Icariin inhibits oral squamous cell carcinoma cell proliferation and induces apoptosis via inhibiting the NF-κB and PI3K/AKT pathways

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Abstract. Oral squamous cell carcinoma (OSCC), one of the most common types of human cancer, has a high mortality rate and a poor prognosis due to its high rates of recurrence and metastasis. In recent years, icariin (ICA) has been reported to play an important role in a variety of malignancies, such as gastric, colorectal, pancreatic and ovarian cancer. However, its role and mechanism in OSCC remains to be elucidated. The present study aimed to investigate the effect of ICA in OSCC cells and to reveal its underlying mechanisms. The OSCC cell lines SCC9 and Cal 27 were used to explore the effect of different concentrations of ICA on the biological behavior of OSCC cells. The effect of ICA on OSCC cell proliferation and apoptosis was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and flow cytometric assays, respectively. Subsequently, the protein expression levels of caspase-3 and cleaved-caspase-3 were detected using western blot analysis. Additionally, the protein and mRNA expression levels of nuclear factor-κB (NF-κB) and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway-related factors were determined using western blot analysis and reverse transcription-quantitative PCR, respectively. The results demonstrated that ICA inhibited OSCC cell proliferation and significantly increased the apoptosis rate in a dose-dependent manner. In addition, treatment of OSCC cells with ICA upregulated the protein expression of cleaved-caspase-3 and increased the cleaved-caspase-3/caspase-3 ratio. The protein expression levels of phosphorylated (p)-p65, p-PI3K and p-AKT were decreased in OSCC cells treated with ICA. The aforementioned findings revealed that ICA could attenuate the proliferation of OSCC cells and induce apoptosis via inhibiting the NF-κB and PI3K/AKT signaling pathways. Therefore, the current study provided a new insight into the clinical treatment of OSCC.

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Introduction

Icariin (ICA), a flavonoid extract of epimedium, has a wide range of pharmacological effects, such as anti-inflammatory, anti-oxidant and antitumor effects in the cardio-cerebral vascular, nervous and urogenital systems and malignant tumors (1-4). Chinese medicine has been widely used to treat several types of malignant tumors. ICA, as a vital active ingredient of epimedium in Chinese medicine, serves an inhibitory role in the occurrence and development of malignant tumors; therefore, it has been widely used in the prevention and treatment of numerous types of cancer, such as cervical, ovarian, colon and triple-negative breast cancer (5-8).

Oral squamous cell carcinoma (OSCC) occurs in the oral mucosa, and is characterized by strong local invasion and easy metastasis to the cervical lymph nodes (9,10). Furthermore, ICA is considered as a novel biological immune modulator and inducer of differentiation, and it has been reported to improve immune function, inhibit tumor cell proliferation, tumor growth and angiogenesis, induce tumor cell apoptosis and alter tumor cell cycle distribution (11,12). Compared with other traditional antitumor drugs, ICA can regulate tumor immunity and reduce the lethality of cells of the surrounding normal tissues. Li *et al* (13) demonstrated that ICA could inhibit hepatocellular carcinoma cell proliferation, promote apoptosis and enhance the antitumor effects of arsenic trioxide *in vitro* and *in vivo*. Additionally, Yang *et al* (14) revealed that ICA not only inhibited the proliferation of glioblastoma cells in a dose-dependent manner, but also enhanced the antitumor effect of temozolomide. Shi *et al* (15) also found that ICA attenuated the proliferation of colorectal cancer (CRC) cells and inhibited the antitumor activity of 5-fluorouracil in CRC via inhibiting the activity of nuclear factor-κB (NF-κB) *in vitro*. The NF-κB signaling pathway is closely associated with tumor cell apoptosis and plays a key role in the immune aging process of tumor cells (16-18). The aforementioned studies indicate that ICA has a wide range

of potential antitumor effects. However, its effects on OSCC remain elusive.

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway has been reported to play critical roles in various types of cancer (19-21). For example, the PI3K/AKT pathway is regarded as one of the key mechanisms involved in lung cancer cell metastasis and the epithelial-mesenchymal transition (19). Besides, the PI3K/AKT signaling pathway is associated with renal cell carcinoma cell proliferation and metastasis (20). Moreover, the PI3K/AKT pathway plays important roles in the regulation of human pharyngeal squamous carcinoma cell apoptosis (21). Activation of the PI3K/AKT signaling pathway has been confirmed in OSCC, and inhibiting this signaling pathway has been reported to attenuate the development of OSCC (22-24). Until now, whether ICA could affect PI3K/AKT signaling pathway activation in OSCC remain unclear. Therefore, the present study aimed to investigate the effects and underlying molecular mechanisms of ICA on OSCC cells to provide a novel theoretical basis for the treatment of OSCC.

Materials and methods

Cell culture and treatment. The OSCC cell lines Cal 27 and SCC9 were obtained from the American Type Culture Collection, and human oral mucosa fibroblasts were purchased from Shanghai Aiyan Biotechnology Co., Ltd. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified incubator containing 5% CO_2. Human normal oral keratinocytes (hNOKs) were obtained from Lifeline® Cell Technology. hNOKs were cultured in DermaLife® K Medium (Lifeline® Cell Technology). Cells were treated with 0, 5, 10, 20 and 40 μM ICA (Shanghai Yuanye Biotechnology Co., Ltd.) for 24, 48 and 72 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. OSCC cell proliferation was evaluated using a MTT assay. Briefly, Cal 27 and SCC9 cells were seeded (10^4 cells per well) into 96-well plates and cultured at 37˚C in a humidified incubator containing 5% CO_2. Subsequently, cells were treated with 0, 5, 10, 20 and 40 μM ICA for 24, 48 or 72 h. Following treatment, cells were supplemented with 10 μl MTT solution and incubated for an additional 4 h, according to the manufacturer's instructions. The formazan crystals were dissolved by the addition of dimethyl sulfoxide. Finally, optical density values were measured at 570 nm using a multifunctional plate reader (BD Biosciences).

Flow cytometric analysis. Cell apoptosis was assessed using an Annexin-V/PI Apoptosis Detection kit (Beyotime Institute of Biotechnology). Cal 27 and SCC9 cells were treated with 0, 5, 10, 20 and 40 μM ICA for 48 h. Subsequently, cells in the logarithmic phase of growth were digested with 0.25% trypsin solution without ethylene diamine tetra acetic acid, centrifuged at 1,000 x g for 5 min at 4˚C and the supernatant was discarded. The cell pellet was then washed twice with pre-chilled PBS and re-suspended in 100 μl of pre-chilled 1X Annexin V binding buffer (Beyotime Institute of Biotechnology). The cells were then incubated with 5 μl Annexin V-FITC and 5 μl propidium iodide (Beyotime Institute of Biotechnology) for 15 min at room temperature in the dark. To detect apoptosis, a BD FACSCalibur flow cytometer (BD Biosciences) was used, and data were analyzed using the Cell Quest software (version 5.1; BD Biosciences).

Western blot analysis. The expression levels of cleaved-caspase-3 (cat. no. ab32042; dilution, 1:500; Cell Signaling Technology, Inc.), pro-caspase-3 (cat. no. ab32150; dilution rate, 1:1,000; Abcam), Bcl-2 (cat. no. 4223; dilution, 1:1,000; Cell Signaling Technology, Inc.), Bax (cat. no. 5023; dilution, 1:1,000), phosphorylated (p)-p65 (cat. no. 3033; dilution, 1:1,000), p65 (cat. no. 8242; dilution, 1:1,000) and p-AKT (cat. no. 4060; dilution, 1:1,000) and AKT (cat. no. 4685; dilution, 1:1,000) (all Cell Signaling Technology, Inc.) were evaluated using western blot analysis. Total proteins were extracted from OSCC cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), and the protein concentration was determined using a bicinchoninic acid assay kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. Proteins (40 μg per lane) were separated by 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (EMD Millipore). Following blocking with 5% skimmed milk for 1 h at room temperature, the membrane was first incubated with primary antibodies at 4˚C overnight and then with anti-rabbit IgG horseradish peroxidase-linked antibodies (cat. no. 7074; dilution, 1:2,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. Finally, the blot was developed with enhanced chemiluminescence reagent (Cyvita) and visualized. The strips were evaluated by light gray value analysis using ImageJ software (version 1.46; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from OSCC cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was then reverse transcribed into cDNA using the HiScript™ II qRT SuperMix (Vazyme Biotech Co., Ltd.). Reverse transcription reaction condition was as following: 25˚C For 5 min, 42˚C for 60 min and 80˚C for 2 min. cDNA was analyzed by qPCR using the ChamQ™ Universal SYBR qPCR Master mix (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The amplification conditions were as follows: Pre-denaturation at 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 34 sec. Primer sequences were listed as following: GAPDH, forward: 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse: 5'-GTAGAGGCAGGGATGATGTTCT-3'; Bcl-2, forward: 5'-GATCCTCGAGATGGCGCACGCTGGGAGAAC-3' and reverse: 5'-GATCGGATCCTCATGGCTGAGCGCAG-3'; Bax, forward: 5'-GGACGAACTGGACAGTAACATGG-3' and reverse: 5'-GCAAAGTAGAA-AAGGGCGACAAC-3'; p65, forward: 5'-ACAACAACCCCTTCCAAGAAGA-3' and reverse: 5'-CAGCCTGGTCCCGTGAAATA-3'; AKT, forward: 5'-TAAAGAAGGAGGTCATCGTGG-3' and reverse: 5'-CGGGACAGGTGGAAGAAAA-3'. The relative gene expression levels were analyzed using

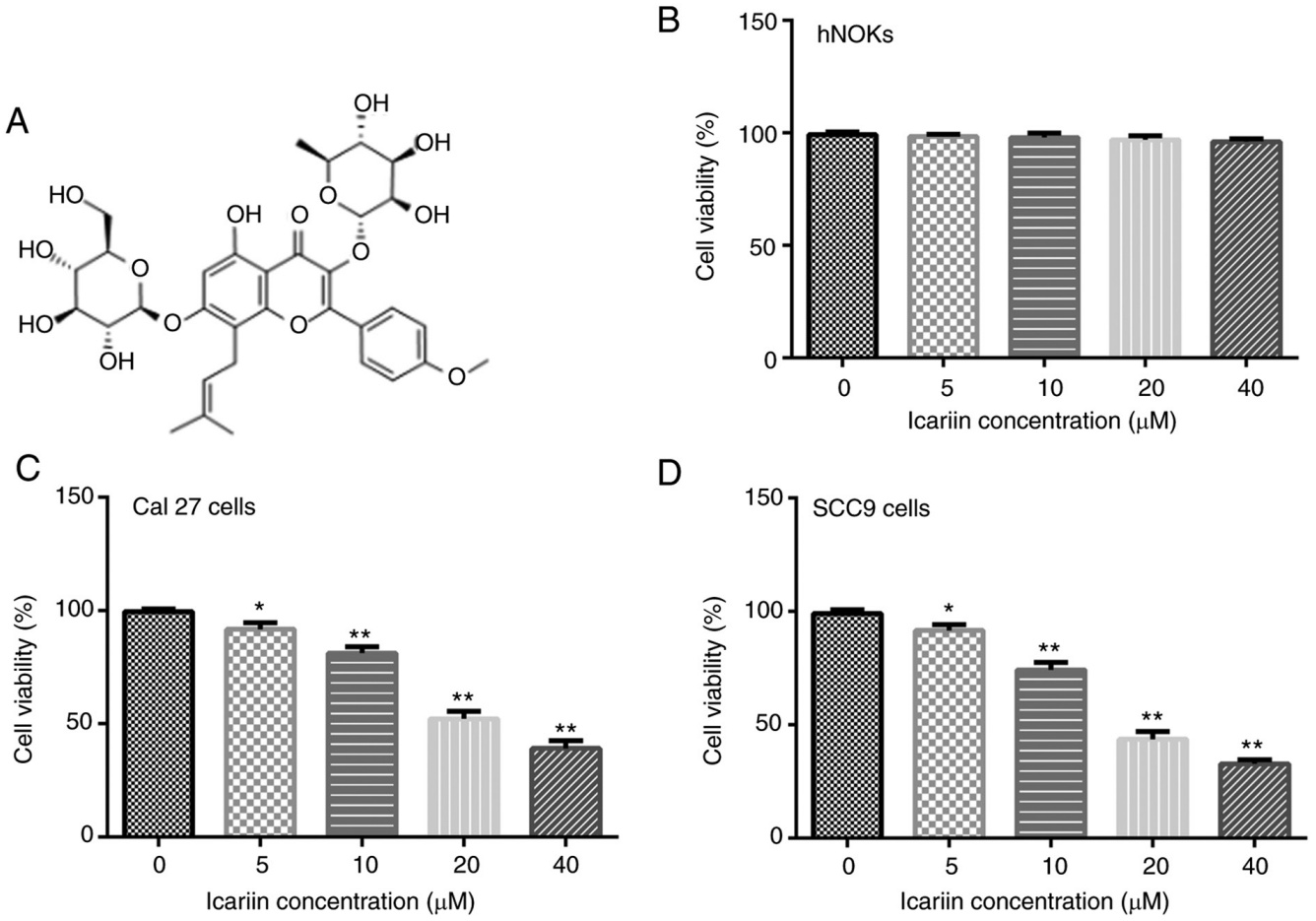

Figure 1. Effect of ICA on OSCC cell viability. (A) Chemical molecular structure of ICA. (B) hNOKs were treated with 0, 5, 10, 20 or 40 μM ICA for 48 h, and then a MTT assay was performed to measure cell viability. (C and D) OSCC cell lines, Cal 27 and SCC9, were treated with 0, 5, 10, 20 or 40 μM ICA for 48 h, and then a MTT assay was performed to measure cell viability. *P<0.05 and **P<0.01 vs. 0 μM ICA control group. ICA, icariin; OSCC, oral squamous cell carcinoma; hNOKs, human normal oral keratinocytes.

the $2^{-\Delta\Delta Cq}$ method (25). All experiments were performed in triplicate.

Statistical analysis. The data from three independent experiments are expressed as the mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, Inc.). The statistical differences among different groups were analyzed by one-way ANOVA followed by Tukey's post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of ICA on the OSCC cell lines, Cal 27 and SCC9. The chemical molecular structure of ICA is shown in Fig. 1A. First, the effect of ICA on the proliferation of human normal oral keratinocytes (hNOKs) was determined, and the results indicated that there was no significant effect of ICA on the viability of hNOKs (Fig. 1B). Then, the effect of ICA on OSCC cells investigated and Cal 27 and SCC9 cell lines were treated with 0, 5, 10, 20 or 40 μM ICA for 48 h. The effect of ICA on cell viability was determined using a MTT assay. The results demonstrated that ICA inhibited Cal 27 and SCC9 cell viability in a dose-dependent manner (Fig. 1C and D).

Effect of ICA on OSCC cell apoptosis and expression of apoptosis-related proteins. The results revealed that following treatment of Cal 27 cells with ICA (0, 5, 10, 20 or 40 μM) for 48 h, the apoptosis rate was significantly increased with increasing concentrations of ICA (Fig. 2A and B). Furthermore, the protein expression levels of cleaved-caspase-3 in Cal 27 cells were upregulated, those of pro-caspase-3 were down-regulated and the ratio of cleaved-caspase-3/pro-caspase-3 was increased, in a dose-dependent manner (Fig. 2C and D). Besides, it was demonstrated that the protein and mRNA expression levels of Bax (Fig. 2E and F) in Cal 27 cells were upregulated, and those of Bcl-2 (Fig. 2E and G) were downregulated in a dose-dependent manner. Additionally, the apoptosis rate results (Fig. 3A and B) and the expression levels of the apoptosis-related proteins (Fig. 3C-G) in SCC9 cells were consistent with those observed in the Cal 27 cell line.

Effect of ICA on the NF-κB and PI3K/AKT signaling pathways in OSCC cell lines. Cal 27 cells were treated with ICA (0, 5, 10, 20 or 40 μM) for 48 h, and the protein expression levels of p-p65, p65, p-AKT and AKT were detected using western blot analysis. The protein expression levels of p-p65 and p-AKT in Cal 27 cells were decreased with increasing concentrations of ICA (Fig. 4A). In addition, the p-p65/p65 and p-AKT/AKT

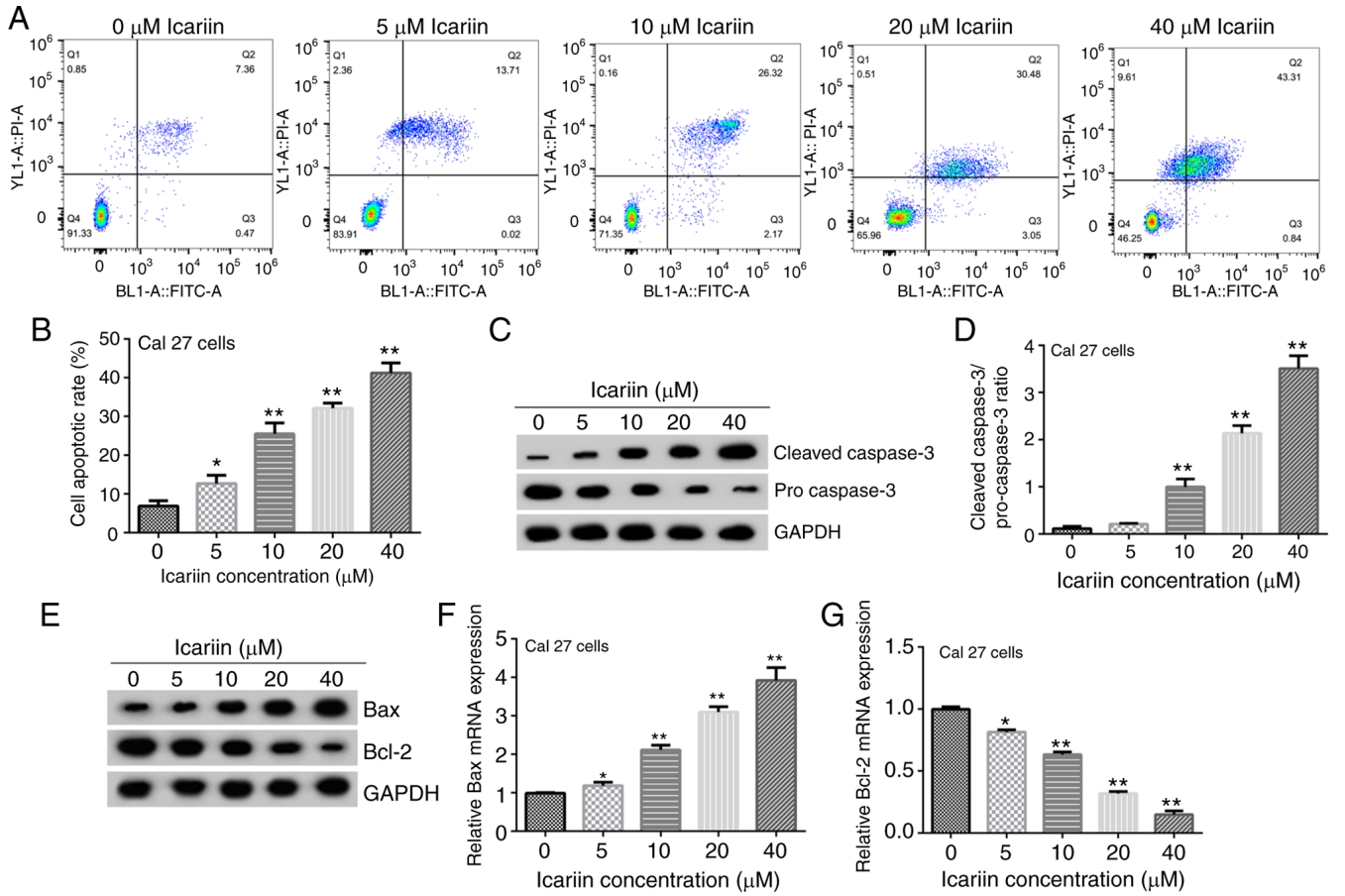

Figure 2. Apoptosis rate of the ICA-induced oral squamous cell carcinoma cell line, Cal 27. (A and B) Apoptosis rate of Cal 27 cells treated with 0, 5, 10, 20 or 40 μM ICA for 48 h was determined by flow cytometric analysis. (C) Protein expression levels of cleaved-caspase-3 and pro-caspase-3 were detected using western blot analysis. (D) Cleaved-caspase-3/pro-caspase-3 ratio in Cal 27 cells is shown. (E) Protein expression levels of Bax and Bcl-2 were detected using western blot analysis. (F) mRNA expression levels of Bax were detected using RT-qPCR. (G) mRNA expression levels of Bcl-2 were detected using RT-qPCR. *P<0.05 and **P<0.01 vs. 0 μM ICA control group. ICA, icariin; RT-q, reverse transcription-quantitative.

protein ratios were reduced in Cal 27 cells treated with ICA (Fig. 4B and C). The RT-qPCR results revealed no significant changes in the mRNA expression levels of p65 and AKT (Fig. 4D and E). Similar results were obtained in SCC9 cells (Fig. 5). The aforementioned findings indicated that treatment of OSCC cells with ICA could attenuate cell proliferation and induce apoptosis via inhibiting the NF-κB and PI3K/AKT signaling pathways.

Discussion

The present study aimed to investigate the effects of ICA in OSCC cells. The results showed that treatment with ICA significantly increased the apoptosis rate in a dose- and time-dependent manner. In OSCC cells treated with different concentrations of ICA, the protein expression of cleaved-caspase-3 and pro-caspase-3 were up- and downregulated, respectively, in a dose-dependent manner. Furthermore, ICA could attenuate OSCC cell proliferation via inhibiting the NF-κB and PI3K/AKT signaling pathways. These findings provided novel insights into the role of ICA and potential for the management and clinical treatment of OSCC.

With increasing research in tumor biology and immunology, it has been gradually considered that the occurrence and development of malignant tumors depend not only on the tumor cells themselves, but also on their regulation by the immune system (26). Therefore, ICA can regulate the function of immune organs and cells, and enhance the activity of immune cytokines, thus improving tumor immune function (27). It has been reported that the Bax/Bcl-2 ratio is increased with increasing concentrations of ICA (3). In addition, a study demonstrated that ICA is involved in the human immune regulation via increasing the activity of NF-κB in anti-inflammatory- and antiviral-related pathways, inducing the expression of p65, accelerating lymphocyte apoptosis and delaying immune aging (28). Furthermore, ICA could inhibit the proliferation of acute promyelocytic leukemia cells in a dose-dependent manner, thereby promoting their apoptosis (4). Additionally, a study revealed that ICA could block the NF-κB signaling pathway, which in turn could hinder the mRNA expression of the inflammation-related factor inducible nitric oxide synthase and delay the onset of inflammatory diseases, thus suggesting that ICA has strong anti-inflammatory effects (2). Therefore, it was hypothesized that ICA could also inhibit the NF-κB and PI3K/AKT signaling pathways in OSCC, thereby attenuating OSCC cell proliferation and inducing apoptosis.

In the current study, the effect of ICA on Cal 27 and SCC9 cell viability and apoptosis was assessed by MTT and

Figure 3. Apoptosis rate of the ICA-induced oral squamous cell carcinoma cell line, SCC9. SCC9 cells were treated with 0, 5, 10, 20 or 40 μM ICA for 48 h. (A and B) Apoptosis rate of SCC9 cells treated with 0, 5, 10, 20 or 40 μM ICA for 48 h was determined using flow cytometric analysis. (C) Protein expression levels of cleaved-caspase-3 and pro-caspase-3 were detected using western blot analysis. (D) Cleaved-caspase-3/pro-caspase-3 ratio in SCC9 cells is shown. (E) Protein expression levels of Bax and Bcl-2 were detected using western blot analysis. (F) mRNA expression levels of Bax were detected using RT-qPCR. (G) mRNA expression levels of Bcl-2 were detected using RT-qPCR. *P<0.05 and **P<0.01 vs. 0 μM ICA control group. ICA, icariin; RT-q, reverse transcription-quantitative.

Figure 4. Effect of ICA on the of nuclear factor-κB and phosphatidylinositol-3-kinase/AKT signaling pathways in Cal 27 cells. (A) Protein expression levels of p-p65, p65, p-AKT and AKT were determined using western blot analysis. (B) p-p65/p65 ratio. (C) p-AKT/AKT. (D) mRNA expression levels of p65. (E) mRNA expression levels of AKT. *P<0.05, **P<0.01 vs. 0 μM ICA control group. ICA, icariin; p-, phosphorylated; AKT, protein kinase B.

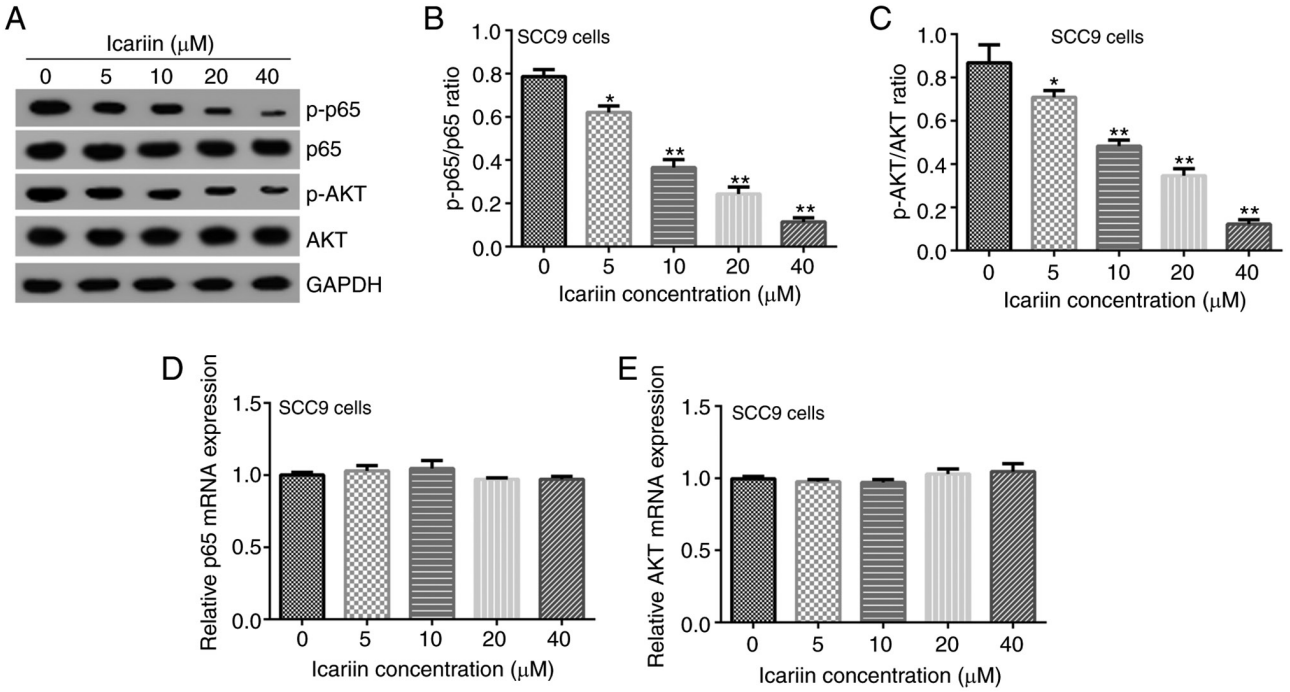

Figure 5. Effect of ICA on the nuclear factor-κB and phosphatidylinositol-3-kinase/AKT signaling pathways in SCC9 cells. (A) Protein expression levels of p-p65, p65, p-AKT and AKT were determined using western blot analysis. (B) p-p65/p65 ratio. (C) p-AKT/AKT ratio. (D) mRNA expression levels of p65. (E) mRNA expression levels of AKT. *P<0.05 and **P<0.01, compared with the 0 μM ICA control group. ICA, icariin; p-, phosphorylated; AKT, protein kinase B.

western blot assays, respectively. The results revealed that ICA could attenuate cell viability and induce apoptosis in the OSCC cell lines in a dose-dependent manner. Chen *et al* (2) demonstrated that ICA could upregulate the protein expression of sirtuin 6 (SIRT6) and downregulate that of NF-κB (p65) in animal tissues and cell models. These results showed that the ICA-mediated SIRT6 upregulation had an inhibitory effect on the NF-κB inflammatory signaling pathway, since treatment with ICA decreased the mRNA expression levels of the NF-κB downstream target genes, TNF-α, intercellular adhesion molecule 1, IL-2 and IL-6. Herein, the protein expression levels of p-p65 and p-AKT were decreased, following cell treatment with ICA, in a dose-dependent manner. No obvious changes were observed regarding the mRNA expression levels of p65 and AKT in both OSCC cell lines. In addition, the p-p65/p65 and p-AKT/AKT ratios were decreased in OSCC cells in a dose-dependent manner. The aforementioned findings suggested that ICA could attenuate OSCC cell proliferation via inhibiting the NF-κB and PI3K/AKT signaling pathways.

ICA is a novel biological immunomodulator and inducer of differentiation, thus improving immunity function, and inhibiting tumor cell proliferation, tumor growth and tumor angiogenesis (27,29,30). Herein, ICA inhibited the NF-κB and PI3K/AKT signaling pathways to promote OSCC cell proliferation, suggesting that this drug should be considered as a potential monomer for treating OSCC. However, the current study still had some limitations. For example, the effect of ICA was only studied in two OSCC cell lines, and so more cell lines need to be used for verification. Furthermore, the effects of only four concentrations of ICA on OSCC cell lines were studied, thus more concentrations of ICA should be explored. In addition, no *in vivo* studies were conducted. In the future, these issues will be studied in more depth.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LS contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. JZ contributed to data collection, statistical analysis and manuscript preparation. All authors read and approved the final manuscript. LS and JZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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