

# HPV16 E7 increases COX-2 expression and promotes the proliferation of breast cancer

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**Abstract.** Breast cancer remains the leading cause of mortality worldwide. Human papilloma virus 16 (HPV16) may serve a function in the pathogenesis and development of breast cancer. However, the detection rate of HPV16 in breast carcinoma may vary by region. In the present study, the expression of HPV16 E7 in paraffin-embedded tissues from patients with breast cancer from North China was detected. Additionally, the molecular mechanisms underlying the function of HPV16 E7 in the proliferation of breast cancer cells were examined. The results demonstrated that the DNA of HPV16 E7 was detected in 30.5% of the samples, and that HPV16 E7 promoted the proliferation of breast cancer cells *in vitro* and *in vivo*. Additionally, HPV16 E7-mediated proliferation of breast cancer cells was suppressed in response to treatment with cyclooxygenase-2 (COX-2)-specific small interfering RNA and celecoxib. The results of the present study revealed that HPV16 E7 may promote the proliferation of breast cancer cells by upregulating COX-2, suggesting that COX-2 may be a potential therapeutic target for HPV16 E7-mediated progression of breast cancer.

## Introduction

It has been reported that breast cancer is one of the main causes of female mortality from cancer worldwide in 2017 (1). The incidence of human breast cancer is associated with various factors, including lifestyle, medical conditions, oncogenes and virus infection (2-7). It has been reported that human papillomaviruses (HPVs), including high risk-HPV (HR-HPV), may be responsible for the pathogenesis and development of

breast cancer. HPV16 is the most prevalent type of HR-HPVs. The integration of the DNA of HPV16 into host cells promotes a constitutive high expression of E7 oncoprotein, leading to carcinogenesis *in vivo* (8). A number of HPV vaccines have been developed to prevent HPV16-mediated cancer progression (9,10). However, these vaccines have been proven to be inefficient for the treatment of HPV16-mediated cancer. Polymerase chain reaction (PCR) has been employed for the diagnosis of HR-HPVs (11). However, the detection rate of HPV16 in breast carcinoma may vary by region (12,13). As ~80% of all breast cancers are invasive ductal carcinoma, the expression of HPV16 E7 was examined in samples from patients with invasive breast ductal carcinoma from North China in the present study (14). Additionally, novel therapeutic targets for HPV16 E7-mediated cancer were investigated.

Cyclooxygenase-2 (COX-2) is a rate-limiting catalyzer in the conversion of arachidonic acid to prostaglandins (15). Furthermore, COX-2 is highly modulated in various malignancies, including breast carcinoma (16,17). Several reports have demonstrated that the suppression of COX-2 by selective inhibitors has antiviral effects on viral agents, including herpes simplex virus, avian influenza A (H5N1) and hepatitis C virus (18-20). Celecoxib is a selective COX-2 inhibitor and has been reported to be used for the treatment of various types of cancer (21-23). Evidence suggests that COX-2 is overexpressed in various HPV-induced lesions (24,25). However, it remains unknown whether the upregulation of COX-2 results from HPV E7-mediated progression in breast carcinoma.

In the present study, the expression of HPV16 E7 was detected in samples from patients with invasive breast ductal carcinomas from North China. Additionally, the molecular mechanisms underlying the function of HPV16 E7 in the proliferation of breast cancer cells were examined.

## Materials and methods

**Tissue specimens.** A total of 59 cases of invasive breast ductal carcinoma were collected from the patients who underwent resection in the Department of Pathology, Third Affiliated Hospital of Xinxiang Medical University (Henan, China) between January 2010 and March 2012. All patients were females and did not receive chemotherapy, radiotherapy or immunotherapy prior to surgery. All specimens were subjected to haematoxylin and eosin staining and diagnosed as invasive

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ductal breast carcinoma by two pathologists. All participants provided written informed consent and the tissue acquisition protocol was approved by the Institutional Board of Xinxiang Medical University (Xinxiang, China).

**DNA isolation and quantitative (q)PCR.** Invasive breast ductal carcinoma tissues were fixed in neutral buffered formalin, embedded in paraffin and stored at room temperature. Paraffin-embedded tissue samples were cut into 4  $\mu$ m-thick sections. The sections were deparaffinized in xylene and then were rehydrated in a descending ethanol series. The sections were dried at room temperature and digested with Proteinase K (Qiagen GmbH, Hilden, Germany) overnight at 56°C. The following day, the samples were incubated for 1 h at 90°C to inactivate Proteinase K and total DNA was extracted from paraffin-embedded tissues using QIAamp DNA FFPE Tissue kit (Qiagen GmbH), according to the manufacturer's protocol. The primers for HPV16 E7 and COX-2 are presented in the Table I. The thermocycling conditions were as follows: Initial denaturation for 1 min at 95°C followed by 40 cycles of denaturation at 95°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. DNA content was measured using Applied Biosystems 7500 Sequence Detection system and SYBR Green I (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The data were normalized to the geometric mean of the housekeeping gene GAPDH and quantified using the  $2^{-\Delta\Delta C_q}$  method (26).

**Immunohistochemistry.** The expression of HPV16 E7 and COX-2 in invasive breast ductal carcinoma tissues were detected using immunohistochemistry streptavidin peroxidase (SP) method. IHC SP-9000 kit which includes 3% H<sub>2</sub>O<sub>2</sub>, goat serum, biotinylated second antibody and avidin-biotin complex reagent. (SP-9000, Ready to use, ZSGB-BIO; OriGene Technologies, Inc., Beijing, China). Paraffin-embedded tissue samples were cut into 4 mm-thick sections and incubated at 60°C for 1 h. The sections were deparaffinized in xylene and then were rehydrated in a descending ethanol series. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide at room temperature for 30 min. Antigens were retrieved with citrate buffer (0.01 M; pH 6). The tissue sections were blocked for 20 min with normal non-immune serum and then incubated with mouse anti-HPV16 E7 (sc-51951; 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or rabbit anti-COX-2 (12375-1-AP; 1:100; ProteinTech Group, Inc., Chicago, IL, USA) primary antibodies overnight at 4°C. Following the primary incubation, the sections were incubated with pre-diluted biotinylated second antibody and avidin-biotin complex reagent according to the manufacturer's instructions at room temperature for 20 min. The staining was visualized using diaminobenzidine. In negative controls, the primary antibody was replaced by PBS. The results of immunohistochemical staining were evaluated by two independent pathologists blind to the clinical findings. The staining intensity of COX2 was assessed with a standard immunoreactive score (27).

**Cell culture.** MCF-7 cells stably expressing HPV16 E7 (MCF-7/HPV16 E7) or negative control (MCF-7/vector) were established previously (28). Cells were maintained

in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) with the temperature of 37°C and 5% CO<sub>2</sub>.

**Western blot analysis.** Total protein was extracted from cells with 1X sample buffer (3.8 g Tris base, 2.5 ml 10% SDS, 50 ml Glycerin, 75 ml 20% SDS, then ddH<sub>2</sub>O added to a total volume of 500 ml). Protein lysates obtained from the cells and protein determination was measured by BCA protein assay kit (20201ES86; Shanghai Qcbio Science and Technologies Co., Ltd, Shanghai, China). A total of 20  $\mu$ l protein lysates was loaded per lane and then was separated by SDS-PAGE (10.5% gels) and transferred onto a polyvinylidene difluoride membrane (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The membranes were blocked in 5% non-fat milk, which was suspended in tris-buffered saline with Tween 20 in room temperature for 2 h and then were incubated with mouse anti-HPV16 E7 (1:200) or rabbit anti-COX-2 (1:200) primary antibodies overnight at 4°C. The membranes were incubated with the HRP-conjugated secondary antibody (7076s; anti-mouse IgG; 1:2,000; 7074s; anti-rabbit IgG; 1:2,000; CST Biological Reagent Co., Ltd., Shanghai, China) in room temperature for 1 h. The protein bands were visualized by enhanced chemiluminescence (Tanon Science and Technology Co., Ltd., Shanghai, China).

**MTT assay.** A total of 1x10<sup>3</sup> cells were seeded onto 96-well plates and cultured for 24 h. Then, 20  $\mu$ l MTT solution (Merck KGaA, Darmstadt, Germany) was added to each well prior to incubation at 37°C for an additional 4 h. Following careful removal of the medium, 150  $\mu$ l dimethyl sulfoxide (Merck KGaA) was added to the wells. The absorbance was measured at 450 nm using a microplate autoreader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Three individual experiments were performed.

**Colony formation assay.** Cells were trypsinized, plated on 6-well plates (200 cells/well) and cultured for 2 weeks. Subsequently, the cells were fixed with 4% paraformaldehyde for 5 min and stained with hematoxylin for 30 min. Visible colonies consisting of >50 cells were counted. Three independent experiments were performed.

**Soft agar assay.** A total of 1x10<sup>3</sup> cells were resuspended in 2 ml 0.3% low melting point agarose in DMEM/20% FBS and plated on top of 1 ml 0.6% agarose in the same medium in 6-well culture plates. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 2 weeks. The cell colonies were photographed using light microscope (magnification, x200).

**In vivo proliferation assay.** Female BALB/c nude mice aged 4-5 weeks (weight, 15-18 g) were obtained from the Center of Laboratory Animal Science of Guangdong (Guangzhou, China). The mice were kept in the plastic cage with sealed air filter at 27°C, in *ad libitum* feeding and maintained 10 h of light and 14 h of dark daily. All animal experiments were conducted in accordance with the China Guidelines

Table I. Primer sequences.

Gene	Forward primer	Reverse primer
HPV16E7	5'-GCATGGAGATACACCTACATTG-3'	5'-TGGTTTCTGAGAACAGATGG-3
COX-2	5'-CGAGGTGTATGTATGAGTGT-3	5'-AGTGGGTAAAGTATGTAGTGC-3
GAPDH	5'-GACTCATGACCACAGTCCATGC-3	5'-AGAGGCAGGGATGATGTTCTG-3

HPV, human papilloma virus; COX-2, cyclooxygenase-2.

for Animal Care and Ethic for Animal Experiments. The study was approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University (Henan, China). For the *in vivo* proliferation assay, a total of  $2 \times 10^6$  MCF-7/HPV16 E7 or MCF-7/vector cells were injected subcutaneously into the hind limb ( $n=6/\text{group}$ ) of the mice. Tumor size was measured using a slide caliper twice weekly (volume = length  $\times$  width  $\times$  height). After 3 weeks, mice were euthanized and tumors were excised, fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin-embedded tissue samples were cut into 4- $\mu\text{m}$  thick sections. Sections were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of ethanol (100, 100, 95, 95, 85, 70 and 0%). The sections were then stained with hematoxylin and eosin. Additionally, the tissue sections were washed three times in PBS. Citrate buffer (0.01 M, pH 6.0) was applied to the sections for 5 min under 95°C for antigen retrieval. Then IHC SP-9000 kit (SP-9000, Ready to use, ZSGB-BIO: OriGene Technologies, Inc.) was used in the detection of ki-67 expression according to the manufacturer's protocol. Firstly, the sections were incubated in 3%  $\text{H}_2\text{O}_2$  and goat serum at room temperature for 30 min respectively and then were incubated in 4°C for 10 h with primary antibody against mouse ki-67 (MX006; 1:100; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China). Subsequently, the slides were incubated with biotinylated second antibody and avidin-biotin complex reagent (SP-9000 kit) at room temperature for 20 min.

**Treatment with COX-2-specific small interfering RNA (siRNA) and celecoxib.** COX-2 siRNA (sequence, 5'-GCTCAGCCATACAGCAAAT-3') was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were transfected Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol (siRNA, 100 nmol/l; 37°C for 24 h). The control siRNA was provided by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) which was a random sequence of siRNA and incompatible with the target gene. Cells were treated with celecoxib (Selleck Chemicals, Houston, TX, USA) at the concentration of 1 mol/l (diluted in DMSO) at 37°C for 48 h (DMSO without celecoxib as the control).

**Statistical analysis.** Data were analyzed using SPSS software (v20.0; IBM Corp., Armonk, NY, USA). Data are expressed as the mean  $\pm$  standard deviation. Three individual experiments were performed. One-way analysis of variance followed by least significant difference (LSD) test was used

to examine differences between more than two group means. Mann-Whitney U-test was used to examine differences between two groups medians. The association between HPV16 E7 and COX-2 expression was analyzed using Chi-square test.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of HPV16 E7 DNA in invasive ductal breast cancer.** The DNA of HPV16 E7 was detected in 18 out of 59 cases of paraffin-embedded invasive ductal breast carcinoma samples by qPCR (Table II). Therefore, HPV16 E7 was positive for 30.5% cases of invasive ductal breast carcinoma from North China.

**HPV16 E7 promotes the proliferation of breast cancer cells in vitro and in vivo.** To further explore the function of HPV16 E7 in breast cancer, MCF-7 cells with stable expression of HPV16 E7 were used. MCF-7/vector cells were used as a control (Fig. 1A). Cell proliferation was assessed using a MTT (Fig. 1B), colony formation (Fig. 1C and D) and soft agar (Fig. 1E). The results demonstrated that stable expression of HPV16 E7 in MCF-7 cells promoted the proliferation of MCF-7 cells (Fig. 1B-F). Additionally, female BALB/c nude mice injected with MCF7/HPV16 E7 cells exhibited an increased tumor volume (Fig. 1G) and increased cellular proliferation (Fig. 1H, I) compared with control mice.

**HPV16 E7 promotes the proliferation of breast cancer cells by upregulating COX-2.** Western blot analysis demonstrated that the expression of COX-2 increased with stable expression of HPV16 E7 in MCF-7 cells (Fig. 2A). Next, MCF-7/HPV16 cells were treated with COX-2-specific siRNA (siCOX-2) and celecoxib to inhibit the expression of COX-2 (Fig. 2B). Proliferation was assessed in response to siCOX-2 and celecoxib treatment using a MTT (Fig. 2C), colony formation (Fig. 2D and E) and soft agar (Fig. 2F, G) and revealed that downregulation of COX-2 significantly inhibited proliferation of MCF-7/HPV16 cells (Fig. 2B-G). Therefore, these results suggest that HPV16 E7 may promote the proliferation of breast cancer cells by upregulating the expression of COX-2.

**The association between the expression of HPV16 E7 and COX- in invasive breast ductal carcinoma.** According to the results of IHC, immunopositive cells for HPV16 E7 were detected in the nucleus and the immunopositive cells for

Table II. Expression of HPV16 E7 and COX-2 in invasive ductal breast cancer.

Sample	Sex	Ct GAPDH (mean $\pm$ SD)	Ct of HPV16E7 (mean $\pm$ SD)	Ct of COX-2 (mean $\pm$ SD)
1	Female	25.15 $\pm$ 0.07	Undetermined	27.56 $\pm$ 0.43
2	Female	24.27 $\pm$ 0.18	25.53 $\pm$ 0.29	23.62 $\pm$ 0.23
3	Female	25.51 $\pm$ 0.32	Undetermined	27.68 $\pm$ 0.39
4	Female	23.55 $\pm$ 0.18	24.29 $\pm$ 0.09	21.38 $\pm$ 0.38
5	Female	26.42 $\pm$ 0.23	27.58 $\pm$ 0.31	25.36 $\pm$ 0.24
6	Female	26.09 $\pm$ 0.32	Undetermined	28.29 $\pm$ 0.10
7	Female	28.07 $\pm$ 0.07	Undetermined	27.18 $\pm$ 0.11
8	Female	24.58 $\pm$ 0.20	22.22 $\pm$ 0.04	23.22 $\pm$ 0.19
9	Female	26.94 $\pm$ 0.05	Undetermined	29.52 $\pm$ 0.18
10	Female	27.59 $\pm$ 0.50	Undetermined	25.67 $\pm$ 0.34
11	Female	25.07 $\pm$ 0.27	Undetermined	27.77 $\pm$ 0.35
12	Female	29.35 $\pm$ 0.16	Undetermined	28.77 $\pm$ 0.44
13	Female	25.06 $\pm$ 0.12	Undetermined	27.53 $\pm$ 0.41
14	Female	21.30 $\pm$ 0.21	Undetermined	26.64 $\pm$ 0.91
15	Female	25.44 $\pm$ 0.26	Undetermined	27.56 $\pm$ 0.29
16	Female	24.33 $\pm$ 0.15	32.17 $\pm$ 0.03	24.21 $\pm$ 0.17
17	Female	26.49 $\pm$ 0.30	Undetermined	28.32 $\pm$ 0.21
18	Female	26.28 $\pm$ 0.19	28.16 $\pm$ 0.05	24.42 $\pm$ 0.15
19	Female	28.28 $\pm$ 0.22	Undetermined	27.30 $\pm$ 0.10
20	Female	27.51 $\pm$ 0.40	Undetermined	28.52 $\pm$ 0.18
21	Female	26.62 $\pm$ 0.20	33.21 $\pm$ 0.05	26.44 $\pm$ 0.20
22	Female	27.60 $\pm$ 0.50	Undetermined	26.52 $\pm$ 0.23
23	Female	24.57 $\pm$ 0.23	Undetermined	27.58 $\pm$ 0.21
24	Female	29.44 $\pm$ 0.23	Undetermined	29.13 $\pm$ 0.18
25	Female	25.67 $\pm$ 0.17	Undetermined	24.55 $\pm$ 0.37
26	Female	24.67 $\pm$ 0.20	25.32 $\pm$ 0.27	22.33 $\pm$ 0.17
27	Female	27.36 $\pm$ 0.15	Undetermined	27.52 $\pm$ 0.37
28	Female	25.19 $\pm$ 0.13	30.27 $\pm$ 0.13	24.12 $\pm$ 0.03
29	Female	29.34 $\pm$ 0.27	Undetermined	27.19 $\pm$ 0.29
30	Female	25.52 $\pm$ 0.26	28.13 $\pm$ 0.10	23.56 $\pm$ 0.28
31	Female	24.81 $\pm$ 0.15	Undetermined	24.15 $\pm$ 0.09
32	Female	26.41 $\pm$ 0.19	Undetermined	27.45 $\pm$ 0.31
33	Female	23.28 $\pm$ 0.10	Undetermined	21.67 $\pm$ 0.10
34	Female	28.76 $\pm$ 0.30	Undetermined	28.29 $\pm$ 0.15
35	Female	24.54 $\pm$ 0.28	24.18 $\pm$ 0.03	22.59 $\pm$ 0.13
36	Female	29.60 $\pm$ 0.36	Undetermined	29.33 $\pm$ 0.29
37	Female	27.57 $\pm$ 0.31	Undetermined	26.47 $\pm$ 0.15
38	Female	29.82 $\pm$ 0.03	Undetermined	29.21 $\pm$ 0.04
39	Female	24.70 $\pm$ 0.18	33.20 $\pm$ 0.08	22.49 $\pm$ 0.35
40	Female	22.80 $\pm$ 0.16	26.53 $\pm$ 0.29	21.59 $\pm$ 0.11
41	Female	26.29 $\pm$ 0.18	Undetermined	25.67 $\pm$ 0.10
42	Female	27.37 $\pm$ 0.48	Undetermined	26.29 $\pm$ 0.15
43	Female	27.95 $\pm$ 0.02	Undetermined	26.59 $\pm$ 0.13
44	Female	30.49 $\pm$ 0.17	Undetermined	30.39 $\pm$ 0.23
45	Female	26.27 $\pm$ 0.42	Undetermined	24.74 $\pm$ 0.55
46	Female	27.44 $\pm$ 0.34	Undetermined	27.24 $\pm$ 0.22
47	Female	29.74 $\pm$ 0.16	Undetermined	29.38 $\pm$ 0.24
48	Female	22.42 $\pm$ 0.25	30.58 $\pm$ 0.31	21.32 $\pm$ 0.22
49	Female	23.71 $\pm$ 0.20	35.29 $\pm$ 0.09	23.45 $\pm$ 0.22
50	Female	23.61 $\pm$ 0.44	27.18 $\pm$ 0.01	25.42 $\pm$ 0.52
51	Female	25.12 $\pm$ 0.05	Undetermined	25.39 $\pm$ 0.10
52	Female	26.46 $\pm$ 0.31	Undetermined	26.72 $\pm$ 0.33



Table II. Continued.

Sample	Sex	Ct GAPDH (mean $\pm$ SD)	Ct of HPV16E7 (mean $\pm$ SD)	Ct of COX-2 (mean $\pm$ SD)
53	Female	27.41 $\pm$ 0.25	Undetermined	27.65 $\pm$ 0.28
54	Female	29.14 $\pm$ 0.12	Undetermined	29.22 $\pm$ 0.14
55	Female	22.97 $\pm$ 0.03	23.37 $\pm$ 0.23	21.61 $\pm$ 0.12
56	Female	26.81 $\pm$ 0.16	29.31 $\pm$ 0.15	25.27 $\pm$ 0.12
57	Female	28.49 $\pm$ 0.22	Undetermined	30.46 $\pm$ 0.46
58	Female	26.10 $\pm$ 0.02	Undetermined	24.57 $\pm$ 0.31
59	Female	25.44 $\pm$ 0.43	Undetermined	26.15 $\pm$ 0.18

HPV, human papilloma virus; COX-2, cyclooxygenase-2; SD, standard deviation.

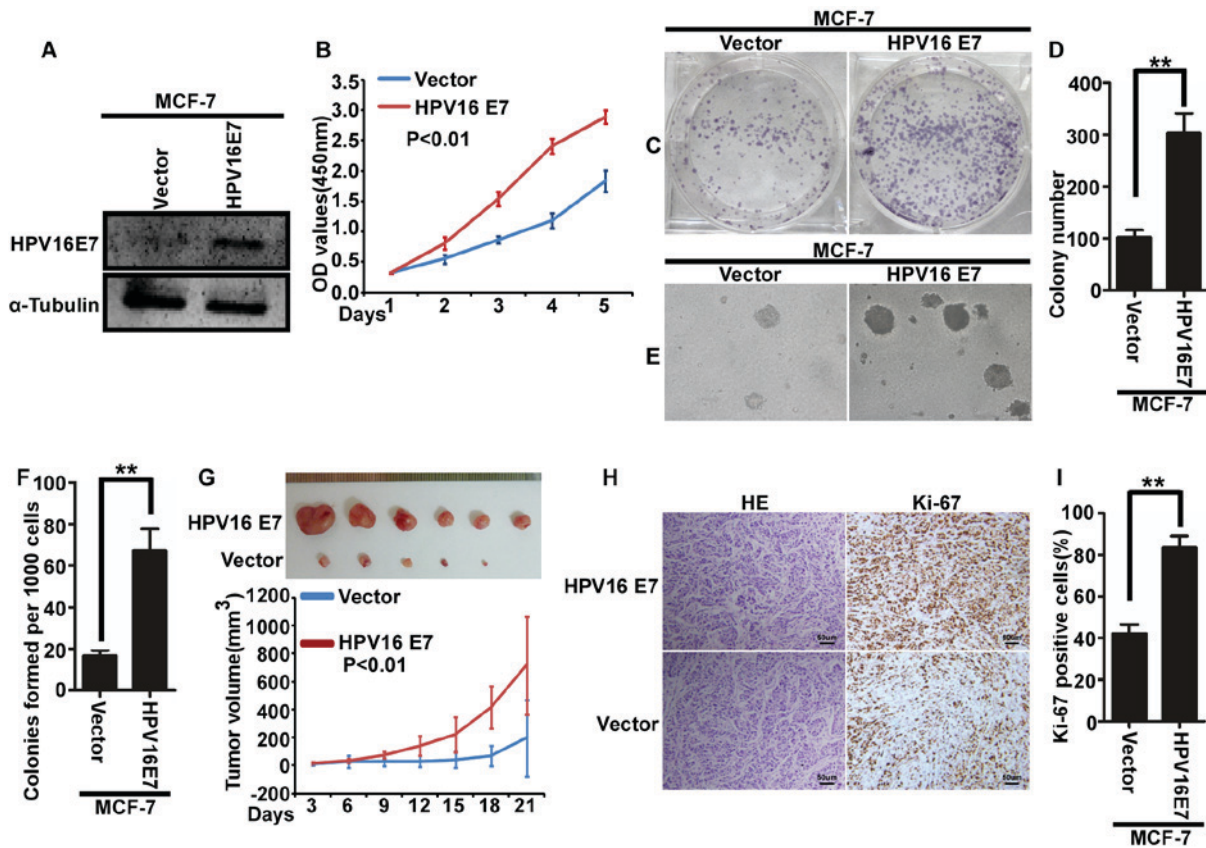


Figure 1. HPV16 E7 promotes the proliferation of breast cancer cells *in vitro* and *in vivo*. (A) The expression of HPV16 E7 in MCF-7/HPV16 E7 and MCF-7/vector cells. (B) Cell proliferation as assessed using a MTT assay. (C) Representative images and (D) quantification of colony formation. (E) Representative images and (F) quantification of soft agar colony formation. (G) Tumor volume in mice injected with MCF-7/HPV16 E7 and MCF-7/vector cells (n=6/group). Tumor volume was evaluated at indicated days. (H) Tumor sections were stained with hematoxylin and eosin or with anti-ki-67 to evaluate proliferation, or subjected to IHC staining using an antibody against ki-67. (I) Quantification of ki-67 immunopositive cells in mice injected with MCF-7/HPV16 E7 and MCF-7/vector cells. \*\*P<0.01. HPV, human papilloma virus; OD, optical density; H&E, hematoxylin and eosin.

COX-2 were detected in the cytoplasm. The expression level of COX-2 DNA in HPV16 E7-positive samples was significantly increased compared with that in HPV16 E7-negative samples (Fig. 3A, Table II). Additionally, the expression of HPV16 E7 and COX-2 was also detected using immunohistochemistry. The results demonstrated that HPV16 E7 protein was expressed in 18 cases of invasive breast ductal carcinoma (Fig. 3B). Additionally, there was an association between HPV16 E7 and COX-2 expression in invasive breast ductal carcinoma (Table III).

## Discussion

Breast cancer remains the leading cause of mortality in females worldwide. Breast cancer accounts for 1.7 million new cases and 0.5 million cases of mortality worldwide in 2012 (29). Additionally, the incidence and mortality rates of breast cancer have been increasing in the developing countries (1). HPV is a small, double-stranded and circular DNA virus that infects epithelial cells. Accumulating evidence has indicated that various HPVs, including HPV16, may

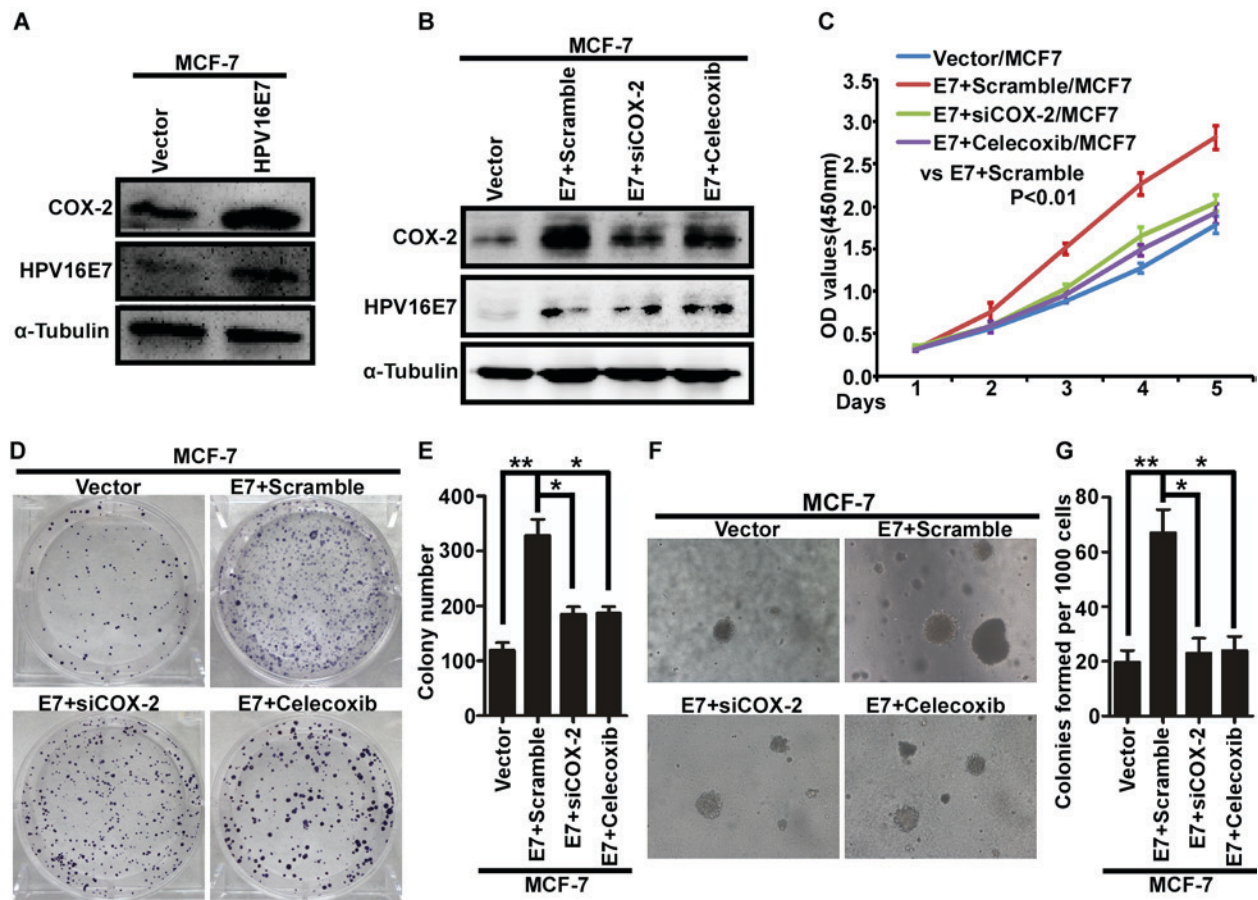


Figure 2. HPV16 E7 promotes the proliferation of breast cancer cells by upregulating COX-2. (A) The expression of COX-2 in MCF-7/HPV16 E7 and MCF-7/vector cells using western blot analysis. (B) The expression of HPV16 E7 and COX-2 in response to siCOX-2 or celecoxib treatment using western blot analysis. (C) Analysis of cell proliferation using an MTT assay. (D) Representative images and (E) quantification of colony formation. (F) Representative images and (G) quantification of soft agar colony formation. \* $P < 0.05$ , \*\* $P < 0.01$ . HPV, human papilloma virus; COX-2, cyclooxygenase-2; siCOX-2, COX-2-specific small interfering RNA.

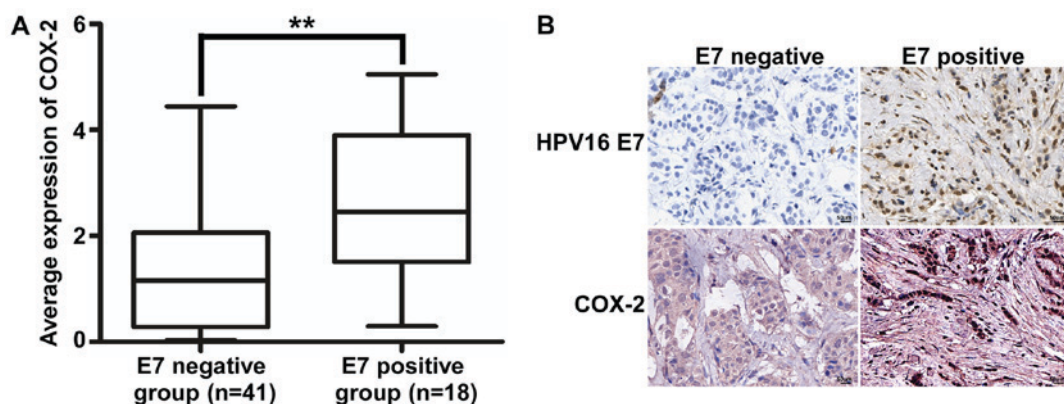


Figure 3. The association between the expression of HPV16 E7 and COX-2 in invasive breast ductal carcinoma. (A) The expression of COX-2 in HPV16 E7-positive and negative samples. \*\* $P < 0.01$ . (B) Representative images of the expression of HPV16 E7 and COX-2 in invasive ductal breast carcinoma tissues by immunohistochemistry (magnification,  $\times 400$ ; scale bar,  $50 \mu\text{m}$ ). HPV, human papilloma virus; COX-2, cyclooxygenase-2.

be involved in the pathogenesis and development of breast cancer (12,30). However, the prevalence of HPV16 may vary widely. The detection rate of HPV16 in breast carcinoma varies from 0-86%, indicating an inconsistent association between HPV16 and the progression of breast cancer. A recent meta-analysis suggested an increased risk of breast cancer associated with HPV infection (31). HPV16 encodes

a vital oncoprotein E7. HPV16 E7 serves an important function in the viral life cycle by regulating cellular proliferation and differentiation in the epithelium, and thus causing the virus to replicate and be constitutively expressed in differentiating epithelial cells (32). Additionally, HPV16 E7 may be a promising therapeutic target for cervical cancer (33).

Table III. Association between the expression of HPV16 E7 and COX-2 in invasive ductal breast cancer tissues by immunohistochemistry.

HPV16 E7 expression	COX-2 expression		$\chi^2$ value	P-value
	High	Low		
Positive	17	1	4.625	0.023
Negative	26	15		

HPV, human papilloma virus; COX-2, cyclooxygenase-2.

In the present study, the expression of HPV16 E7 DNA was detected in patients with invasive breast ductal carcinoma from North China. The results demonstrated that HPV16 E7 DNA was detected in 30.5% cases of invasive breast ductal carcinoma, suggesting that HPV16 E7 may serve an important function in the pathogenesis of breast cancer. Previous studies demonstrated that infection with HPV16 E7 increased the proliferation of breast cancer cells (34,35). The results of the present study demonstrated that the expression of COX-2 in HPV16 E7-positive samples was increased compared with that in HPV16 E7-negative samples, suggesting that the expression of COX-2 may be upregulated by the expression of HPV16 E7.

Recent studies have investigated the function of COX-2 in tumorigenesis (36). COX-2 is an important COX isoform, which is usually undetectable in normal tissues. Various factors, including hormones, cytokines and oncogenes may induce the expression of COX-2. It has been reported that COX-2 serve important functions in carcinogenesis and development of various tumors, including breast cancer (37,38). *In vivo*, it has been demonstrated that increased expression of COX-2 is associated with tumorigenesis (39). Recently, it has been demonstrated that the combination of specific COX-2 inhibitors with conventional chemotherapy may be used as a novel approach for the treatment of breast cancer. Celecoxib is a selective COX-2 inhibitor that has been used for the treatment of osteoarthritis and rheumatoid arthritis. Recently, it has been demonstrated that celecoxib prevented carcinogenesis, delayed cancer progression and enhanced the efficacy of conventional cancer therapies, including chemotherapy and radiation therapy (40-43). Considering the lack of effective therapeutic treatments for HPV-associated breast cancer, the use of celecoxib may be a promising approach. In the present study, the effect of HPV16 E7 on the expression of COX-2 was examined and it was demonstrated that COX-2 may be regulated by HPV16 E7. Additionally, *in vitro* experiments using COX-2 inhibitors demonstrated that HPV16 E7 promoted the proliferation of breast cancer cells by increasing the expression of COX-2.

The expression of HPV16 E7 and COX-2 was detected in 59 cases of invasive breast ductal carcinoma from North China using immunohistochemistry. The results revealed a positive association between the expression of HPV16 E7 and COX-2. These results suggest that COX-2

may be upregulated by HPV16 E7 and are in accordance with previous studies (24,44). Currently, there is no effective therapeutic approach for HPV-associated cancer (45). Thus, the development of therapeutic strategies for the treatment of HPV-induced cancer is required. It has been reported that celecoxib-treated HPV16+/- animals showed a lower incidence of epidermal dysplasia compared with untreated mice (46). Additionally, celecoxib was reported to significantly decrease breast tumor volume in rats (47). In conclusion, the use of celecoxib may be an effective therapeutic approach for the treatment of HPV-associated breast cancer.

In conclusion, the present study confirmed that HPV16 E7 may be an important factor in the pathogenesis of breast cancer in North China, and HPV16 E7 promoted the proliferation of breast cancer cells by upregulating the expression of COX-2. Additionally, the inhibition of COX-2 by siCOX-2 or celecoxib attenuated the HPV16 E7-mediated proliferation of breast cancer cells. Therefore, the present study may provide a potent therapeutic target for HPV16 E7-associated breast cancer.

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#### Availability of data and materials

The data generated or analyzed during this study are available from the corresponding author on reasonable request.

#### Author's contributions

XLQ and JC designed the experiments. YXW and ZYZ conducted experiments and wrote the manuscript. JQW provided research materials and methods and analyzed data.

#### Ethics approval and consent to participate

All participants provided written informed consent to participate and the tissue acquisition protocol was approved by the Ethic Institutional Board of Xinxiang Medical University.

#### Consent for publication

The relevant patients were informed and agreed to publication.

#### Competing interests

The authors declare that they have no competing interests.



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