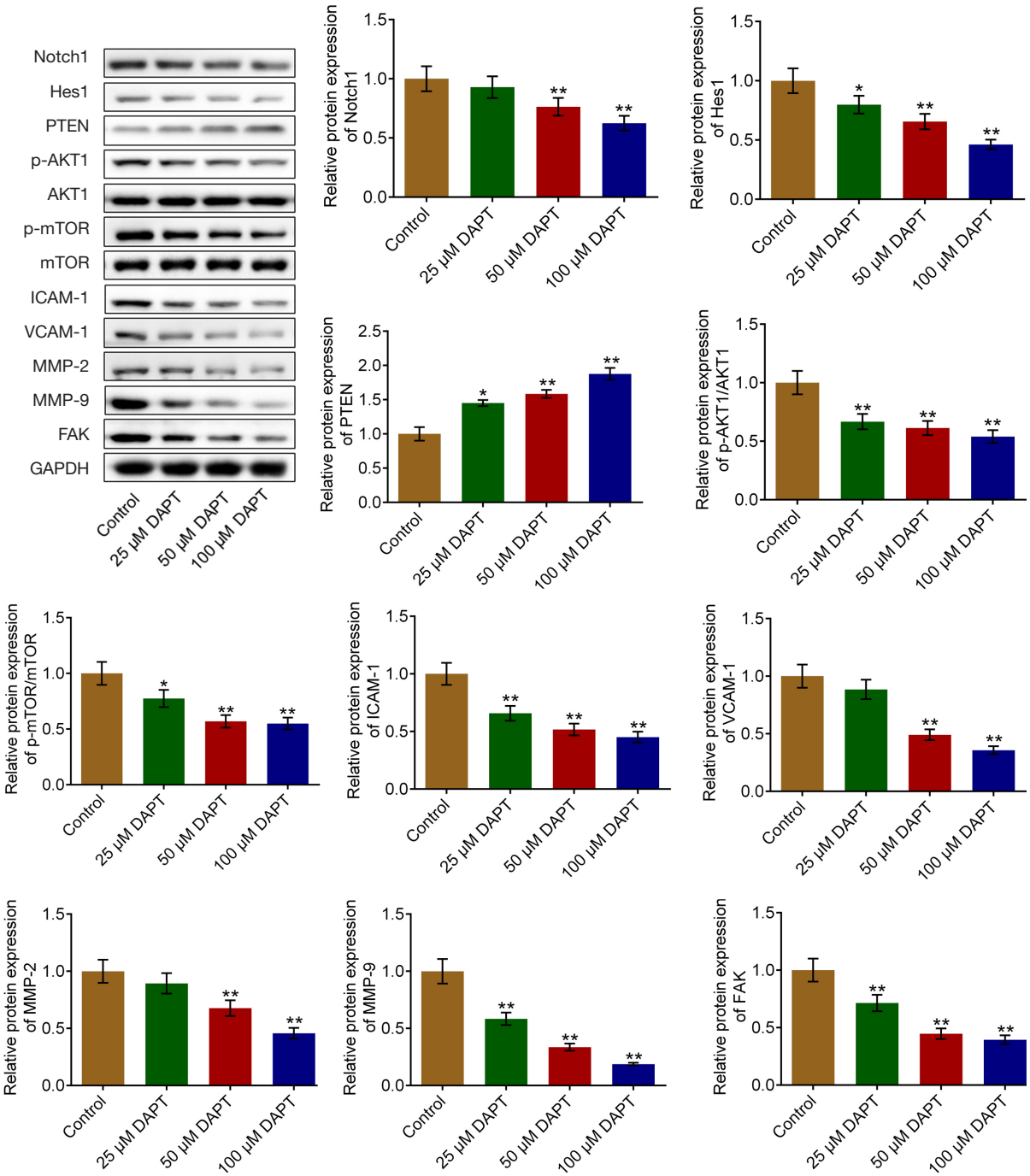

Figure 3 N-(N-[3,5-Difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) significantly inhibited Notch1/Hes1/ Phosphatase and tensin homolog (PTEN) and protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathways, and the expression of matrix metalloproteinases (MMPs) and adhesion molecules. Expression level of Notch1, Hes1, PTEN, AKT1, phosphorylated AKT1, mTOR, phosphorylated mTOR, intracellular adhesion molecule 1, vascular cell adhesion protein 1, MMP-2, MMP-9, and focal adhesion kinase in the DAPT-treated HepG2 cells was evaluated by Western blot assay. *P<0.05 *vs.* control; **P<0.01 *vs.* control.

properties of DAPT on the ECM.

Abolition of DAPT biofunction by activation of the Hes1/PTEN/AKT/mTOR signaling pathway

To verify the involvement of the Hes1/PTEN/AKT/mTOR signaling pathway in the antitumor function of DAPT, Notch1-overexpressed, Hes1-overexpressed HepG2 cells, and MHY-1485, an mTOR activator, were introduced. As shown in *Figure 4A*,*B*, cell viability and the colony number were significantly suppressed in the DAPT-treated HepG2 cells transfected with pcDNA3.1-NC; these were significantly reversed in the DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells, or DAPT and MHY-1485-co-treated HepG2 cells (all P<0.01). As shown in *Figure 4C*, the apoptotic rate in the control, DAPT+pcDNA3.1-NC, DAPT+pcDNA3.1-Notch1, DAPT+pcDNA3.1-Hes1, and DAPT+MHY-1485 groups was 7.29%, 25.85%, 14.86%, 15.61%, and 14.49%, respectively (all P<0.01). Finally, in the Transwell assay, the number of migrated cells was significantly inhibited by the treatment of DAPT (*Figure 4D*), which was significantly elevated in the DAPT-treated HepG2 cells transfected with pcDNA3.1-NC. This was significantly reversed in the DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells or DAPT and MHY-1485-co-treated HepG2 cells (all P<0.01).

Abolishment of the effects of DAPT on the Hes1/PTEN/AKT/mTOR signaling pathway, ECM, and adhesion molecules by activation of the Hes1/PTEN/AKT/mTOR signaling pathway

As shown in *Figure 5*, compared with the control, the expression of Notch1 and Hes1 was significantly inhibited and the expression of PTEN was significantly elevated in DAPT-treated HepG2 cells transfected with pcDNA3.1-NC; this was reversed in DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells or DAPT and MHY-1485-co-treated HepG2 cells (P<0.01 *vs.* control, P<0.05 *vs.* DAPT+pcDNA3.1-NC,). Subsequently, the downregulated p-AKT1 and p-mTOR induced by DAPT was significantly upregulated in DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells or DAPT and MHY-1485-co-treated HepG2 cells (all P<0.01). In addition, the suppressed expression of FAK, ICAM-1, and VCAM-1 in HepG2 cells induced by DAPT was significantly increased in DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells or DAPT and MHY-1485-co-treated HepG2 cells (all P<0.01). Finally, the expression of MMP-2 and MMP-9 was significantly inhibited by the treatment of DAPT, which was significantly reversed in DAPT-treated Notch1-overexpressed and Hes1-overexpressed HepG2 cells or DAPT and MHY-1485-co-treated HepG2 cells (all P<0.01).

Antitumor effects of DAPT by regulating the Hes1/PTEN/AKT/mTOR signaling pathway and protecting the ECM in vivo

To verify the antitumor properties, as well as the underlying mechanism, of DAPT on HCC, xenograft experiments were performed (n=3). As shown in *Figure 6*, tumor volume and weight were significantly decreased in the DAPT+pcDNA3.1-NC group, and significantly elevated in the DAPT+pcDNA3.1-Notch1, DAPT+pcDNA3.1-Hes1, and DAPT+MHY-1485 groups (all P<0.01). These data indicated that DAPT might exert antitumor properties by inhibiting the Hes1/PTEN/AKT/mTOR signaling pathway. We further verified the expression of related proteins in the tumors. As shown in *Figure 7*, compared with control, the expression of Notch1 and Hes1 was suppressed; the expression of PTEN increased; the AKT/mTOR signaling pathway was activated, and the expression of FAK, ICAM-1, VCAM-1, MMP-2, and MMP-9 was downregulated in the DAPT+pcDNA3.1-NC group; these were all reversed in the DAPT+pcDNA3.1-Notch1, DAPT+pcDNA3.1-Hes1, and DAPT+MHY-1485 groups (all P<0.01). These data confirmed that the antitumor effect of DAPT was related to the regulation of the Hes1/PTEN/AKT/mTOR signaling pathway, ECM, and the expression of adhesion molecules.

Discussion

The Notch signaling pathway has been reported to be involved in the pathogenesis of multiple types of malignant tumors, including HCC (19). The basic helix–loop–helix protein family is one of the main downstream transcriptional factors of notch signaling, including the Hes family, Homocysteine-Induced Endoplasmic Reticulum Protein (HERP) family, and YRPW motif (HEY) family (20-22). Hes inhibits the transcription of target genes by binding with the promoter of specific differentiation effector gene, and plays an important role in Notch signal transduction. The Notch/Hes signaling pathway has

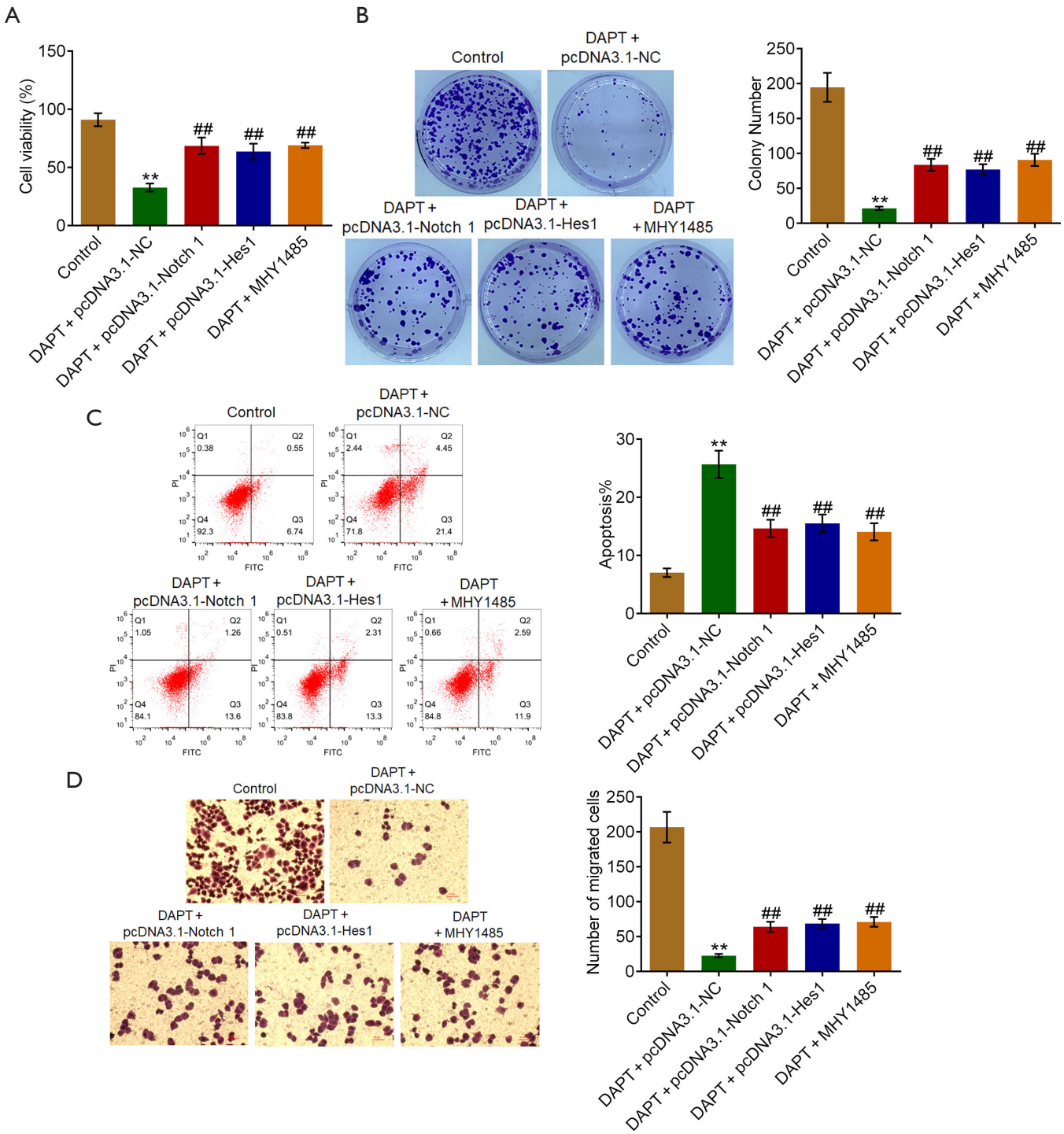

Figure 4 Regulatory effect of N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) on the proliferation, apoptosis, and migration of HepG2 cells was abolished by the activation of Notch1/Hes1 and protein kinase B(AKT)/mammalian target of rapamycin (mTOR) signaling. (A) Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B) Proliferation ability was determined by colony formation assay. (C) Apoptosis was detected by flow cytometry. (D) Migration ability of HepG2 cells was determined by Transwell assay, Scale bar: 40 μm. **P<0.01 *vs.* control, ##P<0.01 *vs.* DAPT+pcDNA3.1- negative control (NC).

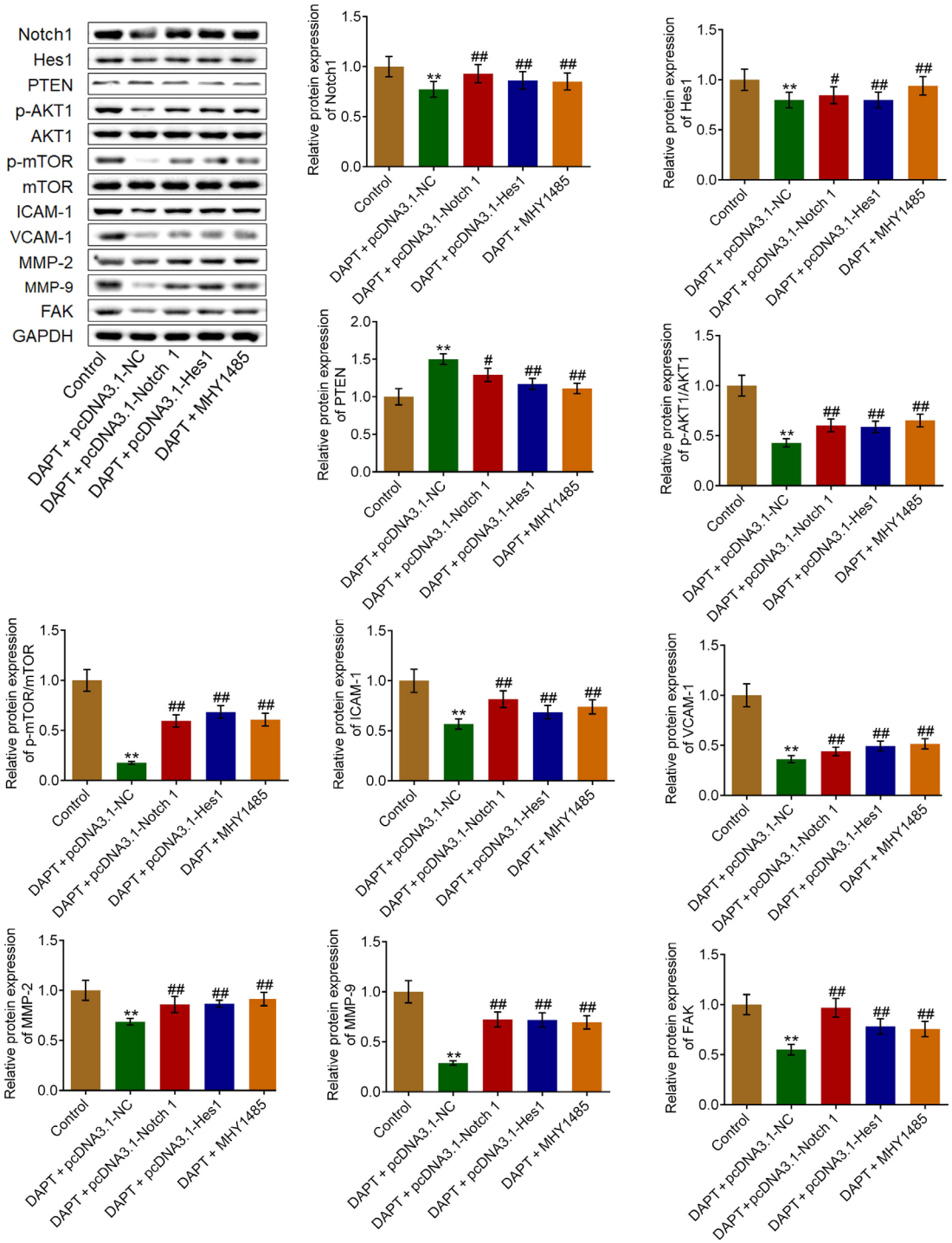

Figure 5 Effects of N-(N-[3,5-difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) on the Hes1/Phosphatase and tensin homolog (PTEN)/protein kinase B(AKT) /mammalian target of rapamycin (mTOR) signaling pathway, ECM, and adhesion molecules were abolished by activating the Hes1/PTEN/AKT/mTOR signaling pathway. Expression level of Notch1, Hes1, PTEN, AKT1, phosphorylated AKT1, mTOR, phosphorylated mTOR, intracellular adhesion molecule 1, vascular cell adhesion protein 1, matrix metalloproteinase (MMP)-2, MMP-9, and focal adhesion kinase in the DAPT-treated HepG2 cells was evaluated by Western blot assay. **P<0.01 *vs.* control; $^{\#}$P<0.05 *vs.* DAPT+pcDNA3.1-negative control (NC); $^{\#\#}$P<0.01 *vs.* DAPT+pcDNA3.1-NC.

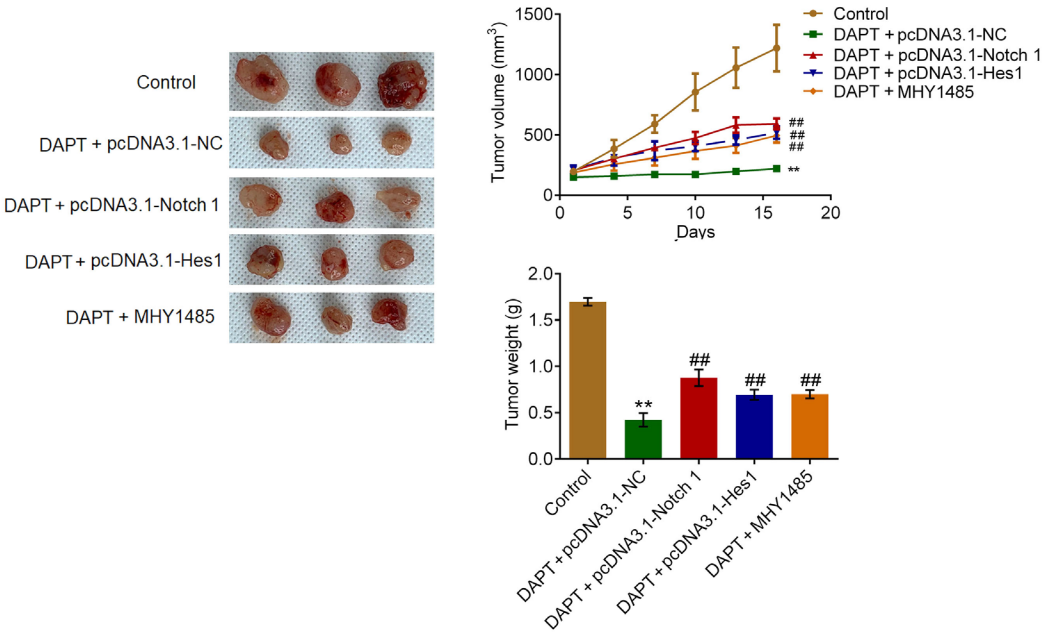

Figure 6 N-(N-[3,5-Difluorophenacetyl]-1-alanyl)-S-phenylglycine t-butyl ester (DAPT) showed promising antitumor properties in a HepG2 xenograft model. Tumors were imaged, tumor volume was recorded every 3 days, and tumor weights were recorded after isolation. **P<0.01 *vs.* control, ##P<0.01 *vs.* DAPT+pcDNA3.1- negative control (NC).

been reported to be involved in the regulation of cellular differentiation and tissue development, and the expression level of the Hes protein is regarded as an important marker for the activation of the Notch signaling pathway (23). Chen *et al.* recently reported that the activation of Notch1/Hes1 signaling was involved in facilitating the progression of HCC (24). The Notch1 signaling pathway is also reported to be activated in HCC and contribute to the development and progression of HCC tumor formation (11). In the present study, we investigated the inhibitory effect of DAPT, a Notch1 inhibitor against HCC cell proliferation and migration. First, we confirmed that Notch1 was indeed highly expressed in both HCC tissues and cell lines. HepG2 cells were chosen as the experimental subject in the present study, as the expression level of Notch1 was found to be highest in the selected HCC cell lines. Subsequently, the *in vitro* antitumor effects of DAPT was evidenced by the decreased proliferation and migration ability, as well as the increased apoptotic rate. Further signaling investigations revealed that Notch1/Hes1 signaling was significantly blocked by DAPT, which was consistent with Zhang *et al.*'s finding (25). Promising antitumor properties were subsequently verified by the significantly decreased tumor volume in the DAPT-treated HepG2 xenograft animals. To further confirm that the antitumor properties of DAPT were mediated by the Notch1/Hes1 signaling pathway, we established the Notch1-overexpressed and Hes1-overexpressed HepG2 cell lines. We found that the *in vitro* regulation of DAPT on the proliferation, migration, and apoptosis of HepG2 cells, and the *in vivo* tumor growth inhibitory effects, were reversed by the overexpression of Notch1 or Hes1. These data support the involvement of Notch1/Hes1 signaling in the antitumor properties of DAPT. In our future work, the impact of DAPT on more downstream molecules of Notch signaling will be investigated, such as eIF3a, SRY-related high-mobility-group-box protein-2 (SOX2), and Nrf2 to fully understand the antitumor mechanism of DAPT (26-28).

PTEN is an important tumor suppressor that has been reported to be downregulated in multiple types of malignant tumor cells (29). It has also been verified to have a low expression in HCC cells, and its degradation contributes to HCC progression (30). PTEN expression has been reported to be negatively regulated by Notch1/Hes1 signaling (31,32). PTEN downregulation is regarded as an important inducer of the activation of the AKT/mTOR signaling pathway, which plays an important role in regulating the proliferation of multiple types of tumor cell lines (33,34).

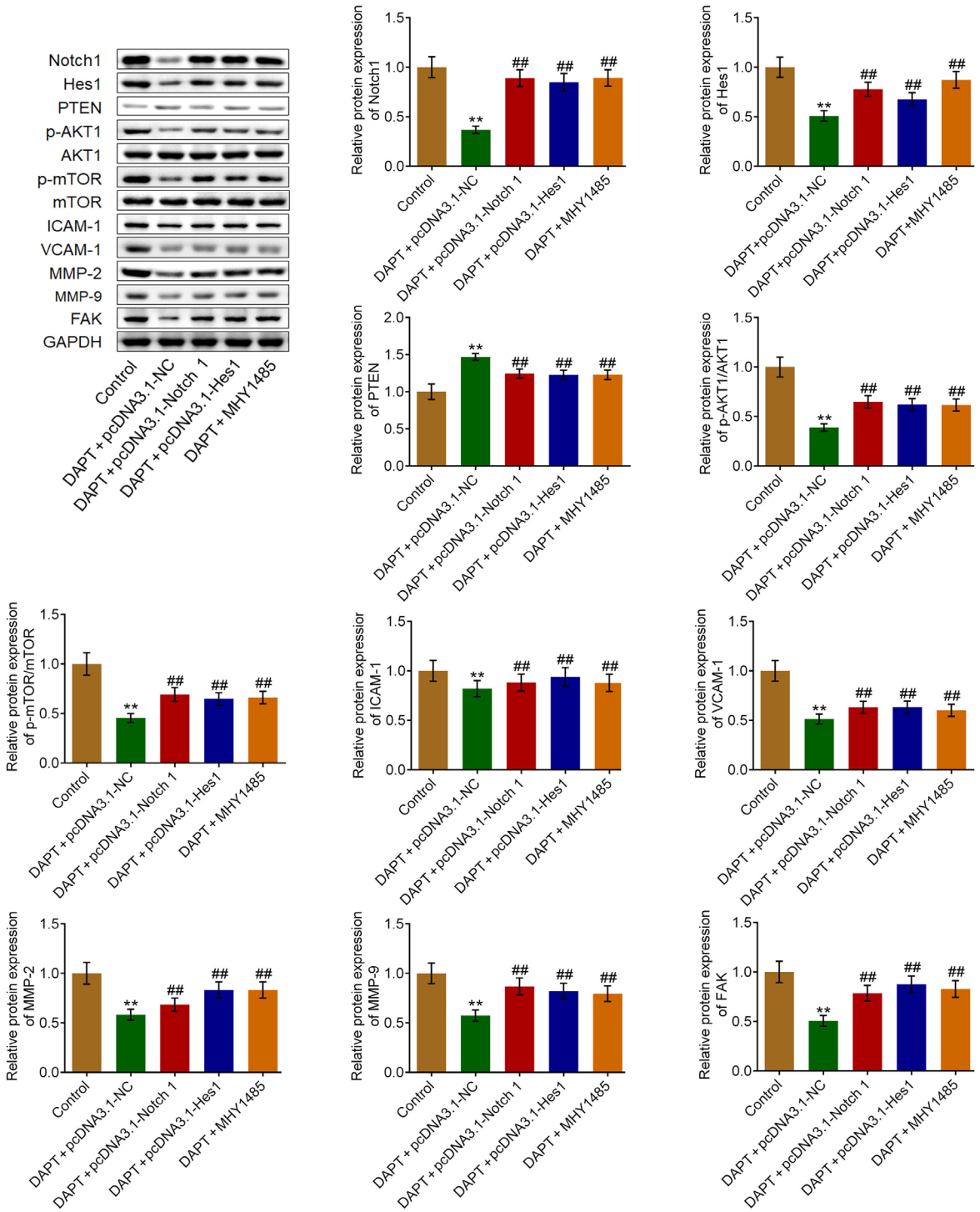

Figure 7 The effects of DAPT on Hes1/PTEN/AKT/mTOR signal pathway, ECM, and adhesion molecules in the xenograft tumor tissues were abolished by activating the Hes1/PTEN/AKT/mTOR signal pathway. The expression level of Notch1, Hes1, PTEN, AKT1, p-AKT1, mTOR, p-mTOR, ICAM-1, VCAM-1, MMP-2, MMP-9, and FAK in the tumor tissues was evaluated by Western blot assay. **P<0.01 *vs.* control, ##P<0.01 *vs.* DAPT+pcDNA3.1-negative control (NC).

In the present study, we found that DAPT elevated the expression of PTEN by inhibiting Notch1/Hes1 signaling, further suppressing the AKT/mTOR signaling pathway. As a consequence, the proliferation and migration ability of HepG2 cells were inhibited, and the apoptotic rate of HepG2 cells increased. The effects of DAPT on Notch1/Hes1/PTEN/AKT/mTOR signaling was further verified by introducing the activator of mTOR. In our future work, the PTEN knockdown of HepG2 cells will be introduced to further verify the signaling transduction route of DAPT.

The degradation of the ECM has been reported to be involved in the development and progression of tumor metastasis, which could be induced by the function of MMPs (35). In addition, the upregulation of adhesion molecules is also regarded as an important inducer for the migration of tumor cells (36). In the present study, we found that DAPT protected the ECM from degradation by downregulating MMP-2 and MMP-9, accompanied by the downregulation of adhesion molecules, which were all reversed by the activation of the Notch1/Hes1 signaling pathway or the activation of the AKT/mTOR signaling pathway. It is a novel finding that blocking Notch1/Hes1 signaling pathway suppressed the migration of tumor cells by preventing the ECM from degradation and downregulating adhesion molecules. Our future work will involve more detailed investigation into the regulatory effects of DAPT on the ECM and the expression of adhesion molecules to provide more support for the therapeutic application of DAPT in the treatment of HCC.

Taken together, our data indicated that DAPT could suppress the proliferation and migration of HCC by regulating the ECM and inhibiting the Hes1/PTEN/AKT/mTOR signaling pathway.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee Board of Ningbo Medical Center Lihuili Hospital (NO: KY2020PJ190). Because of the retrospective nature of the study, the requirement for informed consent was waived. A protocol related to animal experiments was prepared before the study, which can be accessed by contacting the corresponding author. It was approved by the Animal Ethics committee of Ningbo University School of Medicine (NO: 2019-381) and guidelines for the care and use of animals were followed.

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