

Original Article

Isocorydine induces cell differentiation through reversed epithelial mesenchymal transition in SiHa cells

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Abstract: Isocorydine (ICD) can be isolated from many plant materials and has been validated as a potential antitumor agent in some carcinomas. However, its role in cervical carcinoma remains unknown. Therefore, in this study, the mechanisms involved in isocorydine-induced suppression of cervical cancer were examined. MTT assay was used to examine the effects of ICD on proliferation of SiHa cells. Differential gene expression profiles in ICD-treated SiHa cells were analyzed with cDNA microarrays. Expression of HPV16E6, p53, NDRG1, CLCA2, and CASP14 in SiHa cells and tumor tissues was examined and Western blotting was used to analyze protein expression of epithelioid cell markers, such as E-cadherin and Vimentin. ICD reduced proliferation of SiHa cells, *in vitro* and *in vivo*, perhaps through p53-related pathways. Additionally, ICD regulated NDRG1, CLCA2, and CASP14 to suppress invasion and metastasis of SiHa cells and nude mice tumors. Histopathological examination of ICD-treated SiHa cells and nude mice tumors revealed morphologic changes suggesting differentiation, whereas Western blotting showed upregulated E-cadherin and downregulated vimentin. In conclusion, this study's findings reveal that ICD could suppress invasion and metastasis of SiHa cells by inducing G₁/S cell cycle arrest and reversed epithelial mesenchymal transition. ICD might be a potential therapeutic for treatment of cervical cancer.

Keywords: Isocorydine, cell cycle arrest, epithelial mesenchymal transition, mesenchymal-epithelial transition, antitumor, SiHa cells

Introduction

Cervical cancer is one of the most common primary malignant cancers, resulting in the death of women. In 2012, cervical cancer was responsible for the deaths of 266,000 women worldwide [1]. Human papilloma virus (HPV) infection is a key factor leading to occurrence of cervical cancer [2]. However, prevention and treatment of HPV infection in cervical cancer has remained limited. For example, the adaptive population of HPV vaccine has been restricted and most women in resource-poor areas have had few opportunities to access implementation of HPV vaccination [3, 4]. Moreover, most anticancer drugs have serious toxic side effects. Thus, it is urgent that new therapeutic drugs with increased efficacy and decreased toxicity are found.

Isocorydine (ICD) is widely distributed in nature. *Dicranostigma leptopodum* (Maxim) Fedde, *Ste-*

phania brachyandra Diels, and *Dactylicapnos scandens* Hutch have exhibited many obviously biological activities, such as antimicrobial, antiviral, antitumor, anti-liver fibrosis, and anti-inflammatory activity [5, 6]. In 2010, ICD was authorized by China State Food and Drug Administration (SFDA) to act as spasmolytic analgesics to cure pain. Due to its attractive biological activities, many studies have focused on its potential medicinal value. Moreover, the anticancer activity of ICD has become a hot pharmaceutical research area in recent years. For example, recent research has demonstrated that ICD and its derivate d-ICD can inhibit cell proliferation by inducing G₂/M cell-cycle arrest and apoptosis, along with targeting drug-resistant cellular side population through PDCD4-related apoptosis [7-9]. Epithelial-mesenchymal transition (EMT) is a process transforming tightly connected epithelial cells into spindle-like fibroblast mesenchymal stem cells,

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Table 1. Gene sequences of primers

| Gene | Primers | Product |
|---------|---|---------|
| GAPDH | 5'-CCAGGTGGTCTCCTCTGACTTC-3' (forward) | 91 bp |
| | 5'-GCTTGACAAAGTGGTCGTTGAG-3' (reverse) | |
| p53 | 5'-GGCTCTGACTGTACCACCATCCA-3' (forward) | 152 bp |
| | 5'-GGCACAAACACGCACCTCAAAG-3' (reverse) | |
| HPV16E6 | 5'-AGGAGCGACCCAGAAAGT-3' (forward) | 136 bp |
| | 5'-GCATAATCCCGAAAAGC-3' (reverse) | |
| CASP14 | 5'-GTACCTGGGGTTCAAGAGCATCAT-3' (forward) | 130 bp |
| | 5'-CATGAGCACCAAAAGGCACAG-3' (reverse) | |
| CLCA2 | 5'-AGTGAAGTGGAGGCTTCTCTACAAC-3' (forward) | 153 bp |
| | 5'-AATCCATTATACCCATTGTCTTGAA-3' (reverse) | |
| NDRG1 | 5'-CTCTGTTCACGTCACGCTGT-3' (forward) | 103 bp |
| | 5'-AGAGGGGGTTGTAGCAGGT-3' (reverse) | |

which lose cell polarity, resulting in invasion and metastasis of tumor cells [10]. Diagnosing EMT and targeting key molecules of the process is important in treating tumor metastasis [11].

In this study, *in vivo* and *in vitro* experiments were used to validate the effects of ICD on HPV16E6 in SiHa cells and nude mice xenografts and to analyze the molecular networks of ICD inhibition of cervical cancer using transcriptomic and proteomic methods, clarifying the effects of ICD on cell cycle, apoptosis, and HPV16E6-related signaling pathways. This study demonstrates that ICD significantly suppressed growth of SiHa cells via G₁/S cell cycle arrest with no effects on apoptosis. In addition, ICD induced cell differentiation by increasing the activity of p53-related pathways, *in vivo* and *in vitro*. ICD can suppress the invasion and metastasis of SiHa cells by reversing EMT. The aim of this study was to provide a solid experimental and theoretical foundation for possible application of ICD as a new and effective treatment for HPV infection.

Materials and methods

Cell lines and cell culture

Human SiHa cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone®, sh30022.01B) containing 10% heat-inactivated fetal bovine ser-

um (FBS) (Hyclone®, SV30087.02) at 37°C with 5% CO₂.

Drug source and stocks

ICD, extracted from *Dicranostigma leptopodum* Fedde, was kindly provided by Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, and stored at 4°C, shielded from light.

MTT assay

SiHa cells were seeded in 96-well plates (5000 cells/well), treated with ICD (25, 50, 100, 150, or 200 μmol/L), and incubated for 24, 48,

and 72 hours. Complete medium was used as the blank. MTT (20 μL, 5 mg/mL) was added to ICD-treated cells and cells were incubated for 4 hours in the dark. The supernatant was replaced with 100 μL DMSO and the plate was shaken for 10 minutes to dissolve formazan. Absorbance was measured by an ELISA reader at 490 nm.

Cell cycle analysis

After confirming cell adherence (coverage of approximately 50% of the culture flask surface), the medium was replaced with 50, 100, or 200 μmol/L of ICD (diluted in high-glucose DMEM with 10% FBS) for 48 hours. Cells were collected, washed three times with PBS, and suspended in precooled 70% ethanol at -20°C overnight. Next, cells were washed with PBS three times and resuspended in 500 μL PBS containing 50 μg/mL propidium iodide (PI) (Sigma, P4170), 0.2% TritonX-100 (Takara Bio Inc. Japan), and 100 μg/mL RNAase (Takara Bio Inc. Japan). They were then incubated at 23°C in the dark for 30 minutes and analyzed by flow cytometry (BD FACSVerser, BD Biosciences, San Jose, CA, USA).

Apoptosis analysis

SiHa cells were treated with ICD (50, 100, 150, and 200 μmol/L for 24, 48, or 72 hours). Cells were collected with a cell scraper, washed three times with precooled PBS, and resuspended in 400 μL of 1 × binding buffer. The cell number was adjusted to 1 × 10⁹/L, and cell suspension (100 μL) was aspirated before add-

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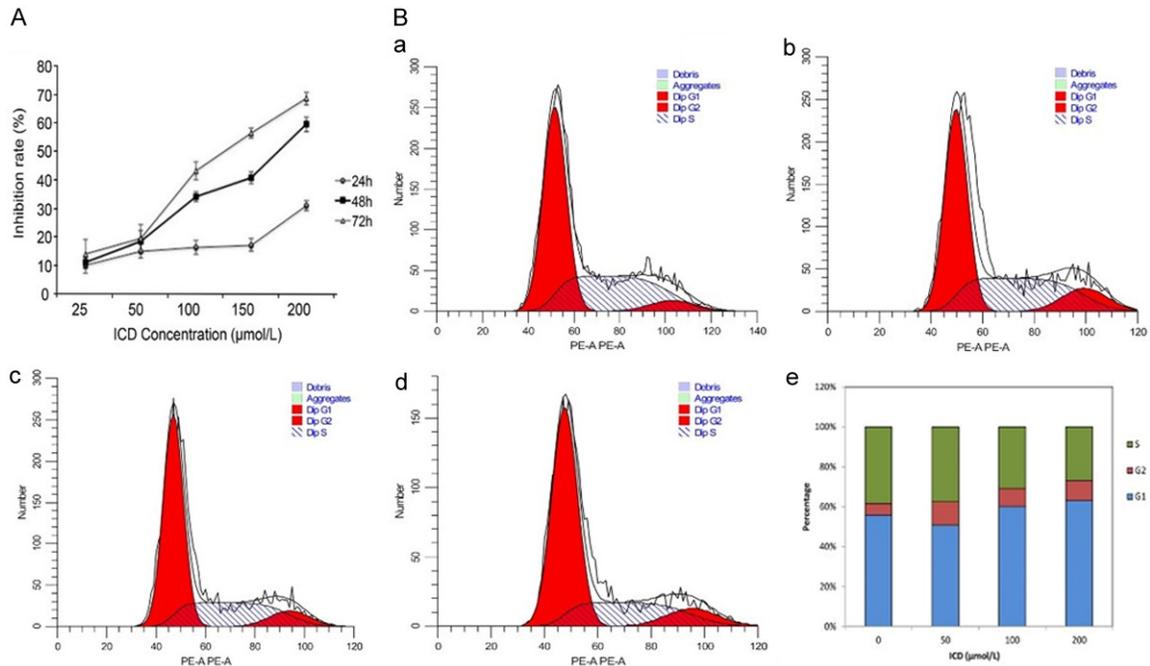


Figure 1. ICD suppressed proliferation of SiHa cells by G_1/S cell cycle arrest. A. MTT assay: growth inhibition rate of SiHa cells after ICD treatment (25, 50, 100, 150, and 200 $\mu\text{mol/L}$ for 24, 48, and 72 h). B. Flow cytometry analysis after 48-h ICD treatment (0, 50, 100, and 200 $\mu\text{mol/L}$). a. 0 $\mu\text{mol/L}$. b. 50 $\mu\text{mol/L}$. c. 100 $\mu\text{mol/L}$. d. 200 $\mu\text{mol/L}$. e. Cell cycle distribution of SiHa cells after ICD treatment for for 48 h.

ing 5 μL annexin V and 10 μL PI (Invitrogen, V13-241, Alexa Fluor® 488 Annexin V/Dead cell Apoptosis Kit). Samples were incubated for 10 minutes at room temperature in the dark and analyzed using flow cytometry (BD FACS verse).

cDNA microarray

Cells were exposed to 200 $\mu\text{mol/L}$ ICD for 12, 24, or 48 hours, in order to identify specific ICD-targeted genes and to analyze (Affymetrix GeneChip® Human Transcriptome Array 2.0, analysis performed by Shanghai Biotechnology Corporation, Shanghai, China).

Real-time RT-PCR

Expression of p53, HPV16E6, CLCA2, CASP14, and NDRG1 in SiHa cells were detected via Q-PCR after exposure to 200 $\mu\text{mol/L}$ ICD for 12, 24, or 48 hours. Total RNA was reverse transcribed using Power RT kit (BioTeke Corporation, Beijing, China) with random primers. Real-time RT-PCR was performed using BioEasy SYBR Green I Real Time PCR Kit (Bioer Technology, Hangzhou, China) with FTC3000 Real-time Quantitative Thermal Cycler (Funglyn Biotech, Shanghai, China). Gene expression levels

were quantified with the $2^{-\Delta\Delta\text{Ct}}$ method. Data are represented as mean \pm standard errors of three independent experiments and normalized to respective controls. Primers used are listed in **Table 1**.

Western blotting

Homogenized tumor tissues and SiHa cells treated with 25, 50, 100, and 200 $\mu\text{mol/L}$ ICD for 72 hours were lysed in RIPA lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to PVDF membranes (Millipore). The blots were probed with specific primary antibodies and HRP-conjugated secondary antibodies, diluted proportionally as follows: HPV16E6 (1:50, Santa Cruz Biotechnology Sc-1584, America), P53 (1:200, Santa Cruz Biotechnology sc-126, America), CASP14 (1:1000, ABCAM ab45415, America), NDRG1 (1:1000, ABCAM ab124689, America), CLCA2 (1:1000, SIGMA HPA047192, America), PARP (1:1000, CST Cst5625, America), E-cadherin (1:1000, Santa Cruz Biotechnology sc7870, America), Vimentin (1:1000, CST, Cst5741, America), and P21 (1:1000).

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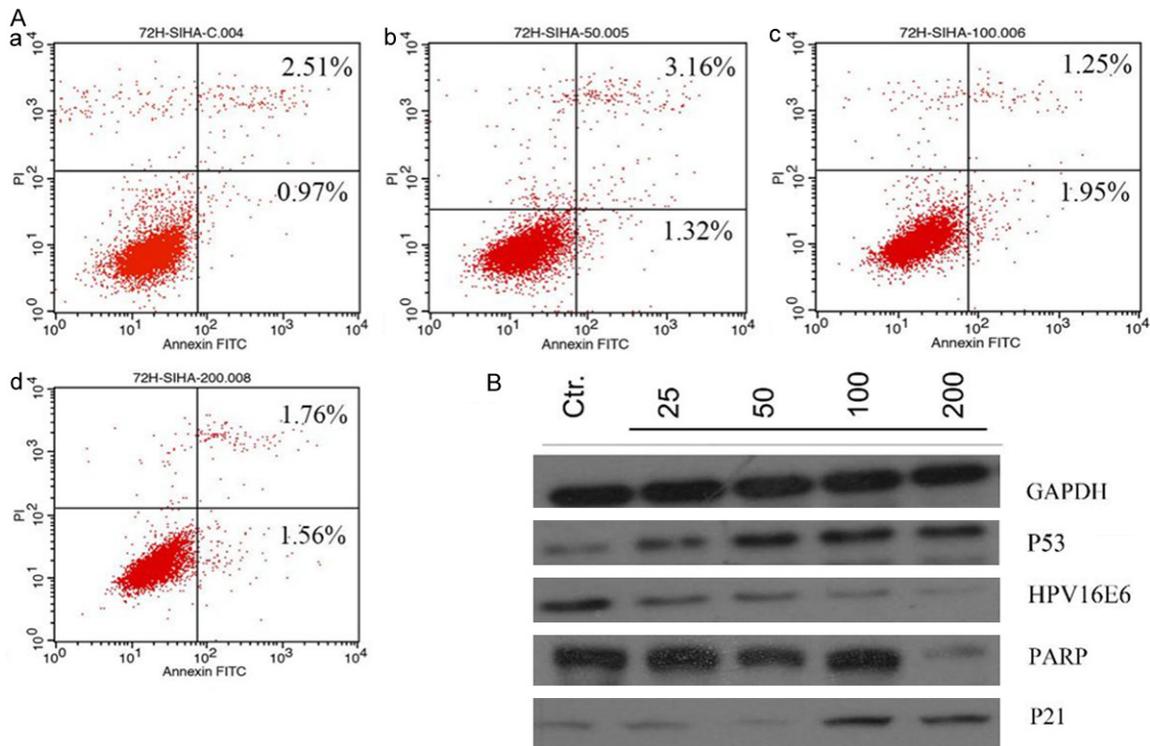


Figure 2. ICD had no effect on apoptosis of SiHa cells. A. Apoptosis analysis: SiHa cells were treated with ICD (200 $\mu\text{mol/L}$) for 72 h and the apoptotic rate was detected by annexin V and PI double staining. B. Western blotting analysis of p53, HPV16E6, PARP, and p21 expression in ICD-treated SiHa cells.

Protein signals were detected by an ECL system (Pierce, Rockford, IL, USA).

Xenograft experiments

Female BALB/C (nu/nu) mice (5-6 weeks old, 18-20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and kept in a dedicated specific-pathogen free facility at the Laboratory Animals Center of Gansu University of Traditional Chinese Medicine. SiHa cells (1×10^7) were suspended in 0.2 mL PBS and inoculated subcutaneously into the backsides of mice. After 5 weeks, the mice were randomly divided into 5 groups: negative control group treated with physiological saline (0.9%) ($n = 8$), groups treated with 0.1, 0.2, and 0.4 mg/kg ICD ($n = 7$ per group), and a positive control group treated with 12 mg/kg paclitaxel (Taxol) ($n = 7$). ICD-treated groups and negative control received intraperitoneal injections 5 times per week, whereas positive control animals were continuously injected with Taxol, intraperitoneally, for 5 days. Tumor dimensions were regularly measured every 48 hours using calipers. Volume was calculated with the following

formula: $V (\text{mm}^3) = L \times W \times H \times 0.523$. Mice health status and body weight were recorded every 2 days. After 4 weeks, the mice were sacrificed and subcutaneous tumors were surgically excised, weighed, photographed, and sliced for further analysis. All experiments with animals were approved by the Animal Experimental Ethical Committee of Gansu University of Traditional Chinese Medicine and conducted in accordance with institutional guidelines for laboratory animal care. The effects of ICD treatment on tumor growth were evaluated by statistically analyzing tumor weights.

Histopathological examination and immunohistochemistry

Cervical cancer xenograft tumor tissues were fixed in neutral 10% formalin solution at room temperature, dehydrated in ethanol, washed with xylene, embedded in paraffin, sectioned at a thickness of 4 μm , and stained with hematoxylin-eosin. Sections were observed and photographed under an optical microscope.

Isocorydine and SiHa cells

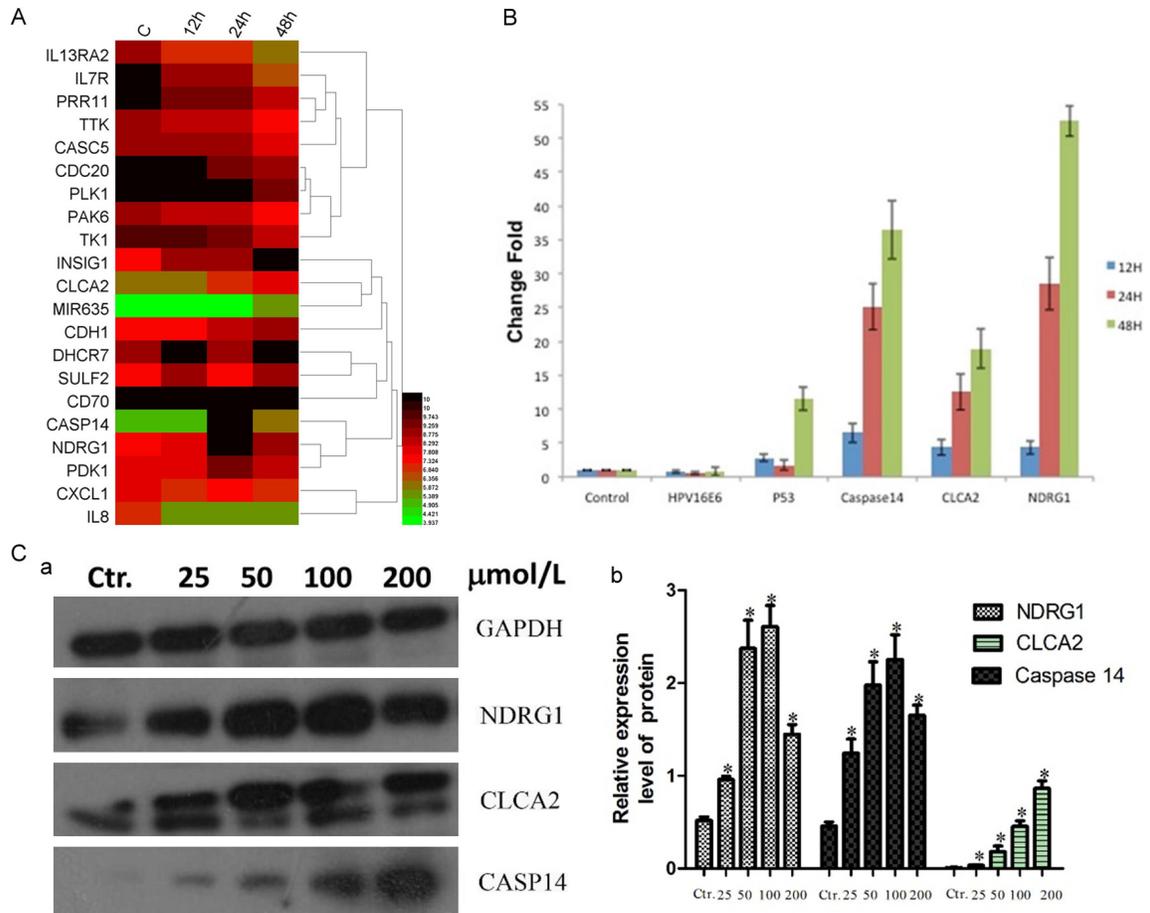


Figure 3. ICD reduced proliferation of SiHa cells by increasing the activity of p53-related pathways. A. Heatmap of an Affymetrix Genechip after treatment with 200 μmol/L ICD for 12, 24, and 48 hours. B. Expression of p53, HPV16E6, CLCA2, CASP14, and NDRG1 (Q-PCR) after exposure to 200 μmol/L ICD for 12, 24, and 48 hours. C. Western blotting analysis of CLCA2, CASP14, and NDRG1 expression in SiHa cells.

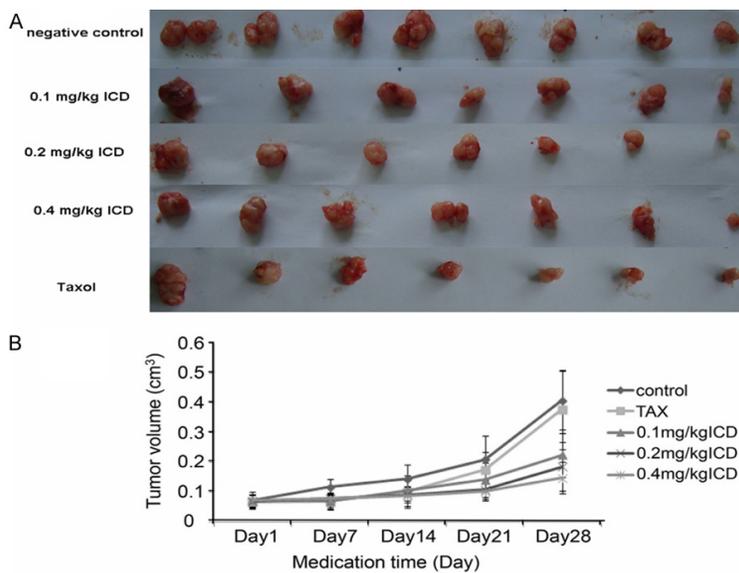


Figure 4. Xenograft experiment. A. Nude-mouse xenograft model: a) negative control (PBS); b) 0.1 mg/kg ICD; c) 0.2 mg/kg ICD; d) 0.4 mg/kg ICD;

e) positive control (Taxol). B. Tumor sizes after 1, 7, 14, 21, and 28 days of ICD treatment. Data are represented as mean ± SD of five independent experiments. *Significant differences ($P < 0.05$).

Immunohistochemical staining was used to evaluate HPV16E6, p53, CASP14, NDRG1, CLCA2, cleaved PARP, and E-cadherin expression in cervical cancer xenograft tumor tissues. Briefly, formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm. Sections were warmed up and deparaffinized in EZ Prep (Roche) for 4 minutes. After quenching of endogenous per-

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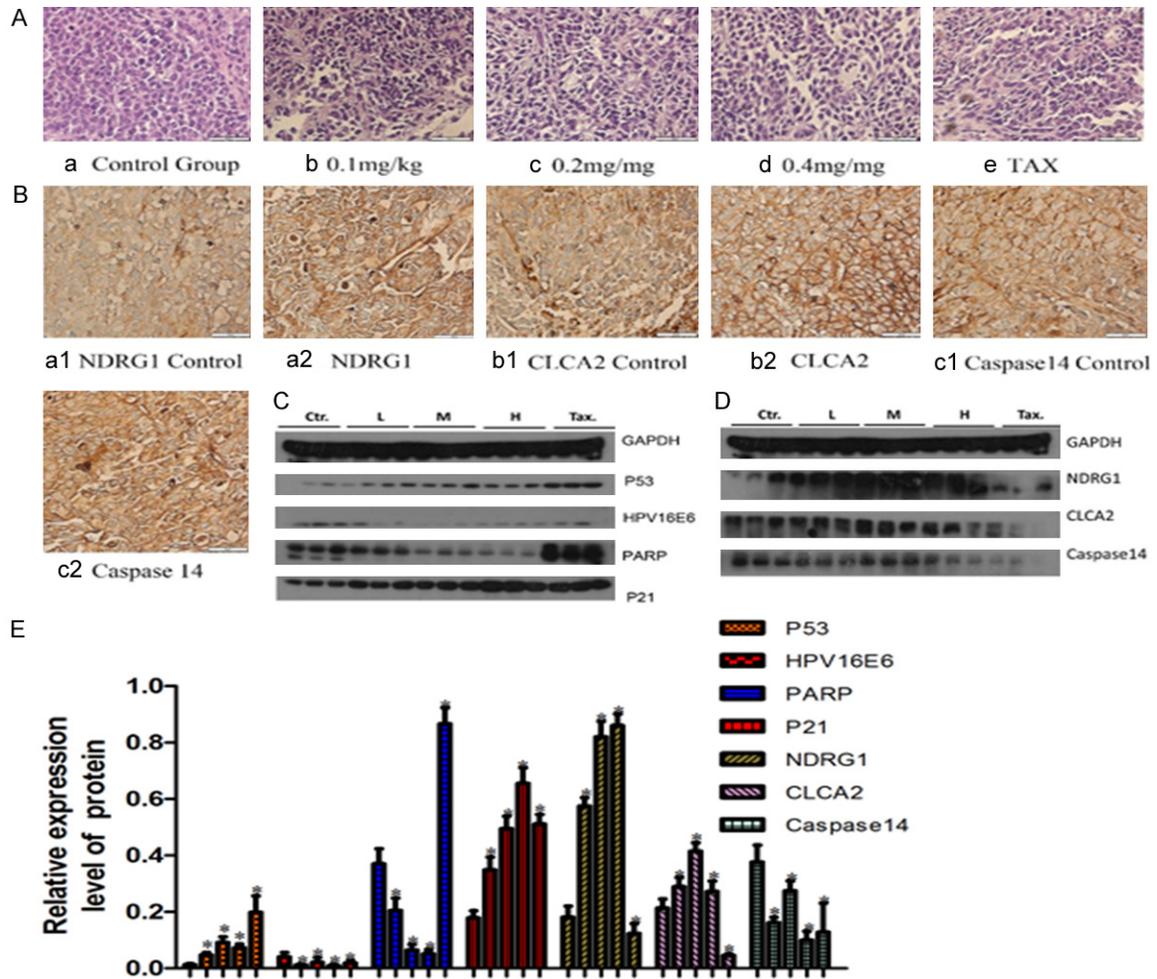


Figure 5. A. Histopathological examination of tumor tissues: a) negative control (PBS); b) 0.1 mg/kg ICD; c) 0.2 mg/kg ICD; d) 0.4 mg/kg ICD; e) positive control (Taxol); B. Immunohistochemical analysis of selected genes in tumor tissues after PBS or 0.2 mg/kg ICD treatment: NDRG1 (a1, a2), CLCA2 (b1, b2), and CASP14 (c1, c2) in negative control and 0.2 mg/kg ICD groups, respectively; C. Western blotting analysis of p53, HPV16E6, PARP, and p21 expression in tumor tissue. D. Western blotting analysis of NDRG1, CLCA2, and CASP14 expression in tumor tissues. Ctr., L, M, and H represent negative control and 0.1, 0.2, and 0.4 mg/kg ICD groups, respectively. E. Western blotting analysis of p53, HPV16E6, PARP, P21, NDRG1, CLCA2, and CASP14 expression in tumor tissues.

oxidase with 3% hydrogen peroxide for 3 minutes, Cell Conditioning Solution (CC1) was added for 30 minutes to induce epitope retrieval. The slides were incubated with monoclonal anti-HPV16E6 (1:150), p53 (1:150), CASP14 (1:250), NDRG1 (1:250), CLCA2 (1:500), PARP (1:150), and E-cadherin (1:400) at 37°C for 32 minutes, and signals were developed with streptavidin-peroxidase method. Secondary antibodies have already been listed above. Antibodies were visualized using UltraView Universal DAB Detection Kit (Roche) as a chromogen for 8 minutes. Sections were counterstained with hematoxylin and examined under an optical microscope.

For histopathological examination, paraffin sections of xenografts were deparaffinized in xylene and rehydrated with descending concentrations of ethanol. Three slide tumor samples per group were stained with hematoxylin and eosin to select tumor areas and other tissues were immunohistochemically stained.

Results

ICD significantly suppressed growth of SiHa cells via G₂/S cell cycle arrest with no effects on apoptosis

To analyze the effects of ICD on development of cervical cancer, MTT assay was performed on

Table 2. Mice body weight and tumor mass

| Group | Body weight (g) | | Tumor mass (g) |
|--------------------------|--------------------|------------------|----------------|
| | Initial weight (g) | Final weight (g) | |
| Negative control | 23.86 ± 0.67 | 24.08 ± 0.98 | 0.44 ± 0.14* |
| 0.1 mg/kg ICD | 23.14 ± 0.98 | 24.03 ± 1.55 | 0.43 ± 0.20* |
| 0.2 mg/kg ICD | 23.56 ± 1.45 | 24.11 ± 1.42 | 0.25 ± 0.19* |
| 0.4 mg/kg ICD | 24.13 ± 1.01 | 25.01 ± 0.81 | 0.25 ± 0.11* |
| Positive control (Taxol) | 23.73 ± 1.00 | 25.06 ± 0.94 | 0.34 ± 0.19* |

Data are represented as mean ± SD of five independent experiments. *Significant differences (P < 0.05).

ICD-treated SiHa cells. The results suggested that ICD significantly inhibited growth of SiHa cells in a dose- and time-dependent manner (Figure 1A). ICD treatment at 25, 50, 100, 150, and 200 µmol/L dose- and time-dependently inhibited SiHa cell growth. Western blot analysis of gene and protein expression of HPV16E6 and p53 in SiHa cells showed that HPV16E6 was downregulated and p53 upregulated after 72 hours of ICD treatment (Figure 2B). Effects of ICD on SiHa cells cell cycle were examined after 48 hours of treatment using flow cytometry (Figure 1B). ICD caused G₁/S cell cycle arrest (Table S1), with 35.72, 32.94, and 27.14% of cells in S phase after treatment with 50, 100, and 200 µmol/L by ICD, respectively, significantly decreased compared to control (37.92%). The proportion of cells in G₁ increased slightly (55.66 to 63.82%). In contrast, the percentage of cells in G₂ phase was unchanged.

This study examined the mechanisms by which ICD suppresses proliferation of SiHa cells using annexin V/PI double staining and flow cytometry (Figure 2A). Less than 5% of apoptotic cells were observed, suggesting the lack of apoptosis. In addition, Western blotting indicated that the important apoptosis indicator PARP was unchanged, whereas P21 was upregulated (Figure 2B), indicating that this suppression was via cycle arrest, not via cell apoptosis.

ICD induced cell differentiation by increasing activity of p53-related pathways in vitro and in vivo

cDNA microarray indicated that NDRG1, CLCA2, and CASP14 were significantly upregulated (Figure 3A). Q-PCR (Figure 3B) supported the cDNA microarray results. It should be noted that CDH1, which provides instructions for making a protein called epithelial cadherin or

E-cadherin, showed temporary upregulation after exposure to ICD for 24 hours (Figure 3A). In addition, Western blotting also indicated that CLCA2, NDRG1, and CASP14 were upregulated (Figure 3C).

Histopathological analysis and immunohistochemical staining revealed that ICD treatment induced characteristic morphologic changes (Figure 5A, 5B).

Western blotting indicated that HPV16E6 was downregulated and p53 was upregulated. Additionally, the PARP was unchanged and P21 was upregulated in tumor tissue after ICD treatment, consistent with *in vivo* studies (Figure 5C). In addition, Western blotting analysis of NDRG1, CLCA2, and CASP14 in tumor tissues supported the *in vitro* data (Figure 5D).

ICD suppressed SiHa tumor growth in vivo

ICD has efficiently inhibited cell proliferation *in vitro*. This study, therefore, examined its effects *in vivo* using a nude mouse xenograft model (Figure 4A). Tumor weights indicated that ICD significantly inhibited tumorigenicity of SiHa cells (P < 0.05) in this model system (Table 2). Final body weights of ICD-treated mice showed no significant differences compared to control (P > 0.05) (Table 2), indicating ICD lack of treatment toxicity.

ICD induced SiHa cell differentiation to epithelial cells to suppress tumor growth

ICD-induced differentiation of SiHa cells was examined using histopathological (Figure 6A) and immunohistochemical methods (Figure 6C). Upregulation of E-cadherin and downregulation of vimentin in SiHa cells (Figure 6B) and tumor tissues (Figure 6D), detected in Western blotting, indicated that ICD-treated SiHa cells differentiated into epithelial cells through a reverse-EMT-like process. The cells displayed morphological changes suggesting the loss of stem-like tumor cell characteristics.

Discussion

ICD has been used for treatment as a spasmolytic analgesic, antiplasmodial, and antiarrhythmic. Recently, it has been proven to act as a potential antitumor agent in a wide variety of

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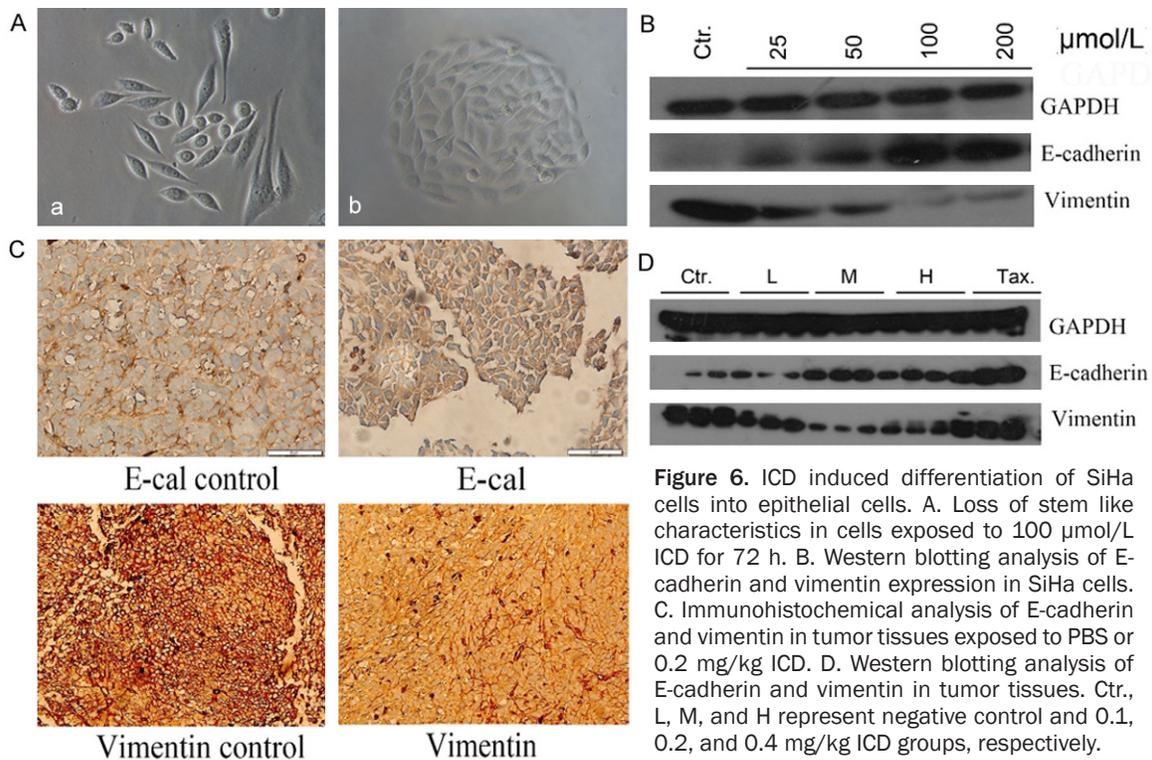


Figure 6. ICD induced differentiation of SiHa cells into epithelial cells. A. Loss of stem like characteristics in cells exposed to 100 $\mu\text{mol/L}$ ICD for 72 h. B. Western blotting analysis of E-cadherin and vimentin expression in SiHa cells. C. Immunohistochemical analysis of E-cadherin and vimentin in tumor tissues exposed to PBS or 0.2 mg/kg ICD. D. Western blotting analysis of E-cadherin and vimentin in tumor tissues. Ctr., L, M, and H represent negative control and 0.1, 0.2, and 0.4 mg/kg ICD groups, respectively.

tumors, such as liver, gastric, and lung cancer [12, 13]. However, its potential role in cervical carcinoma remains unknown. The extent of ICD's antitumor effects in additional cancer types requires further study.

In this study, ICD effectively inhibited the growth of cervical cancer SiHa cell lines. MTT assay indicated that 72 hours of treatment with 200 μM ICD reduced the survival of SiHa cells whereas flow cytometry revealed that ICD induced cell cycle arrest at the G_1/S phase. In addition, ICD significantly reduced HPV16 E6 protein expression and upregulated p53 and p21. Therefore, it is speculated that these ICD effects may lead to reduced SiHa cell proliferation by P53-P21 pathways. However, annexin staining and analysis showed that less than 5% of apoptotic cells were observed. Meanwhile, Western blotting indicated that PARP, which plays an important role in maintenance of genomic integrity, chromatin remodeling, and transcriptional control, was unchanged [14]. These results indicate that ICD suppressed proliferation of SiHa cells via cycle arrest, not via cell apoptosis.

After 48 hours of treatment with ICD, 101 upregulated and 150 downregulated genes

were observed in cDNA microarray of SiHa cells (double fold change), of which 6 genes had significant changes. K-means clustering indicated gradual downregulation of genes associated with cell division. Microarray results were corroborated by real-time PCR and Western blotting. CLCA2, NDRG1, and Caspase 14 were upregulated in the microarray. CLCA2 belongs to the CLCA gene family and has been discovered as a tumor suppressor gene. Its downregulation will induce EMT and lead to tumorigenicity, invasiveness, and metastasis [15]. In 2012, Tanikawa et al. [16] reported on the role of CLCA2 in mediating induced tumor cell senescence by p53, demonstrating that CLCA2 significantly reduced cell proliferation and promoted oxidative stress in a p53-dependent manner. In 2015, Sasaki et al. [17] examined gene expression in human osteosarcoma Saos-2 cells and analyzed p53-induced genes involved in tumor growth inhibition, identifying CLCA2 as a downstream mediator of p53, regulating tumor spread and invasion. NDRG1 (N-myc downstream regulated gene1), a member of the NDRG gene family, also plays an important role in carcinogenesis and tumor progression, especially in invasion and metastasis [14]. Wangpu et al. [18] have shown the involvement

of NDRG1 in FAK/Paxillin pathways and its role in inhibiting tumor metastases and adhesion. Moreover, NDRG1 has been shown to inhibit invasion of cervical cancer Ca Ski and ovary cancer HO-8910PM cells by regulating CLCA2 gene expression [14]. Caspase-14 is a member of conserved family of cysteinyl aspartate-specific proteinases, traditionally regarded as being involved in inflammation and apoptosis. However, Denecker G et al. argued that, although most caspases were expressed in many tissues, caspase-14 expression was confined mainly to cornifying epithelia, such as the skin [19]. Moreover, recent evidence has indicated that caspase-14 plays a major role in epidermal differentiation [20].

In this study's xenograft mouse model, after 28 days of ICD treatment, tumor weight and diameter reduced significantly ($P < 0.05$). Hematoxylin-eosin staining revealed cytomorphosis changes, suggesting that ICD might have reversed EMT, leading researchers to analyze epithelioid cell marker E-cadherin using histochemistry and Western blotting. Upregulated E-cadherin and downregulated vimentin expression was detected, confirming the observation from hematoxylin-eosin staining, whereas immunohistochemistry revealed significant differences in treated cells compared to epithelial cells.

Recently, Pattabiraman et al. [21] examined cholera toxin and forskolin regarding inducing benign transformation of malignant breast cancer via PKA pathways, indicating two approaches for targeting mesenchymal tumor-initiating cells: development of agents with specific or preferential cytotoxicity and inducing mesenchymal-epithelial transition (MET). This present study indicates that the antitumor effects of ICD are based on induced MET. Inducing MET has the potential to efficiently suppress invasion and metastasis, reduce side effects, and improve patient quality of life [22].

Taken together, these results suggest that ICD could induce G₁/S cell cycle arrest by p53-p21 pathways and induce reversed EMT by regulating antitumor pathways involving NDRG1, CLCA2, and CASP14 to suppress invasion and metastasis of SiHa cells. This present research group has investigated a lot of research based on ICD, including isolation and purification in industrialized production, the content determinable method, pharmacokinetics and tissue

distribution, and chemical structural modification and bioactivity research of ICD derivatives [12, 23-25].

In summary, the data of this present study shows that ICD fights SiHa cells by inducing cell cycle arrest and inhibiting tumor proliferation and migration. ICD would be an effective antitumor agent or a potential lead compound for the development of an effective anticancer agent. Consequently, it may be considered as useful for treatment of cervical cancer patients. Further investigation should be performed to confirm how clinical utility of ICD can be maximized in cervical cancer patients.

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Disclosure of conflict of interest

None.

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Isocorydine and SiHa cells

Table S1. Cell cycle distribution of SiHa cells after ICD treatment for 48 h

| Cell cycle | Distribution (%) | | | |
|------------|-------------------------|--------------------------|---------------------------|---------------------------|
| | ICD 0 $\mu\text{mol/L}$ | ICD 50 $\mu\text{mol/L}$ | ICD 100 $\mu\text{mol/L}$ | ICD 200 $\mu\text{mol/L}$ |
| G1 | 55.66 \pm 0.42 | 52.46 \pm 1.00 | 58.97 \pm 0.89 | 63.82 \pm 0.60 |
| S | 37.92 \pm 0.53 | 35.72 \pm 1.07 | 32.94 \pm 1.56 | 27.14 \pm 0.57 |
| G2 | 6.42 \pm 0.38 | 11.82 \pm 0.19 | 8.09 \pm 0.77 | 9.04 \pm 0.89 |

Data are represented as means \pm SD of three independent experiments. $P < 0.05$.