Doxycycline as an Inhibitor of the Epithelial-to-Mesenchymal Transition and Vasculogenic Mimicry in Hepatocellular Carcinoma

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Abbreviations

HCC, hepatocellular carcinoma; EMT, epithelial-to-mesenchymal transition; VM, vasculogenic mimicry; MV, mosaic vessels; MMPs, matrix metalloproteinases; DNMTs, DNA methyltransferases; ECM, extracellular matrix; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PAS, periodic acid Schiff; PBS, Phosphate-buffered saline; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcription-polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; E-cadherin, epithelial-cadherin; VE-cadherin, vascular endothelial-cadherin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ip, intraperitoneally; IR, inhibition rate; OD, optical density; 3D, Three-dimensional

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Abstract

This study was conducted to examine the effects of doxycycline on the survival time and proliferation of hepatocellular carcinoma (HCC) in vivo and on the biological functions of HCC in vitro. This study was also designed to evaluate the effects of doxycycline on epithelial-to-mesenchymal transition (EMT)- and vasculogenic mimicry (VM)-related protein expression and on matrix metalloproteinase (MMP) and DNA methyltransferase (DNMT) activity in vitro. Human MHCC97H cells were injected into BALB/c mice, which were divided into treatment and control groups. Doxycycline treatment prolonged the mouse survival time and partly suppressed the growth of engrafted HCC tumor cells, with an inhibition rate of 43.39%. Higher amounts of VM and endothelium-dependent vessels were found in the control group than the treatment group. Immunohistochemistry indicated that epithelial (E)-cadherin expression was increased in the doxycycline-treated mice compared with the control group. In in vitro experiments, doxycycline promoted HCC cell adhesion but inhibited HCC cell viability, proliferation, migration, and invasion. Western blots, semi-quantitative RT-PCR, quantitative real-time PCR, and immunofluorescence demonstrated that doxycycline inhibited the degradation of the epithelial marker E-cadherin and downregulated the expression levels of EMT promoters, the mesenchymal marker vimentin, and the VM-associated marker vascular endothelial (VE)-cadherin. Furthermore, the activities of MMPs and DNMTs were examined in different groups via gelatin zymography and a DNMT activity assay kit. A methylation-specific polymerase chain reaction (MSP)
was performed to assess the promoter methylation of CDH1 (the gene encoding E-cadherin). Doxycycline prolonged the mouse survival time by inhibiting EMT progression and VM formation.

**Introduction**

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer morbidity and mortality worldwide (1). Despite advancements in treatment options, the five-year survival rate of patients with HCC has not improved. This negative result is primarily attributed to early-stage metastasis, which involves a series of rare, stochastic events. During the stages, the loss of epithelial (E)-cadherin expression and the subsequent loss of homotypic cellular adhesion may be a unique and important step that enables malignant epithelial cells to invade and metastasize normal cells. This mechanism characterizes a developmental regulatory program, the epithelial-to-mesenchymal transition (EMT).

HCC is a hypervascular solid tumor that primarily exhibits aberrant angiogenesis. Previous studies indicated that only endothelial cells could form blood vessels to support the growth of malignant tumors; however, recent studies have revealed several new patterns by which tumor tissues nourish themselves. These patterns include the formation of a pattern of mosaic vessels (MVs) from endothelium and tumor cells (2) and the generation of channels lined exclusively with tumor cells. The latter is also known as vasculogenic mimicry (VM) (3). The molecular mechanisms underlying VM remain unclear; nevertheless, tumor cells lining VM networks secrete matrix metalloproteinases (MMPs) and express vascular endothelial (VE)-cadherin to
induce extracellular matrix (ECM) remodeling (4, 5). Our previous studies also showed that VM occurs in HCC (6) and that EMT may be involved in VM formation in HCC (7). Previous research data indicated that VM was observed in 18 out of 97 HCC patients (19%) (4). Twist, one of the transcription factors that regulates EMT, was detected in 13 of the 18 samples (72%) in the VM-positive group. In vitro, Twist up-regulation leads to increased HCC cell invasion, migration, and VM formation (4). Several studies have also demonstrated that EMT and VM are associated with poor clinical prognosis in patients (8, 9). However, few studies have investigated inhibitors of these processes (10).

Doxycycline belongs to the tetracycline family of antibiotics; this drug has been used in first-line therapy for decades. Studies have revealed that doxycycline has a wide range of highly valuable pharmacological properties. One of the well-characterized non-antimicrobial properties of doxycycline is its ability to inhibit members of the MMP family of endopeptidases (11). MMPs are associated with angiogenesis and tumor metastasis. Hence, the inhibition of EMT and VM by doxycycline is of great interest in many fields of research.

**Materials and Methods**

**Cell culture and animals** The following cell lines were obtained from the American Type Culture Collection and from Zhongshan Hospital Affiliated to Fudan University (Shanghai, China) in 2012: HepG2, PLC, SMMC7721, MHCC97H, and 293T. All of them were authenticated using the STR analysis by Genewiz Inc. in May 2014. The STR analysis results showed that the submitted samples were in good
agreement with the reference cell lines. The cell culture conditions for the proliferation and functional assays are described in the Supplementary Data.

Forty-two male athymic BALB/c nu/nu mice, weighing 18 g to 20 g and four to six weeks of age, were obtained from HFK Bioscience Co., Ltd., Beijing, China. The mice were brought to the Animal Center of Tianjin Medical University one week prior to the experiment and were bred under specific pathogen-free conditions. All of the mice were handled according to the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Drugs, antibodies, and reagents** Doxycycline-HCl powder (99.99% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), stored at 4 °C, and dissolved in sterile 0.9% NaCl prior to use.

The primary antibodies used in this study were rabbit anti-E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-vimentin (Epitomics, Burlingame, CA, USA), rabbit anti-Snail (Abcam), rabbit anti-Twist (Santa Cruz Biotechnology), and rabbit anti-VE-cadherin (Abcam). Transwell cell inserts were purchased from Costar. Matrigel was purchased from BD Biosciences.

**Mouse tumor model, doxycycline administration and tumor growth analysis** An MHCC97H cell suspension containing $5 \times 10^6$ cells was subcutaneously injected into the upper right flank region at 0.2 ml/mouse. Twelve mice were randomly divided into a treatment group (six mice) and a control group (six mice). At 7 d after the tumor cells were engrafted, tumor growth was evaluated by measuring the length and width of the tumor mass at the inoculation site. A doxycycline solution (0.1 ml/mouse/day) was administered intraperitoneally (ip) in the treatment group, and a 0.9% NaCl
solution was administered ip in the control group. At four weeks after the treatment was initiated, the tumor-bearing mice were euthanized. The tumor masses were removed and weighed. A portion of each tumor without necrosis was collected and stored at -80°C, and the remainder of the tumor was fixed in 4% formaldehyde and embedded in paraffin. Another 30 nude mice received the same subcutaneous xenografts and the same doxycycline treatment, and we determined the survival time of the mice in the treatment group (15 mice) and the control group (15 mice). Survival time was defined as the interval between the day of inoculation and the time of death.

The length and width of each tumor were measured with a Vernier caliper each day from 8 d to 35 d after inoculation. The tumor size was determined using the following formula: [volume (mm$^3$) = (length × width$^2$)/2]. The inhibition rate (IR) of the engrafted tumors was determined using the following formula: IR (%) = 

$$\left(1 - \frac{\text{tumor weight of treatment group}}{\text{tumor weight of control group}}\right) \times 100\%$$

**Immunohistochemistry and endomucin/Periodic Acid Schiff (PAS) double staining** Sections (4 µm) were pretreated in a microwave, blocked, and incubated with a series of antibodies (Supplementary Table S1). The PicTure PV6000 staining system (Zhongshan Chemical Co., Beijing, China) was used. Negative controls were incubated with phosphate-buffered saline (PBS) instead of primary antibodies. After immunohistochemical staining for endomucin was performed, the sections were washed with running water for 5 min and incubated with PAS for 15 min. All of the sections were counterstained with hematoxylin, dehydrated, and mounted. The results were quantified according to the method described by Bittner et al.

**Cytotoxicity assay** Exponentially growing HepG2, PLC, SMMC7721, and MHCC97H HCC cells were separately seeded into 96-well plates at $1 \times 10^3$ cells/well
and were incubated for different periods (1, 2, 3, 4, 5, or 6 d). Each cell line was treated with doxycycline at different concentrations. Subsequently, 10 μl of MTT (methylthiazol tetrazolium, 0.5 mg/ml final concentration; Sigma) was added to each well. After a 4 h incubation at 37°C, the supernatant was decanted, and 150 μl of dimethylsulfoxide (DMSO) was added to each well. The optical density (OD) was determined at 490 nm using a BioTek ELx800 (USA). Each experiment was performed in quintuplicate.

**Clonogenic assay** Each well of a 6-well plate was seeded with 1,000 cells in complete DMEM medium. After 24 h of growth at 37°C, the cells were treated with doxycycline at different concentrations. The plates were then incubated at 37°C and 5% CO₂ for 2 weeks. The colonies were fixed with methanol and stained with 0.5% crystal violet. Colonies > 50 μm in size in 10 random fields were counted under an inverted microscope.

**Adhesion assay** HepG₂, PLC, SMMC7721, and MHCC97H HCC cells were briefly trypsinized, plated in 24-well culture plates, and grown in complete medium for 24 h. The medium was then removed, and the cells were washed with PBS. The cells were randomly divided into two groups. One group was treated with doxycycline at different concentrations and then incubated for another 48 h. The other group was treated with doxycycline for 12 h and then treated with 150 mmol/L CoCl₂ for another 48 h. The total protein content of the suspended and adherent cells was determined by measuring the OD using a spectrophotometer (Eppendorf AG). Each experiment was performed in triplicate.

**Transwell assay** HepG₂, PLC, SMMC7721, and MHCC97H HCC cells (1 × 10⁵ cells) in 100 μl of DMEM without FBS were seeded into Matrigel matrix-coated upper 24 wells (1 mg/ml; BD Biosciences) containing polyethylene terephthalate
filters with 8 μm porosity (Invitrogen, Carlsbad, California, USA). The lower chamber was filled with 10% FBS-containing medium. The cells were incubated with or without 10 μg/ml doxycycline for 24 and 48 h, and non-invading cells were removed from the upper surface of the membrane. The cells that invaded the Matrigel matrix and adhered to the bottom surface of the membrane were fixed with methanol and stained with 0.5% crystal violet. The number of invading cells was counted using an inverted light microscope (Nikon, Japan).

**Migration assay** For wound-healing assays (conventional scrape motility assays), HepG2, SMMC7721, and MHCC97H HCC cells were plated into 24-well culture plates 24 h before the assay to form a monolayer. A straight scratch was created in the center of each well using a micropipette tip. Cell motility was assessed by measuring the movement of the cells into the scratch in the well. The speed of wound closure after 12, 24, 36, 48, 60, and 72 h was determined by comparing the length of the wound to its length at 0 h. Each experiment was performed in triplicate.

PLC HCC cells were used as Transwell cell inserts without Matrigel matrix, and a transwell assay was performed according to the above protocol. The numbers of invading cells at 24 and 48 h were counted using an inverted light microscope (Nikon, Japan). Each experiment was performed in triplicate.

**Three-dimensional (3D) culture** VM formation was evaluated in vitro using a 3D culture containing Matrigel (BD, USA). Matrigel matrix (20 μl) was used to coat 96-well plates, and the Matrigel was allowed to transform into gel for 1 h at 37°C. SMMC7721 and MHCC97H HCC cells in complete DMEM medium were seeded onto the gel and were incubated with or without doxycycline at 37°C for 24 h. The formation of capillary-like structures was filmed under a phase-contrast microscope (200×).
Semi-quantitative RT-PCR and quantitative real-time PCR Total cellular RNA was extracted with TRIzol reagent according to the manufacturer’s instructions. For semi-quantitative RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA in a 25 μl reaction using the QuantScript RT Kit (Tiangen Biotech). Semi-quantitative RT-PCR was performed according to the recommended thermal profile: 95°C for 5 min (pre-incubation), followed by 35 cycles at 95°C for 30 s (denaturation), 60°C for 1 min (annealing), and 72°C for 30 s (elongation). The amplified products were subjected to electrophoresis in a 1% agarose gel containing ethidium bromide (Bio-Rad). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to examine the integrity of the RNA in each sample and to standardize the amount of cDNA added to each PCR tube.

The following primers were used for semi-quantitative RT-PCR: CDH1: Forward: 5’-GTCACTGACACCAACGATAATCCT-3’; Reverse: 5’-TTTCAGTGTGGTATTACGACGT-3’; VIM: Forward: 5’-TGGCACGTCTTGACCTTGAA-3’; Reverse: 5’-GGTCATCGTGATGCTGAGAA-3’; CDH5: Forward: 5’-AGCCAGCCCAGCCCTCAC-3’; Reverse: 5’-CCTGTCAGCCGACCCTC-3’; GAPDH: Forward: 5’-CCTGGCCAAGGTCATCCATGAC-3’; Reverse: 5’-TGTCATACCAGGAAATGAGCCTTG-3’.

For quantitative real-time PCR, 2 μg of total RNA was reverse-transcribed into cDNA using a Reverse Transcription Kit (Takara, RR037A). Real-time PCR analyses were performed with Power SYBR Green (Takara, RR820A). All the experiments were performed according to the manufacturer’s instructions. The results were normalized to the expression of GAPDH.
The following primers were used for quantitative real-time PCR: CDH1:
Forward: 5′-GAGTGCCAACTGGACCATTAGTA-3′; Reverse: 5′-AGTCACCCACCTCTAAGGCCATC-3′; VIM: Forward: 5′-TGACATTGAGATGTGCACTACAG-3′; Reverse: 5′-TCAACCGTCTTAAATGGAATGCCGC-3′; CDH5: Forward: 5′-CCTGACTGTGGAGGCCAAAGA-3′; Reverse: 5′-TTCTCACACACTTTTGGGCTGGTAG-3′; GAPDH: Forward: 5′-GCACCCTCAAGGTGGAAGCAAC-3′; Reverse: 5′-TGTTGAAGACGCCAGTGGA-3′. qPCR and data collection were performed using an ABI 7500.

**Western blot analysis and gelatin zymography** HepG2, PLC, SMMC7721, and MHCC97H cells were washed with PBS, and 10% sodium dodecyl sulfate (SDS) was used to lyse the cells. After SDS-PAGE electrophoresis was performed, the cell lysates were transferred onto polyvinylidene fluoride membranes (Millipore, Temecula, CA, USA). These membranes were blocked with 5% skim milk powder and were incubated overnight at 4°C with primary antibodies (E-cadherin, 1:200; vimentin, 1:500; VE-cadherin, 1:500; Snail, 1:500; and Twist, 1:200). The secondary antibody, goat anti-rabbit IgG-horseradish peroxidase (HRP) (1:2,000; Santa Cruz Biotechnology), was added and incubated at 37°C for 2 h. The enhanced chemiluminescence method was then used to measure the protein expression. Equal sample loading was confirmed by probing the membranes with β-actin antibody.

For gelatin zymography, the media were collected and subjected to SDS-PAGE using 10% polyacrylamide gels containing 0.01% w/v gelatin. After electrophoresis, the gels were equilibrated in 2.5% Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, 150 mM NaCl, and 1 mM ZnCl₂ for 42 h at 37°C. The gels
were then stained with 0.1% Coomassie Brilliant Blue G250 and were destained until the wash buffer became clear and the clear bands associated with MMP activity became apparent.

**Immunofluorescence and confocal microscopy** Cells adhering to coverslips were grown until they reached 50% to 60% confluence. The cells were then fixed with cold methanol for 10 min and were blocked with 5% fetal calf serum. The coverslips were incubated with primary antibodies (E-cadherin 1:50 and vimentin 1:250) overnight at 4°C. Secondary antibodies were added and incubated at 37°C for 1 h. Fluorescein isothiocyanate (FITC)-conjugated rabbit IgG antibodies (Santa Cruz) were used as labels for the immunofluorescence assay. The cells were immunolabeled, washed, stained with 4’, 6-diamidino-2-phenylindole (DAPI, Zhongshan), mounted, and viewed under a fluorescence microscope (Nikon, Japan).

**DNA methyltransferase (DNMT) activity** Nuclear protein fractions were prepared using a nuclear extraction kit (KeyGen Biotech) and quantified using a bicinchoninic acid protein assay kit (KeyGen Biotech). DNMT activity was measured in nuclear proteins (10 μg) using the EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Ultra Kit according to the manufacturer’s protocol. The results were quantified by measuring the OD at 450 nm of an ELISA-like reaction.

**DNA methylation assay and methylation-specific polymerase chain reaction (MSP)** Genomic DNA was extracted from various cell lines using the TIANamp Genomic DNA Kit (Tiangen Biotech) according to standard procedures and was treated with sodium bisulfite. The genomic DNA (2 μg) was diluted with distilled H₂O to obtain a final volume of 50 μl, and 5.5 μl of 2 M NaOH was added. The DNA was incubated at 37°C for 10 min to obtain single-stranded DNA. Subsequently, 30 μl of 10 mM hydroquinone (Sigma) and 520 μl of freshly prepared 3 M sodium bisulfate
at pH 5.0 (Sigma) were added, and the solution was incubated at 55°C for 16 h. The DNA samples were purified using Wizard DNA purification resin (Tiangen Biotech), and the DNA was eluted with 50 μl of deionized water. Desulfonation was performed by adding 5.5 μl of 3 M NaOH, and the DNA was then incubated at room temperature for 5 min. The DNA was precipitated in 3 volumes of ethanol overnight at -20°C and was resuspended in 20 μl of water (12). The treated DNA was used as a template to amplify the E-cadherin promoter region via a nested PCR assay. A methylation and unmethylation analyses were performed according to the protocol described by Caldeira et al. (13).

E-cadherin gene silencing and transfection OmicsLink™ shRNA Expression Clones containing E-cadherin short hairpin RNA (shRNA) lentiviral expression plasmids were purchased from GeneCopoeia, Inc. (catalog no. HSH023466-HIVU6). The shRNA targeted the following nucleotide sequence: 5′-GGTAACCGATCAGAATGAC-3′. A non-silencing shRNA sequence without the E-cadherin shRNA component was used as a negative control. We transfected HepG2, PLC, SMMC7721, and MHCC97H cells with the E-cadherin shRNA expression plasmid or the shRNA control vector using the Lenti-Pac™ HIV Expression Packaging Kit (catalog no. HPK-LvTR-40) according to the manufacturer’s protocol. The transfection efficiency was confirmed via a western blot analysis. Doxycycline treatment was then performed for 24 h. The functional effects of the downregulated E-cadherin expression and doxycycline were studied using western blots, semi-quantitative RT-PCR, quantitative real-time PCR, and immunofluorescence assays using previously described methods.

Statistical analysis The data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant. A one-way analysis of
variance (ANOVA) and the Bonferroni correction were used to compare the three
groups treated with different drug concentrations. A Kaplan-Meier survival analysis
was also performed. Differences in the survival curves were assessed using a log-rank
test. Student’s t-test was performed to determine differences between two groups in
terms of immunohistochemical markers and invasion assay results.

Results

1 Doxycycline prolonged the survival of nude mice and inhibited VM and
EMT in vivo

1.1 Effects of doxycycline on tumor growth and survival time in nude mice

The engrafted MHCC97H tumors in the doxycycline treatment group developed
more slowly than those in the control group, and the tumor sizes in the treatment
group were smaller than those in the control group (Fig. 1A). Four weeks after
doxycycline was administered, the average sizes of the tumors in the treatment and
control groups were 1.083 and 1.991 cm³, respectively. The tumor mass weights
indicated that the IR of doxycycline was 43.39%. The Kaplan-Meier survival analysis
results showed that the mice in the control group exhibited shorter a survival time
than those in the doxycycline treatment group (Fig. 1B).

1.2 Doxycycline inhibited VM formation

At 7 d after the MHCC97H cells were injected, the right flank region of the mice
contained palpable engrafted tumors, which were removed. The tumors were soft and
globular, and numerous network vessels were found on the tumor surfaces. Some of
the MHCC97H cells had invaded the skeletal muscle. Endothelium-dependent vessels
and VM channels were observed in the tumors (Fig. 1C and 1D). Using
endomucin/PAS double staining, we found that tumor cells had formed in the VM
channels that were lined with PAS-positive substances, but these cells were negative
for endomucin. Red cells remained visible at the center of the VM channels. The number of microcirculation patterns in the tumors indicated that the treatment group had fewer VM channels and endothelium-dependent vessels than the control group. The treatment group had $15.33 \pm 2.066$ endothelium-dependent vessels and $1.17 \pm 1.329$ VM channels, whereas the control group had $17.67 \pm 1.211$ endothelium-dependent vessels and $1.67 \pm 0.816$ VM channels ($P < 0.05$). In the xenograft model, VE-cadherin expression was examined via immunohistochemical staining and was compared between the treatment and control groups. The treatment and control groups contained cytoplasmic VE-cadherin-positive tumor cells (Fig. 1E). Supplementary Data Table S2 shows that the tumors in the treatment group expressed less VE-cadherin protein than those in the control group ($P < 0.05$).

1.3 Doxycycline inhibited EMT in vivo

To determine whether doxycycline inhibits EMT in MHCC97H cells, we examined the expression levels of E-cadherin, vimentin, Snail, and Twist in the xenograft model via immunohistochemical staining. The treatment and control groups contained E-cadherin-, vimentin-, Snail-, and Twist-positive tumor cells. Both groups exhibited positive staining for E-cadherin in the plasma membrane, vimentin in the cytoplasm, Snail in the nucleus, and Twist in the nucleus and/or cytoplasm (Fig. 1E). Supplementary Data Table S2 further shows that the tumors in the treatment group expressed the E-cadherin protein at a higher level than in the control group, and other markers were expressed at a lower level in the treatment group compared with the control group ($P < 0.05$).

2 Effects of doxycycline in assays of tumor biological function

2.1 Doxycycline inhibited the viability and proliferation of HCC cells

To further investigate whether doxycycline can induce cell death and inhibit cell
proliferation, cytotoxicity and clonogenic assays were performed. HCC cells were treated with doxycycline at different concentrations. Significant differences in growth were observed between the cells in the treatment groups and the control group. In an MTT assay, the suppression of growth in the doxycycline-treated groups was observed as early as the third day (Fig. 1F). In the clonogenic assay, the higher drug concentrations (5 and 10 μg/ml) resulted in the significant inhibition of cell proliferation (P < 0.05) (Fig. 1G).

2.2 Doxycycline promoted the adhesion of HCC cells

In a conventional drug cytotoxicity assay, the drug solution is added before cell adhesion occurs. In this study, the effect of doxycycline on HCC cell adhesion was investigated using two methods: (a) the doxycycline solution was added after cell adhesion occurred and (b) CoCl₂ was added after doxycycline pretreatment and after cell adhesion had occurred. To investigate the effect of doxycycline on HCC cell adhesion, we used four cell lines: HepG₂, PLC, SMMC7721, and MHCC97H. In the first experiment, the cells in the control group exhibited morphological characteristics such as suspension and fragmentation; the doxycycline-pretreated cells contained a higher number of normal cell junctions (Fig. 2A). Suspended and anchorage-dependent cells were collected, and the total protein concentration was determined by measuring the OD. The doxycycline-treated group contained a higher proportion of anchorage-dependent cells compared with the control group (Fig. 2B). This result indicated that cell adhesion promotion may be an important means by which doxycycline inhibits EMT. Our previous studies showed that CoCl₂ could simulate hypoxic conditions to induce VM formation (14). In the present study, 150 mmol/L CoCl₂ was added to each well after the cells were pretreated with doxycycline for 12 h. The results were then compared with those from the previous
study, revealing a stronger cytotoxic effect in the present work (Fig. 3). This result indicated that doxycycline may inhibit hypoxia induced VM formation.

2.3 Doxycycline inhibited the migration and invasion of HCC cells

The tumor cells that exhibited the ability to undergo EMT and form VM also had a greater ability to invade and metastasize. Fig. 4 further shows that cellular migration was delayed in a time-dependent manner after the addition of 1, 5, and 10 μg/ml doxycycline. In a Transwell chamber without Matrigel matrix, the cell migration of PLC cells was strongly inhibited in the doxycycline-pretreated group. The in vitro invasion assay demonstrated that doxycycline significantly inhibited cell invasion: the numbers of invading cells were reduced by exposure to doxycycline for 24 and 48 h (Fig. 5).

3 Doxycycline inhibited EMT in vitro

3.1 Effects of doxycycline on EMT biomarker expression

E-cadherin and vimentin are characteristic markers of EMT with opposite effects. A western blot analysis, semi-quantitative RT-PCR and quantitative real-time PCR were performed to determine the effects of doxycycline on E-cadherin and vimentin expression levels (Fig. 6A-6C). After cells were exposed to doxycycline at different concentrations, the levels of E-cadherin protein and mRNA were significantly increased in the HepG2, PLC, SMMC7721, and MHCC97H cells. In contrast, the levels of vimentin protein and mRNA were decreased in these cell lines. Previous studies have suggested that E-cadherin is suppressed as a result of the expression of Twist and Snail, two transcriptional suppressors of E-cadherin (15). Doxycycline pretreatment also decreased the expression of Twist and Snail (Fig. 6A). These results indicate that doxycycline controls a series of regulators linked to EMT and metastasis in human HCC cells. Furthermore, immunofluorescence results revealed the
upregulation of the expression of an epithelial marker (E-cadherin) and the downregulation of a mesenchymal marker (vimentin) after cells were treated with doxycycline (Fig. 6D).

3.2 Further analyses of the effects of doxycycline on the specific downregulation of E-cadherin expression

To investigate whether the doxycycline-induced increase in the E-cadherin protein level was due to increased synthesis or decreased degradation, we used shRNA to knock down the expression of E-cadherin mRNA. Doxycycline treatment was then performed for 24 h. Immunofluorescence, western blot, semi-quantitative RT-PCR, and quantitative real-time PCR analyses were performed to assess changes in the cells’ morphology and protein and mRNA expression levels (Fig. 7A-7D). The specific downregulation of E-cadherin expression caused the HepG2, PLC, SMMC7721, and MHCC97H cells to lose their cell junctions and acquire a mesenchymal-like phenotype. After the cells were exposed to doxycycline for 24 h, the levels of E-cadherin protein and mRNA increased. These result indicated that the increase in the E-cadherin protein level was caused by decreased protein degradation.

3.3 Effects of doxycycline on DNMT activity and E-cadherin promoter methylation

E-cadherin expression is regulated by several mechanisms, including methylation. A DNMT activity assay showed that doxycycline at a high concentration significantly reduced DNMT activity. MSP was performed to assess the methylation of CDH1 (the gene encoding E-cadherin). In the the MPS assay for circulating methylated E-cadherin DNA, the doxycycline-treated group showed weaker bands than the control group. In the MPS assay for circulating unmethylated E-cadherin DNA, the doxycycline-treated group showed stronger bands than the control group.
The results showed that a high concentration of doxycycline partially inhibited CpG methylation in the \textit{CDH1} promoter region, thus inhibiting EMT progression (Fig. 7E and 7F).

4 \textbf{Doxycycline inhibited VM formation in vitro}

4.1 Effect of doxycycline on VE-cadherin expression

VE-cadherin is exclusively expressed by highly aggressive tumor cells and functions as an important marker of VM (16). Western blot, semi-quantitative RT-PCR, and quantitative real-time PCR analyses were performed to determine the effect of doxycycline on VE-cadherin expression. After cells were exposed to doxycycline at different concentrations, the protein and mRNA levels of VE-cadherin were significantly decreased (Fig. 8A1 and 8C). Poorly differentiated tumors are characterized by high VE-cadherin expression and low E-cadherin expression. After E-cadherin expression was specifically downregulated, the VE-cadherin expression did not change significantly. After the cells were exposed to doxycycline for 24 h, the VE-cadherin protein and mRNA levels decreased (Fig. 8A2 and 8C). These results indicate that doxycycline controls VM formation in human HCC cells.

4.2 Effects of doxycycline on MMP-2 and MMP-9 expression

The expression and secretion of MMPs are important in ECM remodeling. Gelatin zymography was performed to measure the activity of MMP-2 and MMP-9 (Fig. 8B) in cells grown in DMEM without FBS. The results showed that the activities of MMP-2 (62 kDa) and MMP-9 (92 kDa) were downregulated in the doxycycline-pretreated group. The activities of MMP-2 and MMP-9 in the doxycycline group were significantly lower than those in the control group.

4.3 Doxycycline inhibited VM channel formation in vitro

A Matrigel 3D culture was used as a well-established in vitro model to
investigate VM formation. SMMC-7721 cells formed typical tube-like structures on the surface of the Matrigel medium. MHCC97H cells appeared to form tube-like structures (Fig. 8D). After pretreatment with doxycycline, SMMC-7721 and MHCC97H cells lost the ability to undergo VM formation in vitro compared with the control group.

Discussion

Tetracyclines, which were first discovered in 1948 by Duggar, are a well-known and widely used family of antibiotics. These compounds represent a class of chemical agents with multiple therapeutic benefits. Since the early 1950s, studies have shown that tetracyclines are involved in various biological actions that are independent of their antimicrobial activities, including anti-inflammatory, induction of apoptosis, immunomodulation, the inhibition of proteolysis, angiogenesis, and tumor metastasis (17-20). Tetracyclines produce antibiotic effects primarily by binding to the bacterial 30S ribosomal subunit and inhibiting protein synthesis (21). The machineries of prokaryotic protein synthesis and eukaryotic mitochondria are similar; therefore, tetracyclines can interfere with mitochondrial protein synthesis in mammalian cells (22). In terms of non-antibiotic properties, doxycycline, a semisynthetic tetracycline, has been shown to inhibit angiogenesis in both humans (23) and animal models (24). True angiogenesis inhibitors usually display a broad spectrum of activity in tumors, providing a compelling basis for antiangiogenic cancer therapy (25, 26). Some researchers have shown that doxycycline participates in the inhibition of tumor invasion, proliferation, and metastasis (27, 28). Doxycycline was rationally designed to inhibit the activity of MMPs; thus, its use should reduce the risk of tumor cells spreading to distant organs by invasion and metastasis.

EMT is a transdifferentiation process by which epithelial cells lose their
epithelial characteristics and acquire the ability to invade, resist apoptosis, and disseminate. A set of pleiotropically acting transcription factors, such as Snail, Twist, coordinate EMT and related migratory processes. As a result, the regulated cells acquire a fibroblast-like, mesenchymal appearance and exhibit downregulated mRNA expression of epithelial markers (such as E-cadherin) and upregulated mRNA expression of mesenchymal markers (such as vimentin). We hypothesize that doxycycline interferes with the mitochondrial protein synthesis of the transcription factors that downregulate E-cadherin expression, thus blocking EMT progression.

Epigenetic mechanisms play an essential role in the regulation of EMT (29). The term “epigenetics” refers to mitotically and meiotically heritable changes in gene expression that are not encoded in the DNA sequence. Epigenetic regulation involves three types of changes: DNA methylation, histone modification, and RNA-associated silencing. These processes initiate and sustain epigenetic silencing, and each of these changes participates in the regulation of EMT and cancer metastasis (30, 31). DNA methylation also functions in gene silencing, which commonly occurs at CpG sites via the activity of DNMT enzymes (32). The hypermethylation of the E-cadherin promoter in cancer, an important event associated with the loss of E-cadherin gene expression in cancer progression, is due to an epigenetic event, rather than a genetic mutation (33). Our study showed that doxycycline could inhibit the activity of DNMTs. Therefore, the tumor suppressor gene E-cadherin showed higher expression in the treatment group than in the control group. The MSP assay also showed that doxycycline could partially inhibit CDH1 promoter methylation. These results indicated that doxycycline could inhibit EMT. Doxycycline also affected the synthesis and activity of MMPs. MMPs degrade and modify the ECM and cell-ECM and cell-to-cell junctions, which facilitates the detachment of epithelial cells from
surrounding tissues. There is evidence that MMPs can function as promoters and mediators to induce EMT or EMT-related processes in cultured cells (34, 35). By targeting MMPs for catalytic inhibition, it may be possible to interfere in MMP-induced or -mediated EMT. In the present study, gelatin zymography assay results showed that the expression levels and activity of MMP-2 and MMP-9 were lower in the doxycycline treatment group than in the control group. Although the molecular mechanism involved in the inhibitory effects of doxycycline on MMPs remains unclear, several possible explanations can be considered: (a) doxycycline may diminish the stability of MMP mRNA (36); (b) doxycycline chelates Zn\(^{2+}\), which is necessary for MMP activity; (c) doxycycline may eliminate reactive oxygen species secreted by ECM cells and consequently inhibit the activation of pro-MMPs; and (d) doxycycline may block the activation of MMPs via the MT1-MMP pathway (28).

In 1999, Maniotis et al. (3) first reported the presence of VM in highly aggressive uveal melanoma. Our previous studies showed that VM was associated with metastasis in HCC and resulted in a shorter survival period (6). The unique structure of VM channels directly exposes tumor cells to the bloodstream; thus, tumor cells can easily enter the microcirculation and metastasize to other organs. This finding may explain the increased risk of metastasis or tumor recurrence and the shorter survival period of patients with VM-positive HCC. Additionally, our previous studies showed that the activation of EMT may be implicated in VM formation (4). These findings indicate that VM channels can be considered as an additional target in antiangiogenic strategies to treat solid tumors (5, 37). In this study, MHCC97H tumor cell growth was reduced by doxycycline treatment, and the survival period was prolonged. We also observed that the doxycycline-treated group contained fewer endothelium-dependent vessels, fewer VM channels, and larger areas of necrosis.
compared with the control group. The gelatin zymography assay results showed that
the expression levels and activities of MMP-2 and MMP-9 were lower in the
doxycycline-treated group than in the control group. In a 3D culture system, VM
formation was significantly reduced in SMMC7721 and MHCC97H cells after
treatment with doxycycline. Furthermore, VE-cadherin, a significant biological
marker of HCC cell plasticity and EMT in endothelial cells, was highly expressed in
the VM-associated tumor cells. VE-cadherin expression was highly decreased in the
doxycycline-treated group. These findings support the hypothesis that doxycycline
blocks the blood supply from endothelium-dependent vessels and VM channels.

The proposed mechanism of the pharmacokinetic properties of doxycycline
involves this drug’s ability to chelate metal ions, such as Ca\(^{2+}\) and Mg\(^{2+}\), which are
required by enzymes to maintain the proper conformation and hydrolytic activity.
Doxycycline primarily binds to these cations along the lower peripheral regions and
then circulates in the blood plasma, primarily as complexes with Ca\(^{2+}\) or Mg\(^{2+}\). The
chelation of metal ions by doxycycline has important biological implications; for
example, calcium ionophores can transport bound Ca\(^{2+}\) and Mg\(^{2+}\) through lipophilic
areas, such as cellular membranes, to deliver these cations and doxycycline to
intracellular compartments (38). Cadherins depend on calcium for their functions.
Calcium binding is also required for cadherin-mediated cell adhesion; the removal of
calcium abolishes cell adhesion, making cadherins vulnerable to proteases (39).
Calcium binding is also important in E-cadherin dimer formation, and the loss of
calcium induces a remarkable reversible conformational change in the entire
extracellular region of E-cadherin. Therefore, calcium is required, at least in part, to
maintain the integrity of cell junctions. Calcium regulation is possibly one of the
mechanisms underlying the anti-cancer activity of doxycycline.
In conclusion, this study demonstrated that the inhibitory effects of doxycycline on EMT progression and VM formation in HCC cells involved the inhibition of DNMTs and MMP activities. (a) Doxycycline sequentially inhibited DNMTs and MMP activity to influence EMT progression. (b) EMT has been reported to contribute to the formation of VM, and the upregulation of EMT-associated transcription factors has been demonstrated in VM-forming tumor cells. By inhibiting EMT progression, doxycycline inhibited VM formation. (c) EMT progression and VM formation may be significant factors influencing tumor invasion and metastasis. This study shows that doxycycline has the ability to interfere with these processes and suggests that doxycycline may have the ability to inhibit tumor invasion and metastasis. Currently, epigenetics is an accepted, mainstream area of cancer research, which is producing information about the epigenetic mechanisms that underlie EMT and tumor progression. Research in this field is also promoting new treatments by elucidating multiple drug actions and the mechanism of doxycycline. These data may form the basis of further studies involving tetracycline-based antibiotics for tumor treatment.

References
27. Sun T, Zhao N, Ni CS, Zhao XL, Zhang WZ, Su X, et al. Doxycycline inhibits the adhesion and


Figure legends

Fig. 1 (A) Tumor sizes and growth curves in the treatment and control groups. (B) A Kaplan-Meier survival analysis showed that the treatment group exhibited a longer survival time than the control group. (C) Endothelial-dependent vessels lined with shuttle-like endothelial cells were positive for both endomucin and PAS (×400, Bars: 50μm). (D) The VM channels formed by tumor cells were negative for endomucin; the basement membrane-like structure between the red blood cells and the tumor cells was positive for PAS (×400, Bars: 50μm). (E) Expression levels of VM- and EMT-associated markers in engrafted HCC samples. The E-cadherin expression was
higher in the doxycycline-treated group than in the control group. In contrast, VE-cadherin, vimentin, Snail, and Twist exhibited lower expression in the doxycycline-treated group than in the control group (×400, Bars: 50μm). (F and G)

Effects of doxycycline treatment on HCC cell viability and proliferation. (F) An MTT assay indicated the suppression of cell growth in the doxycycline-treated groups. (G) A clonogenic assay showed significantly reduced cell growth in the doxycycline-treated group compared with the control group.

Fig. 2 Effect of doxycycline treatment on HCC cell adhesion. (A) The adhesion assay showed that a single doxycycline treatment promoted HCC cell adhesion. (B) The total protein content (indicated by the OD) of adherent and suspended cells was altered by doxycycline in a dose- and time-dependent manner (P < 0.05) (×200, Bars: 50μm).

Fig. 3 Effect of doxycycline treatment combined with CoCl$_2$ on HCC cell adhesion. (A) Doxycycline combined with CoCl$_2$ also significantly promoted HCC cell adhesion. (B) The treatment of doxycycline produced a dose- and time-dependent adhesion-promoting effect in HCC cells (P < 0.05) (×200, Bars: 50μm).

Fig. 4 Effect of doxycycline treatment on the motility ability of HCC cells. (A) Images of the wound-healing assay in the control group and the treatment group (receiving different concentrations of doxycycline) at 0 and 72 h (×40, Bars: 200μm). PLC cells in a Transwell chamber without Matrigel matrix were observed at 24 and 48 h (×100, Bars: 100μm). (B) The migration ratio and numbers of migrating cells were assessed at different times in the migration assay. Compared with cells from the control group, the cells from the doxycycline treatment group moved more slowly toward the gap in a time- and dose-dependent manner (P < 0.05).

Fig. 5 Effect of doxycycline treatment on the invasion ability of HCC cells. (A)
Stained cells invading the Matrigel-coated Transwell inserts (×100, Bars: 100μm). (B) The invading cells were counted in five predetermined fields. Significantly fewer cells from the doxycycline-treated group invaded the Matrigel matrix compared with the control group (P < 0.05).

Fig. 6 (A) The expression levels of EMT-associated proteins, including E-cadherin, vimentin, Snail, and Twist, were examined via western blot; β-actin was used as the loading control. E-cadherin was upregulated in a dose-dependent manner in the doxycycline-treated group compared with the control group. In contrast, vimentin, Snail, and Twist were downregulated in the doxycycline-treated group. (B) Semi-quantitative RT-PCR was performed to measure the mRNA expression levels of E-cadherin and vimentin. Compared with the control group, the doxycycline-treated group exhibited E-cadherin upregulation and vimentin downregulation. GAPDH was used as the loading control. (C) Quantitative real-time PCR was performed to measure the mRNA expression levels of E-cadherin and vimentin. (D) Immunofluorescence staining of E-cadherin and vimentin. Cells from the doxycycline-treated group showed higher E-cadherin expression but lower vimentin expression than cells from the control group. A green signal indicates staining of the protein of interest, whereas a blue signal indicates nuclear DNA staining with DAPI (×400, Bars: 50μm).

Fig. 7 After the E-cadherin expression was downregulated using short hairpin RNA (shRNA), doxycycline treatment was performed for another 24 h. (A) Western blotting was used to detect the expression of EMT-associated proteins, including E-cadherin, vimentin, Snail, and Twist; β-actin was used as the loading control. E-cadherin was upregulated in the doxycycline-treated group compared with the E-cadherin shRNA group. In contrast, the other proteins were upregulated in the E-cadherin shRNA group but were downregulated in the doxycycline-treated group.
(B) Semi-quantitative RT-PCR was performed to measure the mRNA expression levels of E-cadherin and vimentin. GAPDH was used as the loading control. Compared with the E-cadherin shRNA group, the doxycycline-treated group exhibited higher E-cadherin expression but lower vimentin expression. (C) Quantitative real-time PCR was performed to measure the mRNA expression levels of E-cadherin and vimentin. (D) Immunofluorescence staining revealed higher E-cadherin expression in the doxycycline-treated group than in the E-cadherin shRNA group (×400, Bars: 50μm). (E) DNMT activity was significantly decreased by treatment with doxycycline at high concentrations (P < 0.05). (F) The MSP assay showed that a high concentration of doxycycline partially inhibited CDH1 promoter methylation.

Fig. 8 (A1 and A2) VE-cadherin expression was examined via western blot and semi-quantitative RT-PCR assays in the treatment groups (different doxycycline concentrations) and the E-cadherin shRNA group. Doxycycline downregulated the VE-cadherin protein and mRNA expression levels in the group treated with doxycycline alone and in the group treated with both E-cadherin shRNA and doxycycline. (B) Gelatin zymography revealed that doxycycline strongly decreased the MMP-2 and MMP-9 activities. (C) Quantitative real-time PCR was performed to measure the mRNA expression levels of VE-cadherin. (D) The effect of doxycycline on VM formation in HCC cells cultured on Matrigel matrix. SMMC-7721 (×100, Bars: 100μm) and MHCC97H (×200, Bars: 50μm) cells formed typical tube-like structures in the 3D culture. After doxycycline was administered, VM formation was significantly reduced in the HCC cells. The arrows show the tube-like structures and hollow tubular networks in the 3D culture.
Figure 2

A  Control  Doxycycline

HepG2  

PLC  

SMMC  

97H  

B  Adherent cells

Suspension cells

Control  1µg/ml  5µg/ml  10µg/ml

Time Points (h)

OD

Time Points (h)

OD
Figure 3

A | CoCl₂ | Doxycycline+CoCl₂
---|---|---
HepG₂ | ![Image](image1)
PLC | ![Image](image2)
SMMC | ![Image](image3)
97H | ![Image](image4)

B | Adherent cells | Suspension cells
---|---|---
24h | ![Graph](graph1)
36h | ![Graph](graph2)
48h | ![Graph](graph3)
Control | ![Graph](graph4)
1μg/ml | ![Graph](graph5)
5μg/ml | ![Graph](graph6)
10μg/ml | ![Graph](graph7)
Figure 4

A  

HepG2  
0h  

HepG2  
72h  

SMMC  
0h  

SMMC  
72h  

97H  
0h  

97H  
72h  

PLC  
24h  

PLC  
48h  

B

Graph showing the change in ratio over time for different treatments.

Graph showing the change in cell numbers over time for different treatments.
Figure 5

A

HepG2

PLC

SMMC

97H

Control 10 μg/ml Control 10 μg/ml

24h 48h

B

Control 10 μg/ml

Cell Numbers

24h Time Points (h)

48h

Cell Numbers

24h Time Points (h)

48h

Cell Numbers

24h Time Points (h)

48h

Cell Numbers

24h Time Points (h)

48h

*
**Figure 6**

**A**
- Control
- 1 µg/ml
- 5 µg/ml
- 10 µg/ml

E-cadherin
vimentin
Snail
Twist
Beta-actin

**B**
- Control
- 1 µg/ml
- 5 µg/ml
- 10 µg/ml

E-cadherin
vimentin
GAPDH

**C**

- Control
- 1µg/ml
- 5µg/ml
- 10µg/ml

- **E-cadherin**
- **vimentin**

**D**
- Control
- Doxycycline

- **HepG2**
- **PLC**
- **SMMC**
- **97H**
Figure 8

A1 Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml
VE-cadherin Beta-actin VE-cadherin GAPDH HepG2 PLC SMMC 97H

A2 Control shRNA-Negative Control shRNA-Doxycycline Control shRNA-Negative Control shRNA-Doxycycline Control shRNA-Negative Control shRNA-Doxycycline
VE-cadherin Beta-actin VE-cadherin GAPDH HepG2 PLC SMMC 97H

B Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml Control 1 μg/ml 5 μg/ml 10 μg/ml
MMP-9 MMP-2 HepG2 PLC SMMC 97H

C

Control 1 μg/ml 5 μg/ml 10 μg/ml
VE-cadherin

HepG2 PLC SMMC 97H

D

Control Doxycycline

SMMC 97H
Molecular Cancer Therapeutics

Doxycycline as an Inhibitor of the Epithelial-to-Mesenchymal Transition and Vasculogenic Mimicry in Hepatocellular Carcinoma

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