Liao *et al. Virology Journal* (2025) 22:109
https://doi.org/10.1186/s12985-025-02731-9

Virology Journal

RESEARCH Open Access

HPV16 E7 inhibits HBD2 expression by down-regulation of ASK1-p38 MAPK pathway in cervical cancer

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Abstract

Background Recent researches indicated a down-regulation of Human beta-defensin2 (HBD2) expression in cervical cancer cells, but the mechanism and clinical significance is not clear yet.

Methods In this paper, based on the data from the TCGA database, the bioinformatics analysis provided by the UALCAN server was used. The HBD2 mRNA levels were tested with RT-qPCR in cells and protein concentration in cell cultural supernatant was assayed with ELISA. When the gene of Human papillomavirus type 16 E7 oncoprotein (*HPV16 E7*) overexpression or knockdown, the protein expression of ASK1 and p38 MAPK was detected by Western blot.

Results The bioinformatics analysis results implied that mRNA levels of HBD2 in cervical cancer were lower obviously than healthy people. HBD2 mRNA levels and protein in CaSki and SiHa cells increased obviously under the condition of *HPV16 E7* gene silence. However, HBD2 mRNA and protein levels decreased significantly in C33A and CaCo2 cells not only under the conditions of treatment with *HPV16 E7* gene overexpression, but also the inhibition of ASK1-p38 MAPK pathway by SB-203580 or GS-4997, or shRNA expression plasmid of ASK1 transefction. Moreover, p-ASK1(Thr845), the activity forming protein of ASK1, and p-p38, decreased in C33A and CaCo2 cells accompanied with HPV16 E7 overexpression, while p-ASK1(Ser966) protein, an inhibitory forming protein kept in a same stable levels. The completely opposite patterns of the protein expression in ASK1-p38 MAPK pathway were obtained in CaSki and SiHa cells transfected with HPV16 E7 siRNA sequence. Interestingly, statistical higher levels of phosphorylated p38 and cellular apoptosis rates, were found in SiHa cells exposed in Anisomycin than in DMSO solution. And increased HBD2 protein concentration in cell cultural supernatant and decreased cell survial rates, were confirmed in CaSki and SiHa cells treatment with Anisomycin, at the same time.

Conclusions Our results implied that HPV16 E7 suppresses HBD2 expression via the inhibition of the ASK1-p38 MAPK signaling pathway, and this mechanism might be a key way of anti-tumor effect of Anisomycin. This study provided a novel insight into the expression and regulation mechanism of HBD2 in tumors and offered a possible therapeutic strategy by using defensins for cervical cancer in future.

Keywords Human beta-defensin-2, Cervical cancer, HPV16, Apoptosis, Anisomycin

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Background

Based on the 2020 epidemiological survey, cervical cancer is ranked as the fourth leading cause of cancer-related mortality among women [1]. Globally, there are approximately 604,000 new cases of cervical cancer, resulting in around 342,000 deaths [2]. Persistent HPV infection is widely acknowledged as the primary cause of cervical cancer, and over 5% of all human cancers is reported associated with HPV infection [3]. Notably, HPV16 infection accounts for more than 50% of cervical cancers [4]. HPV vaccine vaccination has demonstrated high efficacy in preventing cervical cancer in health women [5]. However, these vaccines are not effective in clearing preexisting infections and associated preinvasive lesions [6]. Currently, surgery, radiation, and chemotherapy are the most common treatment options for cervical cancer, but the prognosis for patients with metastatic and recurrent disease remains bleak [7, 8]. Further research for exploring the deep mechanism and identifying new therapeutic targets is crucial for improving the treatment and prognosis of cervical cancer.

Human beta-defensin 2 (HBD2) is a low molecular weight antimicrobial peptide derived from oral epithelial cells [9, 10], which has antiviral, antibacterial and antifungal functions [11–13]. HBD2 functions as a chemoattractant for dendritic cells, T cells, and monocytes in a chemokine receptor CCR2/CCR6-dependent manner. Additionally, it plays an effective role in immune cell activation, proliferation, and differentiation, and can also serve as a vaccine adjuvant to boost immune response [14–16]. While the antimicrobial and immune-modulating effects of HBD2 have been extensively studied, the downexpression of HBD2 in cervical cancer and its impact on tumor cell proliferation and invasion remains underexplored [17]. Studies have shown that HBD2 can recruit plasmacytoid DCs to tumor sites, inhibit tumor growth, and increase T lymphocyte infiltration [18]. Decreased levels of HBD2 have been observed in oral cancer compared to healthy oral epithelium [19, 20], research indicates that upregulating HBD2 expression can effectively suppress oral cancer cell proliferation and invasion [21]. Furthermore, higher concentrations of HBD2 have been shown to inhibit epithelial cell activity [22]. In human melanoma cells, HBD2 inhibits tumor cell growth by inhibiting the expression of B-Raf, cyclin D1 and cyclin E, promoting the expression of p21 (WAF1) to activate pRB [23]. Phosphatidylinositol 4, 5-diphosphate (PIP2), is one of seven phosphorylated derivatives of phosphatidylinositol, can lead to cell permeabilization by interacting with HBD2 [15, 24]. HBD2 has been demonstrated to induce non-apoptotic cell death in tumor cells through membrane-related mechanisms, without affecting cell migration [17]. The aforementioned studies suggest that HBD2 exhibits a potent anti-tumor effect, yet the exact mechanism by which HBD2 induces tumor cell death remains unclear.

Up to this point, the role of HBD2 in cervical cancer has not been comprehensively explored. Through bioinformatics analysis, we observed a significant decrease in HBD2 expression in tumor tissues compared to adjacent tissues. Furthermore, our findings indicate that knockdown of HPV16 E7 led to a notable increase in HBD2 expression in cervical cancer cells. Mechanistically, we discovered that the HPV16 E7 protein modulates HBD2 expression by impacting the ASK1-p38 MAPK signaling pathway. Lastly, we conducted further investigations into the impact of HBD2 on the survival of cervical cancer cells. In summary, our study reveals that HBD2 is a downstream target of HPV16 E7 and contributes to the progression of cervical cancer. These results not only enhance our understanding of defensin biology, but also offer insights for potential defensin-based drug development in the future.

Methods

Bioinformatics analysis

The mRNA sequencing data of 305 cervical squamous cell carcinoma (CESC) samples and 3 normal control samples were analyzed using a bioinformatics program provided by the UALCAN server, based on the data from the TCGA database.

Plasmid constructions

The gene sequence of HPV16 E7 (Gene ID: 1489079) was retrieved from NCBI, and primers were designed using the principle of homologous recombination (Supplementary Table 1). The amplified HPV16 E7 fragment was then inserted into the pcDNA3.1-3FLAG vector, and the successful construction of plasmids was confirmed through DNA sequencing. Subsequently, the plasmid was transfected into C33A cells to assess its successful expression.

The gene sequence of ASK1 (Genen ID: 4217) and its corresponding shRNA sequence were obtained from NCBI and the professional website of https://rnaidesigner.thermofisher.com/rnaiexpress/sort.do, respectively (Supplementary Table 1). The synthesized shASK1 sequence was then ligated with the vector pShuttl12-U6-CMV-EGFP, and the successful construction of plasmids was confirmed through sequencing. The knockdown effect was validated by transfecting the shASK1 sequence into C33A and CaCo2 cells.

Cell culture and transfection

C33A and CaSki, the cervical cancer cell lines, were purchased from Fenghuishengwu (Hunan, China), while SiHa and CaCo2 cell lines were obtained from the laboratory's stock. C33A and CaCo2 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham,

MA, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China). SiHa and CaSki cell lines were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640, Gibco, Waltham, MA, USA) also supplemented with 10% FBS (Sijiqing, Hangzhou, China). All cell lines were maintained in a constant temperature incubator with 5% CO2 at 37℃.

C33A and CaCo2 cells were transfected with shASK1 plasmid or HPV16 E7 overexpression plasmid using Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) for 48 h. Western blot analysis was performed by detection of the protein expression of ASK1 and HPV16 E7, to confirm plasmids transfection efficiency. The sequences of the shASK1 plasmid and the primers for the HPV16 E7 overexpressed plasmid can be found in supplementary file 2: Table 1.

HPV16 E6/E7 SiRNA knockdown experiments

Both control siRNA and HPV16 E6 siRNA were procured from HANBI (Shanghai, China), while HPV16 E7 siRNA was obtained from Tsingke (Beijing, China). SiHa and CaSki cells underwent transfection with siRNA utilizing Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) for a duration of 48 h. The transfection efficiency was assessed using a fluorescence inverted microscope, and the knockdown effect was confirmed through RT-qPCR (Supplementary Table 1).

Western blot analysis

Cells were lysed using RIPA lysis buffer (Solarbio Technology, Beijing, China) with a protease inhibitor PMSF (100:1) for protein extraction. The extracted were then loaded onto 8–12% polyacrylamide gels, transferred to NC membranes and blocked with 5% skim milk in TBST. The NC membrane was probed with anti-HPV16 E7 (Santa Cruz, 1:200, Dallas, Texas, USA), anti-ASK1 (Proteintech, 1:1500, Wuhan, China), anti-p-ASK1 (Ser966, Proteintech, 1:500, Wuhan, China), anti-p-ASK1 (Thr845, Immunoway, 1:1500, Dallas, Texas, USA), anti-p38(Proteintech, 1:1000, Wuhan, China), anti-p-p38 (Thr180/Tyr182, Proteintech, 1:1000, Wuhan, China), anti-GAPDH (Proteintech, 1:1000, Wuhan, China) and anti-FLAG (Sigma, 1:1000, St Louis, USA) antibodies followed by overnight incubation at 4 °C. Subsequently, the membranes were washed with TBST and incubated with horseradish peroxidase - (HRP-) conjugated anti-rabbit or anti-mouse IgG (Boster Bio, 1:5000, Wuhan, China) for 2 h at room temperature. Finally, protein bands were visualized using the ECL system as per the manufacturer's instructions.

RNA extraction and real-time quantitative PCR assay (RT-qPCR)

Total RNA was extracted from cells using RNAiso Plus (Takara, Japan) reagent, followed by cDNA synthesis with the reverse transcription kit PrimeScript™ RT Master Mix (Perfect RealTime) (Takara, Japan). RT-qPCR analysis was conducted using Realtime PCR Super mix SYBR green (Mei5bio, Beijing, China) on the StepOne™ PLUS Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are provided in Supplementary Table 2. Gene expression levels were calculated

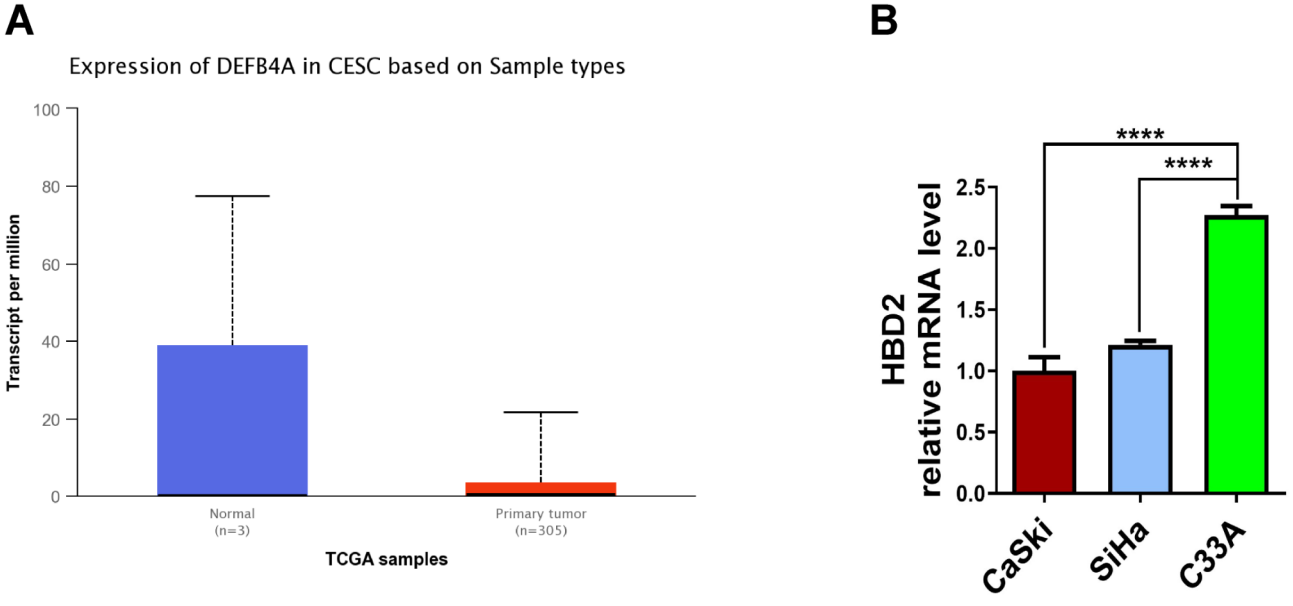

Fig. 1 The HBD2 mRNA kept relatively in lower level state in tissue of cervical cancer or HPV16 + cervical cancer cell lines. (**A**) Box plots comparing HBD2 expression levels in normal and CESC samples were generated using the UALCAN database. (**B**) HBD2 mRNA levels in HPV16 + CaSki and SiHa cell, and in HPV- C33A cells were measured by RT-qPCR.****$P < 0.0001$

relative to GAPDH, with Ct values determined for all genes. The experiment was repeated three times.

ELISA

Cell culture supernatants were collected and HBD2 production was quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) kit from NeoBioscience Technology (Shenzhou, China), following the manufacturer's instructions. The optical density (OD) values of the samples were determined at 450 nm using a microplate reader from Bio-Tek (Vermont, USA). A standard curve was generated using Origin software to calculate the concentration of the specimen.

Cell viability assay

Cell viability was assessed using the CCK-8 Cell Viability Assay Kit (APExBIO, Houston, USA). After exposure to different concentrations of Anisomycin for 24 h, cells were incubated with CCK-8 solution at 37 °C for 2–4 h. Absorbance at 450 nm were measured using a microplate reader (Bio-Tek, Vermont, USA).

Apoptosis assay

The Apoptosis Detection Kit (Bestbio, NanJin, China) was utilized to identify apoptotic cells following the manufacturer's instructions. Cells were exposed to varying concentrations of Anisomycin or DMSO for 24 h, then rinsed twice with ice-cold PBS and reconstituted in 100 µL of Annexin V binding buffer. Subsequently, 5 µL of PI was introduced to the cell suspension and left to incubate at room temperature for 15 min. Following this, 400 µL of Annexin V Binding Buffer was added, and the samples were assessed using a flow cytometer (Bio-Tek, Vermont, USA).

Statistical analysis

GraphPad Prism 6 software was utilized for statistical analysis in this study. Line charts and bar charts were generated to visually represent the data, with all statistical values presented as Mean ± SD. One-way ANOVA was employed to analysis the significance among multiple groups for data keeping a normal distribution, while t-tests were used for assessing the significance in pairwise comparisons. Statistical significance was defined as $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$.

Result

The expression levels of HBD2 in HPV16 positive cancer cell lines or the tissue with cervical squamous cell carcinoma were significantly lower than negative cancer cells or normal tissue

To compare the difference of HBD2 (DEFB4A) gene mRNA levels between normal and cervical squamous cell carcinoma (CESC) tissues, the UALCAN server's bioinformatics program was utilized to analyze TCGA database data related to CESC [25]. Analysis on mRNA data from 305 CESC samples and 3 normal control samples showed that the mRNA levels of HBD2 in CESC tissues were obviously lower than normal tissue adjacent the CESC tumor (Fig. 1A).

Literature suggests that over 50% of cervical cancer cases are associated with HPV16 infection. Building on these findings, we hypothesized whether the typical HPV16 infection in CESC is the genuine reasons to result the expression suppression of HBD2. To test this hypothesis, mRNA levels and protein levels in HPV- cervical cancer cell lines C33A cells and HPV16 + cervical cancer cells including CaSki and SiHa cells were tested by RT-qPCR analysis. The results revealed that levels of HBD2 mRNA in C33A cells were significantly higher than in CaSki and SiHa cells (Fig. 1B), indicating that HPV16 might has the potential inhibition function on HBD2 expression in HPV16 + cervical cancer.

HPV16 E7 had a more stable inhibitory effect on HBD2 expression than HPV16 E6

Since many literatures agree that HPV16 E6/E7 protein plays an important role in the pathogenesis of cervical cancer, this paper focused on the impact of HPV16 E6/E7 on HBD2 expression in cervical cancer cells. At first, we initially silenced HPV16 E6 or E7 in SiHa and CaSki cells using siRNA technology to watch the changes of HBD2 mRNA level and protein expression. The efficacy of HPV16 E6/E7 siRNA knockdown (Fig. 2A and B) was tested with mRNA levels of E6/E7 gene using RT-qPCT, and the effects of E6/E7 on HBD2 expression was identified with the relative HBD2 mRNA expression levels via RT-qPCR and the HBD2 protein expression in cell cultural supernatant by ELISA. The findings revealed that decreased expression of HPV16 E7 led to a significant increase in both HBD2 mRNA and protein levels compared to the NC group (Fig. 2C and D). Conversely, reducing HPV16 E6 expression resulted in a notable increase in the relative HBD2 mRNA expression level, while the protein level remained relatively unchanged (Fig. 2E and F). The results showed that HPV16 E7 was stronger inhibitor of HBD2 expression in cervical cancer cells than E6, so the next continuous research the topic of the study was switched to E7.

To investigate the inhibitory effect of HPV16 E7 on HBD2, an eukaryotic expression plasmid pcDNA3.1(-)-3FLAG-HPV16 E7 was generated and transfected into C33A and CaCo2 cells (Supplementary Fig. 1). The levels of HBD2 mRNA and protein were measured using RT-qPCR and ELISA, respectively. The results demonstrated a significant decrease in HBD2 mRNA and protein levels when HPV16 E7 was overexpressed, compared to the Vector group (Fig. 2G and H). Therefore, it can

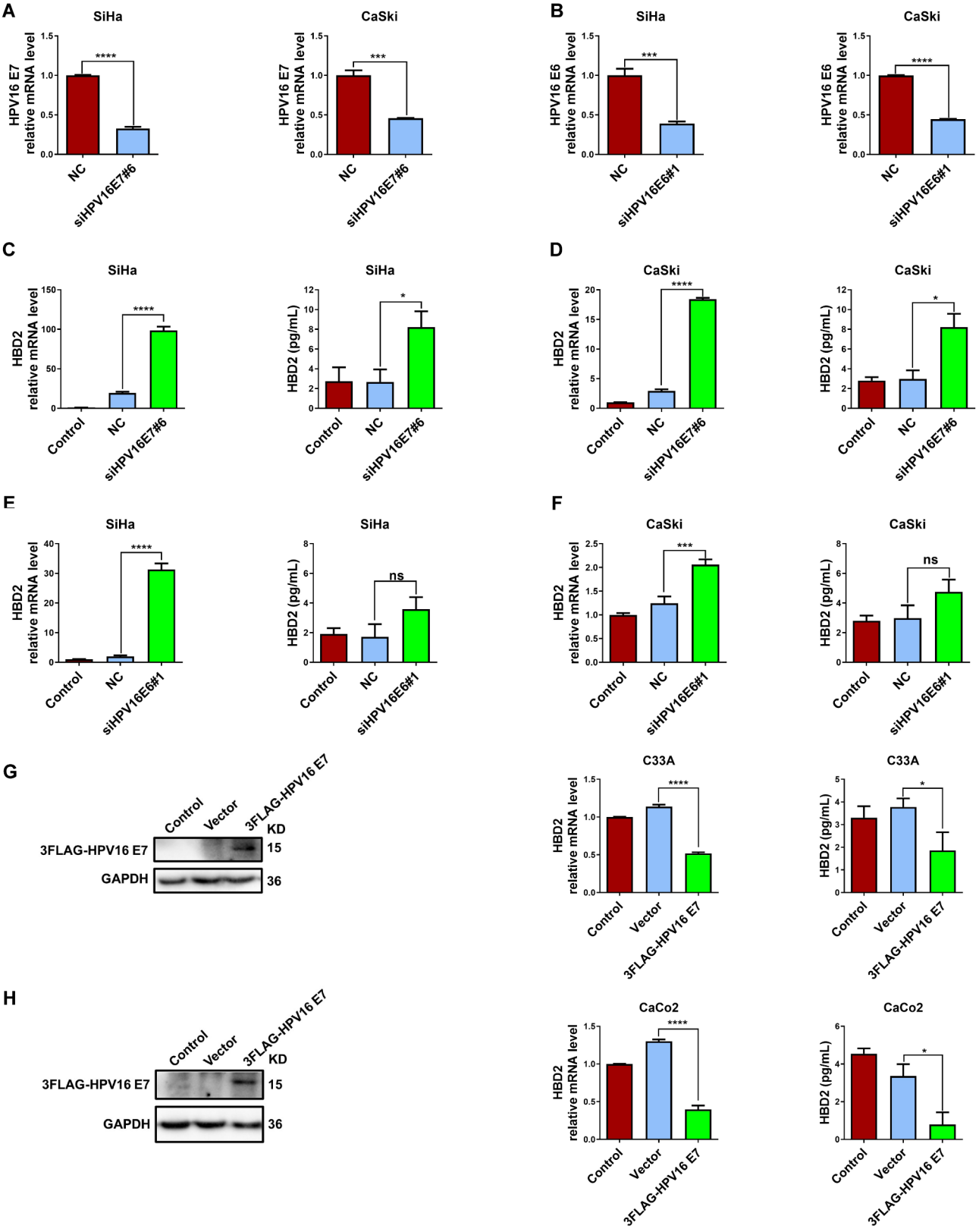

Fig. 2 (See legend on next page.)

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Fig. 2 HPV16 E6/E7 down-regulated HBD2 in cervical cancer cells. (**A**, **B**) SiHa and CaSki cells were transfected with 50 nM HPV16 E6/E7 siRNA or random control RNA sequence, respectively. The tranfection efficiency was determined with E6/E7 mRNA levels measured by RT-qPCR. HBD2 mRNA and protein levels in both cells were assessed 36 h after 50 nM of either random control RNA sequence or HPV16 E7 (**C**, **D**) or E6 (**E**, **F**) siRNA sequences. After C33A (**G**) and CaCo2 (**H**) cells were transfected with 3 μg pcDNA3.1(-)-3FLAG-HPV16 E7 or empty plasmid for 48 h, E7 protein levels was analyzed with Western blot, and HBD2 mRNA and protein levels were determined using RT-qPCR and ELISA, respectively. The data was analyzed with ANOVA to show the differences in all groups and t-tests were used for assessing the significance between any two groups. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$

be concluded primarily that HPV16 E7 inhibits HBD2 expression in cervical cancer cells.

ASK1-p38 MAPK pathway could induce HBD2 expression in cervical cancer cell

Given the close association between ASK1 and p38 MAPK in the induction of HBD2 expression [26, 27], specific inhibitors of p38 MAPK (SB-203580) were used at concentrations of 60 μM and 80 μM to treat C33A and CaCo2 cells for 24 h. The results demonstrated that, compared with the DMSO control group, a significant decrease in HBD2 expression when p38 MAPK activity was inhibited in both C33A and CaCo2 cells (Fig. 3A). Subsequently, C33A and CaCo2 cells were treated with ASK1 specific inhibitor (GS-4997) at concentrations of 60 μM and 50 μM for 24 h, the results consistently revealed a notable decrease of HBD2 expression upon the inhibition of ASK1 activity, compared to the DMSO control group (Fig. 3B). Additionally, the inhibitory regulation of ASK1 on HBD2 expression in cervical cancer cells was further identified by using cellular tranfection with a knockdown plasmid of human ASK1 (pShuttl2-U6-ASK1-CMV-EGFP) (Supplementary Fig. 2). And, as an important downstream molecules, the activation situation of p38 was tested by Western blotting at the same time. The results showed accordantly that the expression of p38 and p-p38 was markedly inhibited, compared to the sh-CK group (Fig. 3C and D). These findings suggested that ASK1-p38 MAPK signaling pathway plays a crucial role in inducing HBD2 expression in cervical cancer cells.

HPV16 E7 gene inhibited the activation of ASK1-p38 MAPK signaling pathway in cervical cancer cell

To investigate the regulation effects of HPV16 E7 on the ASK1-p38 MAPK signaling pathway in cervical cancer cells, activation levels of the key proteins in ASK1-p38 MAPK signaling pathway were tested by western blotting with the treatments both of HPV16 E7 siRNA transfection into SiHa and CaSki cells and of the pcDNA3.1(-)-3FLAG-HPV16 E7 plasmid transfection into C33A and CaCo2 cells. The results indicated that, compared to the NC group, a decreased HPV16 E7 expression led to a significant increase of p-ASK1 (Thr845) and p-p38 protein expression, and a decreased p-ASK1 (Ser966) protein expression (Fig. 4A and B). However, compared to the Vector control group, the over-expression of HPV16 E7 led to a significant decreases of ASK1 and p-p38 protein expression, and a concurrent increase in p-ASK1 (Ser966), a inhibition state of ASK1 protein, in C33A cells (Fig. 4C). Additionally, compared to the Vector group, the protein expression of p-ASK1 (Thr845) and p-p38 decreased significantly in CaCo2 cells with the transfection of pcDNA3.1(-)-3FLAG-HPV16 E7 plasmid (Fig. 4D). These findings imply that HPV16 E7 effectively inhibits the activation of the ASK1-p38 MAPK signaling pathway in cervical cancer cells. Taken together, the results concluded that HPV16 E7 inhibits HBD2 expression through the ASK1-p38 MAPK signaling pathway in cervical cancer cells.

HBD2 might involve the treatment effects of anisomycin on the cervical cancer

Because of the novel finding that the activation of p38 MAPK phosphorylation effectively boosts the expression of HBD2, and Anisomycin, well-known as an antitumor drug and a p38 MAPK activator, did we amaze whether HBD2 plays the important role of Anisomycin in treating cervical cancer. Initially, SiHa cells were exposed to Anisomycin with various concentrations of 0, 0.25, 0.5, 0.75 to 1 μM for 24 h, the results showed a notable increase in p-p38 expression levels (Fig. 5A), while compared to the 0 μM group. These outcomes indicated the capability of Anisomycin to induce p38 MAPK activation in cervical cancer cells. In the Following treatment experiments of SiHa, CaSki and C33A cells with different Anisomycin concentrations for 24 h, the results of RT-qPCR analysis demonstrated a gradual rise in HBD2 expression levels with increasing Anisomycin concentrations, while CCK8 results revealed a gradual decrease in cell viability, compared to the DMSO group (Fig. 5B and C). Flow cytometry analysis of apoptosis illustrated a progressive increase of apoptosis in SiHa and C33A cells treated with 1.0, 2.5, 5.0 μM concentrations of Anisomycin for 24 h, compared to the DMSO group (Fig. 5D and E). In summary, HBD2 mediates the anti-tumor mechanism of Anisomycin in treatment of cervical cancer, highlighting its significant clinical application prospects.

Discussion

Human beta-defensins (HBD1, 2, 3) exhibit antimicrobial properties that protect mucosal surfaces from microbial invasion. The broad-spectrum activity of these defensins in tumor cells treatment has garnered increasing clinical

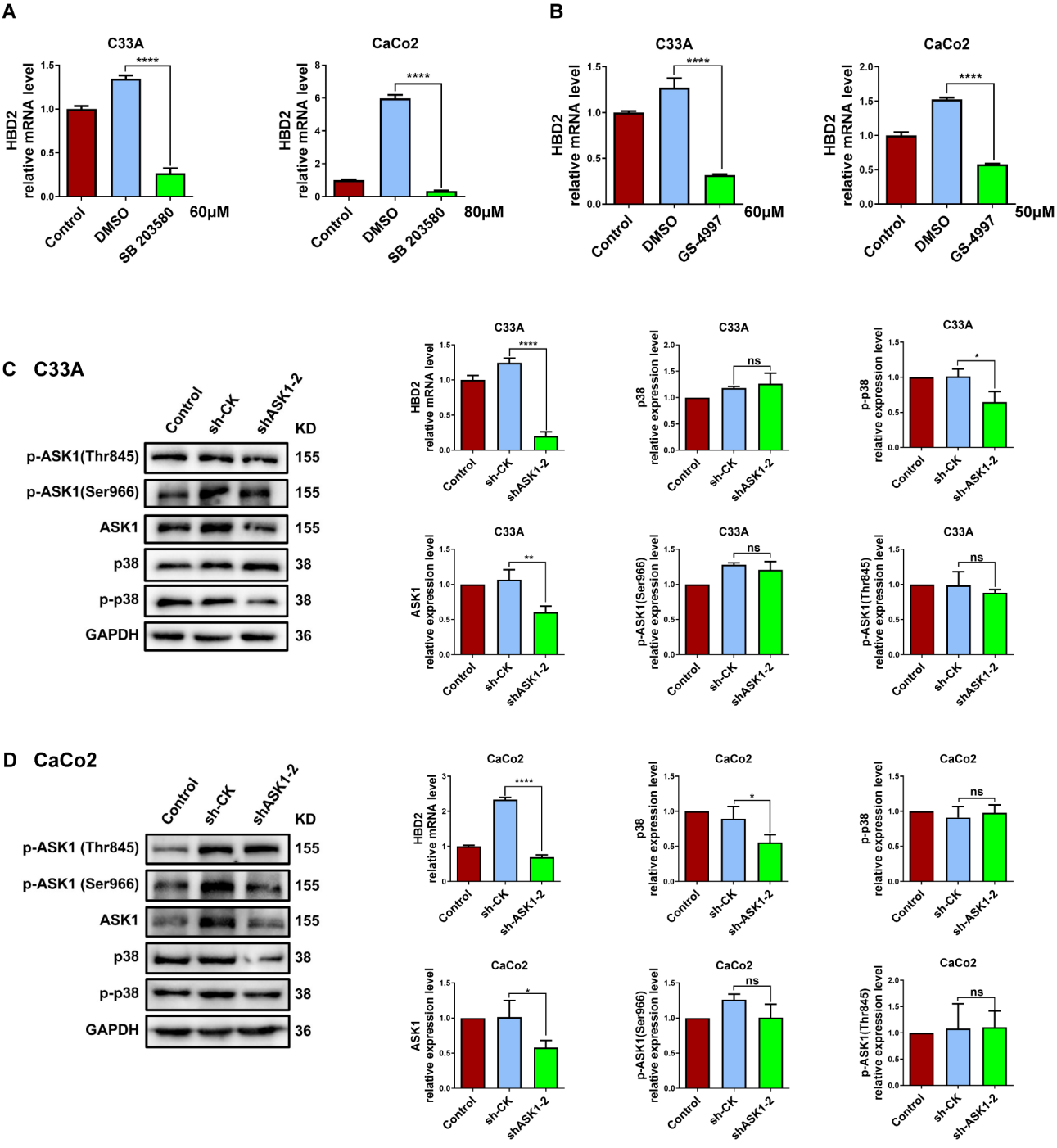

Fig. 3 The inhibition of ASK1-p38 MAPK signaling pathway reduced HBD2 expression in C33A and CaCo2 cells. (**A**) C33A and CaCo2 Cells were treated with 60 μM and 80 μM SB-203580 for 24 h, and the expression of HBD2 mRNA was assessed using RT-qPCR. (**B**) Cells were treated with 60 μM and 50 μM GS-4997 for 24 h, and the expression of HBD2 mRNA was assessed using RT-qPCR. (**C**, **D**) C33A and CaCo2 cells were transfected with pShuttl2-U6-ASK1-CMV-EGFP. Subsequently, the protein levels of ASK1, p-ASK1(Thr845), p-ASK1(Ser966), p38, and p-p38 were analyzed by Western blotting. The relative expression of these proteins was quantified using Image J software with GAPDH serving as an internal control. HBD2 mRNA levels were determined via RT-qPCR. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001

interests [28]. For instance, HBD1 has been identified as a tumor suppressor [29, 30], and the loss of mouse defensin-1 (mBD1) has been associated with heightened susceptibility to HPV16 E6/E7-induced neoplastic transformation in mouse kidney cells [31]. In cervical cancer samples, higher expression level of HBD3 have been observed, compared to healthy tissues, in promoting cervical cancer development and correlating

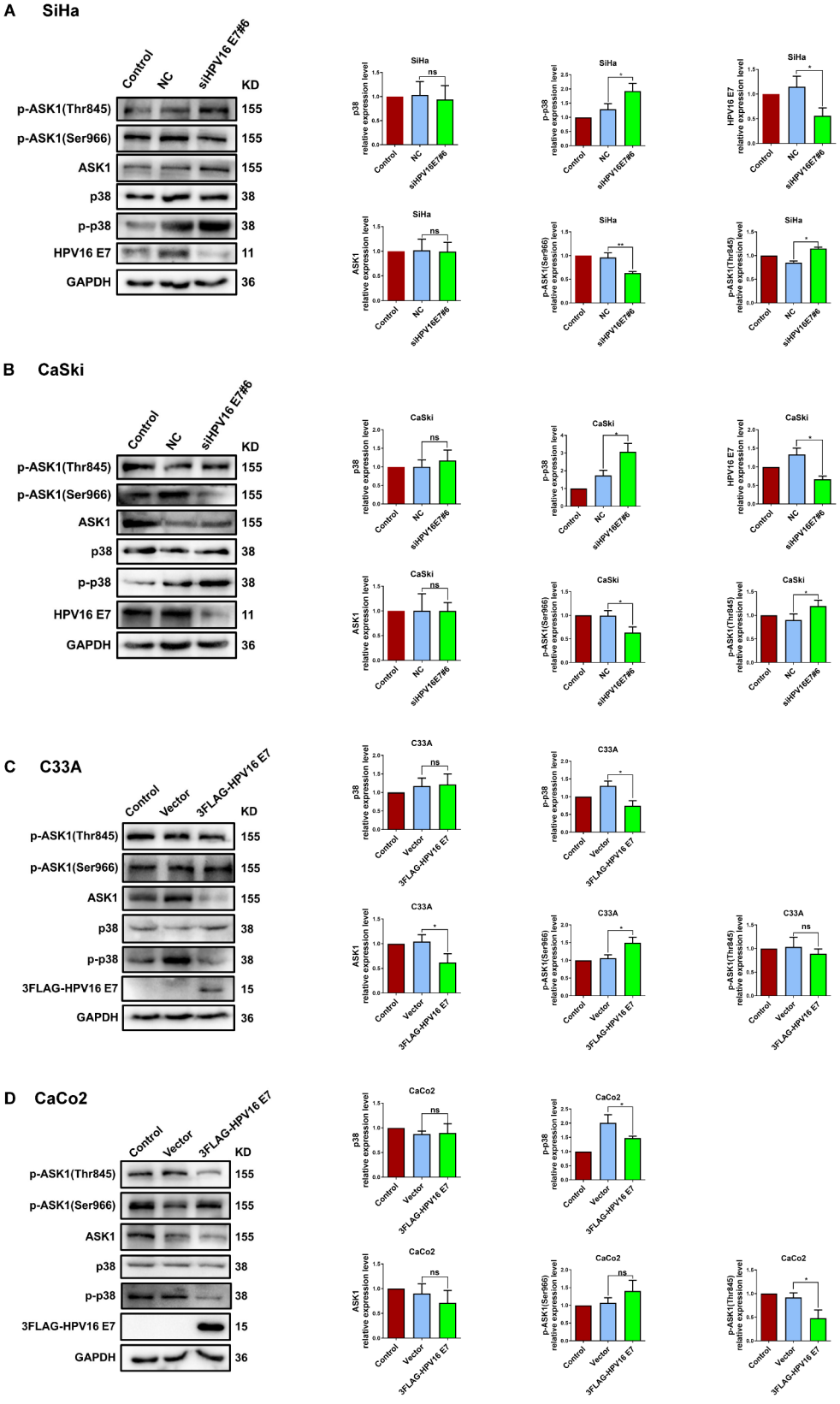

Fig. 4 (See legend on next page.)

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Fig. 4 HPV16 E7 down-regulated the ASK1-p38 MAPK signaling pathway in cervical cancer cells. 50 nM HPV16 E7 siRNA sequence or random control RNA sequence were tranfected into SiHa (**A**) and CaSki cells (**B**) for 36 h, while 3 μg pcDNA3.1(-)-3FLAG-HPV16 E7 plamids or empty control plasmids were transfected into C33A (**C**) and CaCo2 cells (**D**) for 48 h, the protein levels of HPV16 E7, ASK1, p-ASK1(Thr845), p-ASK1(Ser966), p38 and p-p38 in all cells were measured by Western blot analysis. The cells in control groups were not treated with any RNA sequence and plasmid. The relative expression of all proteins was quantified using Image J software, with GAPDH as an internal control. $^{*}P < 0.05$, $^{**}P < 0.01$

positively with disease progression through the induction of NF-κB activation [32]. Research by DasGupt revealed that HPV16 E6 can enhance HBD3 expression by targeting p53 degradation, leading to disrupted cell cycle regulation in cervical cancer [33]. In this paper, based on TCGA database, we used the analysis program provided by UALCAN [25] to explore the role of HBD2 in cervical cancer. The results showed that the expression of HBD2 was obviously decreased in cervical cancer tissues compared with normal individuals.

Although HBD2 has been demonstrated a strong antiviral capability in preventing prolonged viral infections within the host [34]. Research has yet shown a lesser increase in HBD2 levels following HR-HPV infection compared to LR-HPV infection [35], and even studies revealed the absence of HBD2 expression in HPV16-transformed keratinocytes [36]. All these document implied that HPV16 might suppress HBD2 expression by some kind of mechanism in cervical cancer and thus might also participate in cervical cancer occurrence and progression. On the other hand, numerous studies have highlighted the significant role of HPV16 E6/E7 in disrupting both innate and adaptive immune responses, and contributing to the progression of cervical cancer through persistent HPV16 infection [37–39]. Additionally, the documents reported that IL-1β, a potent inducer of HBD2, could be impeded by HPV16 E6 in keratinocytes through IRF6 antagonism [40, 41], and effectively suppressed by HPV16 E7 during various stages of cervical carcinogenesis [40, 42]. Same as the research conclusions in previous, the findings in this study also supported that the oncoproteins HPV16 E6/E7 suppresses HBD2 expression in cervical cancer cells, but HPV16 E7 exhibits a more stable inhibitory effect.

The mitogen-activated protein kinase (MAPK) cascade signaling pathway is a crucial pathway that regulates various cellular processes and plays a significant role in immune response [43]. Apoptosis signal-regulating kinase 1 (ASK1), known as MAP3K5 [44], is a key kinase in the MAPK cascade and is also well-known for its role in inhibiting tumor cell proliferation by promoting apoptosis by activating the c-Jun N-terminal kinase (JNK) and p38 MAP kinase signaling cascades. Research by Zhang revealed a significant down-regulation of ASK1 expression in cervical cancer tissues compared to adjacent non-tumor tissues and inhibit the apoptosis of the cervical cancer cell by this way [45]. By using the ToolKIT in Cistrome DB (https://zhuanlan.zhihu.com/p/687574361) to search for transcription factors related to ASK1 regulation (supplementary material Fig. 6), it was found that lots of proteins may play transcription factor function to ASK1 expression (data not available), for example, the transcription factor E2f1, mainly known as a cell cycle regulator binds directly to the ASKl promoter and up-regulates its expression in cancer cells [46]. Studies by Lin and Lee demonstrated that some cancer-promoting proteins can enhance tumor cell metastasis and proliferation by inhibiting ASK1 phosphorylation in cervical cancer [47, 48], however, ASK1 relationship with HPV infection is not unclear. Sayama's research in epidermal keratinocytes showed that ASK1 phosphorylation activation induces HBD2 expression [27], thus affirmatively aligns with the findings in cervical cancer cells in this study for the first time that HPV16 E7 suppresses the HBD2 expression by inhibiting the activation of ASK1 phosphorylation.

As a member of the MAPK family, p38 phosphorylation has been shown to be crucial in host defense against various DNA virus infections (such as HSV-1, HSV-2 and HBV) [49]. However, the reduced phosphorylated p38 MAPK activity was reported in SiHa cells and also same as our results in SiHa and CaSki cells [50]. Scharf reported that Lactobacillus pneumophila could induce HBD2 secretion by activating p38 MAPK phosphorylation in lung epithelial cells [26]. In this study, by using SB-203580 (specific inhibitor of p38 MAPK) and Anisomycin (specific activator of p38 MAPK) in SiHa, CaSki and C33A cells, respectively, our experiments implied that the activation of p38 MAPK effectively promoted HBD2 expression in cervical cancer cells and vice versa. Obviously, the data in this paper are highly consistent with literature reports in previous.

In addition, ASK1, as an upstream regulator of p38 MAPK, plays a crucial role in immune response, cell proliferation and anti-viral defense by inducing p38 MAPK phosphorylation [51], and it is also an important part of the body's anti-virus and anti-tumor defense [52]. So, it is very important to explore the mechanism of HPV16 E7 decreasing the expression of HBD2 in cervical cancer by investigation on the relationships of regulation among HPV16 E7, ASK1-p38 activation and HBD2 expression. Through many technologies, including HPV16 E7 gene silence in CaSki and SiHa cells, or gene overexpression and ASK1 shRNA expression plasmid transfection in C33A and CaCo2 cells, the findings in this research indicated that HPV16 E7 has the strong ability to suppress

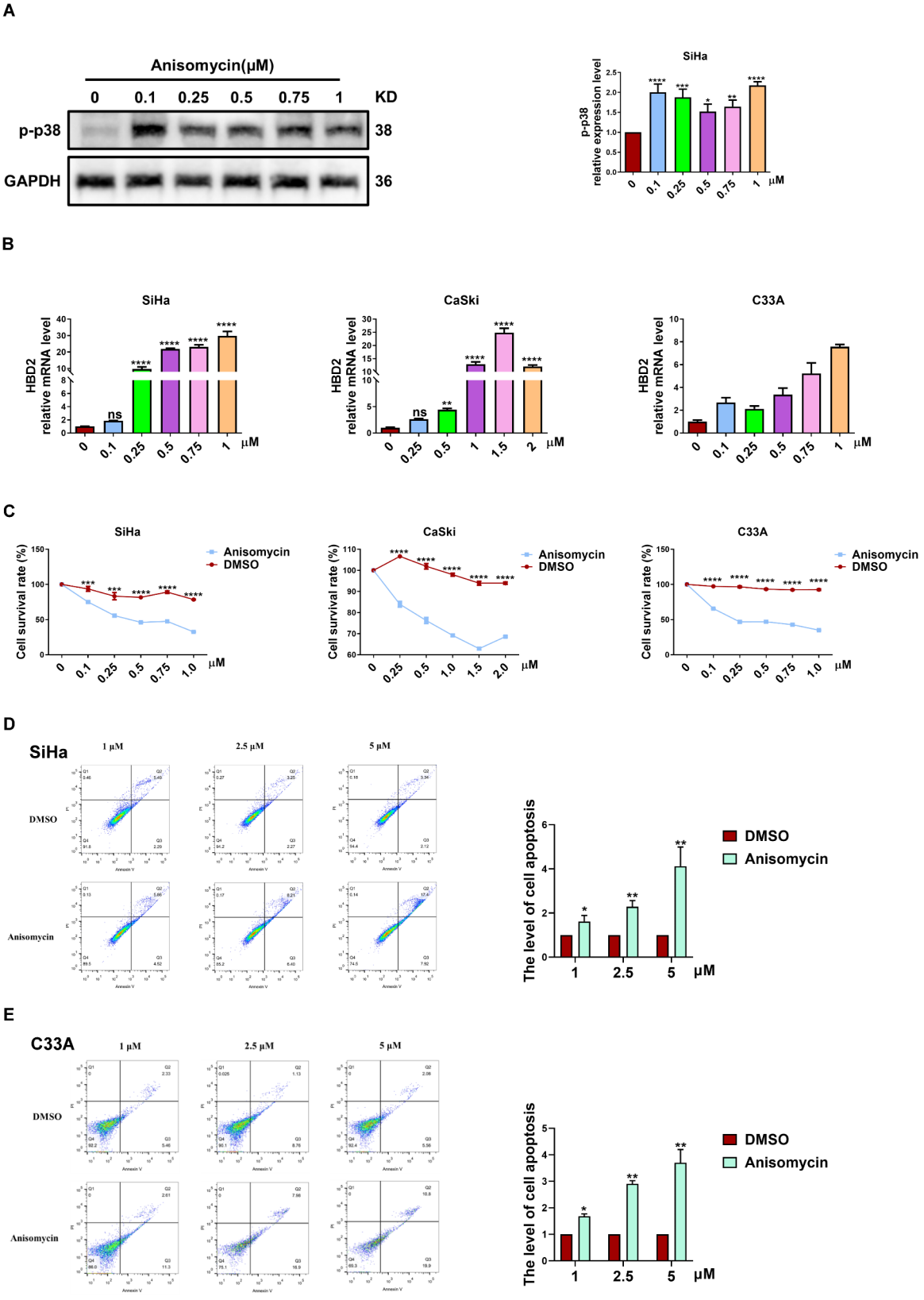

Fig. 5 (See legend on next page.)

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Fig. 5 HBD2 involved deeply in the anti-tumor mechanism of Anisomycin in treatment of cervical cancer. (**A**) SiHa cells were exposed to varying concentrations of Anisomycin from 0, 0.25, 0.5, 0.75 to 1 μM for 24 h, and the levels of p-p38 and GAPDH proteins were assessed using Western blot analysis. The relative expression of these proteins was quantified using Image J software, with GAPDH serving as an internal control. SiHa, CaSki and C33Acells were treated with different concentrations of Anisomycin for 24 h, and HBD2 mRNA levels (**B**) and cell viability (**C**) were measured using RT-qPCR and CCK8 assay, respectively.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, vs. 0 μM group; (**D**) and (**E**) Apoptosis in SiHa and C33A cells treated with various concentrations of Anisomycin for 24 h was evaluated using flow cytometry. ***$P < 0.001$, ****$P < 0.0001$, vs. DMSO group

HBD2 expression via the inhibition of ASK1-p38 MAPK signaling pathway in cervical cancer cells. This mechanism could potentially contribute to HPV16 immune evasion and cervical cancer tumorigenesis.

In cancer, apoptosis constitutes a common tumor suppressor mechanism, a property which is deeply exploited in cancer therapy and is becoming increasingly important as a target for cancer drug development [53, 54]. Apigenin, as a cancer chemopreventive agent, exerts antitumor effects by inducing apoptosis in tumors, including skin cancer, head and neck squamous cell carcinoma, breast cancer, etc [55]. Traditional Chinese medicine research demonstrates that Compound Shougong Powder can restrict tumor growth. Taurinechenodeoxycholic

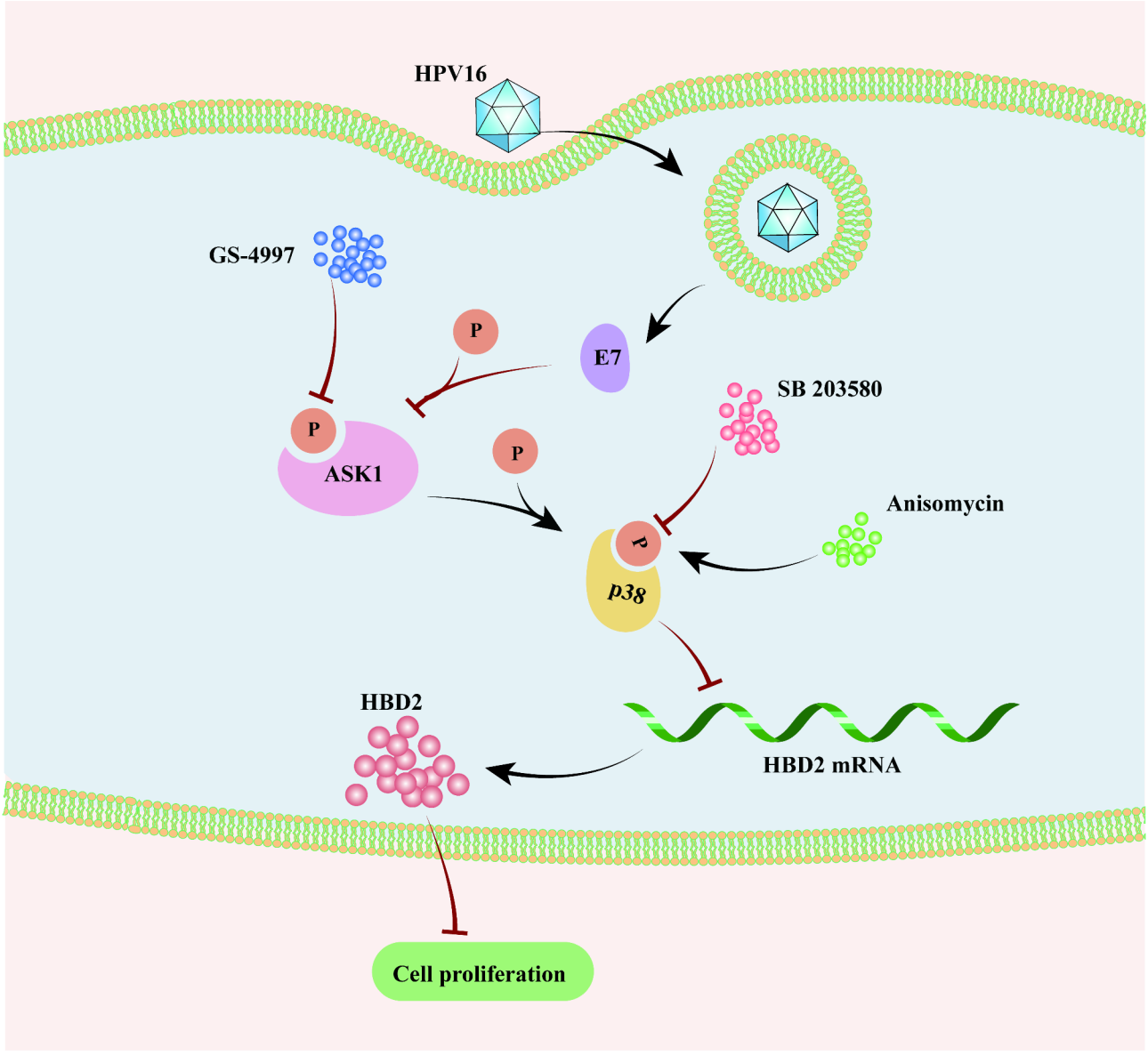

Fig. 6 HBD2 expression was decreased by HPV16 E7 through the inhibition of ASK1-p38 MAPK signaling pathway in cervical cancer

acid (TCDCA), the main component of Compound Shougong Powder, has been shown to inhibit gastric cancer cells proliferation by inducing apoptosis [56]. Continuously, we further tested the apoptosis effect induced by anisomycin to explore the possible clinical significance of the mechanism that the activation of ASK1-p38 MAPK promotes HBD2 expression and the potential important role of HBD2 in tumorigenesis and treatment in cervical cancer with HPV16 infection. The results that anisomycin increased HBD2 protein expression and apoptosis rates, and reduced cellular viability with high level of p38 pathway activation in CaSki and SiHa cells, fully supported that HBD2 may play a very important role in the therapeutic efficacy of anisomycin against cancer, and the mechanism that down-regulation of HBD2 expression by HPV16 E7 through the inhibition of ASK1-p38 signaling pathway and thus is also possibly a key point to the tumorigenesis of cervical cancer post HPV16 infection. These results were also notably consistent with the articles which reported that tumors with low HBD2 expression demonstrate a favorable response to HBD2 treatment [22, 57]. Apart from the ability to induce cervical cancer apoptosis induction by ASK1-p38 MAPK signaling pathway activation, the documents also narrated that HBD2 not only enhances the anti-tumor impact of vaccines as an immune adjuvant [18], but also regulates cell growth by impeding G1/S cell cycle and activating pRb in malignant epithelial cells [28].

Although this article reported for the first time that HPV16 E7 downregulates the HBD2 expression by the inhibition of ASK1-p38 signal pathway in cervical cancer, there were also lot of papers said that E7 proteins deregulates the NF-κB pathway by interaction with IKKα [58]. Thus, HBD2 may also be decreased by this NF-κB pathway inhibition. In fact, Anisomycin activates both p38 and JNK, which regulate HBD2 expression [59]. So, in order to assess the activation profiles and roles of p38 MAPK, JNK and NK-kB pathways by anisomycin in cervical cancer, their specific inhibitors including SB-203580, SP600125 (inhibitor of the JNK) and BAY11-7082 (inhibitor of NF-κB) were used combined with anisomycin or not in SiHa, CasKi, and C33A cells. By comparing with the results from the cells with the treatment of only anisomycin, HBD2 mRNA levels and apoptosis rates in all 3 groups treated with inhibitors were decreased obviously. However the cell proliferation were increased. These results indicated all these pathway play functions in the regulation of HBD2 expression, cell proliferation and cell apoptosis. Obviously, there are lots of defects in this paper including that we can not clarify the exact mechanism how HPV16 E7 does regulate on the ASK1, directly or indirectly, mRNA level or modification post transcription, the answers for all of these questions could not be found in this paper. On the other hand, there is no animal experiments were performed in our paper. So, more continuous researches on the potential anti-tumor effect of HBD2 in cervical cancer and its possible important roles in many other tumor treatment drugs by activation of ASK1-p38 signaling pathway are worth further investigation in future.

Conclusions

The study demonstrated that, in cervical cancer cells with HPV16 infection, HBD2 expression would be suppressed by both its E6 and E7 protein, but HPV16 E7 may exhibit a more stable and effective inhibition. And HPV16 E7 hinders HBD2 expression via the down-regulation of ASK1-p38 MAPK signaling pathway. But Anisomycin could decrease the cell viability and promote cellular apoptosis by enhancing HBD2 expression and p38 pathway activation (Fig. 6). This suggests that the decreased expression of HBD2 by HPV16 E7 through inhibition of ASK1-p38 MAPK signaling pathway is a tumorigenisis of cervical cancer and HBD2 could be an important factor in the therapeutic efficacy of Anisomycin on cervical cancer, highlighting its potential clinical significance.

Abbreviations

HPV	Human papillomavirus
HPV16	Human papillomavirus type 16
HPV16 E7	Human papillomavirus type 16 E7 oncoprotein
HBD2	Human beta-defensin2
ASK1	Apoptosis signal-regulating kinase 1
p38 MAPK	Mitogen-activated protein kinase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02731-9.

Supplementary Material 1

Supplementary File 2

Acknowledgements

The authors would like to thank all the staff for the Department of Medical Microbiology & Immunology, School of Basic Medical Sciences, Jiangxi Medical College for their collaboration and support.

Author contributions

JL and SD designed the experiments. JL performed the experiments and analyzed the data. XH and QS supervised the research, and helped write the paper. BS, MW, YY, YC, RL and LJ contributed to the organization of the manuscript and provided significant input on specific sections. All authors have reviewed the final version of the manuscript.

Funding

This work was funded by grants from the National Natural Science Foundation of China (NO.30760280, 81760472), and funding was also provided by the Natural Science Funding of Jiangxi province, China (No. 20181BAB205064), and Health Commission Project of Jiangxi Province, China (No.202130621).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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Received: 19 December 2024 / Accepted: 9 April 2025
Published online: 19 April 2025

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