ORIGINAL RESEARCH ARTICLE

Effect of zoledronic acid on biological characteristics of cervical cancer cells

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Abstract

Cervical cancer (CC) is a malignant tumor in females characterized by high incidence and mortality rates, often resulting in a poor prognosis for patients. Zoledronic acid (ZA), a third-generation bisphosphonate, exhibits anti-tumor properties across various types of tumors. To further understand the effect of ZA in the treatment of CC, this article included two kinds of human CC cells (CCCs) as the research object, examining the impact of varying levels of ZA on the cells’ biological properties. Hela and Siha were cultivated and exposed to ZA at 0, 50, 100, and 200 μM, and the changes of cell proliferation, clone formation, migration, and invasion characteristics were detected. Cell RNA was extracted to detect epithelial-mesenchymal transition (EMT) and the relative expression (RE) of AKT/GSK3β/β-catenin (β-cat) pathway related proteins. The results show that as against 0 μM, the proliferation rate, clone formation cell number, migration distance, and invasive cell number of Hela and Siha were markedly reduced, while E-cadherin (E-cad) was markedly enhanced. N-cadherin (N-cad), vimentin (Vim), p-AKT, p-GSK3β, and β-cat were markedly decreased at 50, 100, and 200 μM ZA; With the increase of ZA concentration, the biological characteristics and protein expression levels of Hela and Siha changed more markedly, showing concentration dependent characteristics ($P < 0.05$). It was concluded that ZA can influence the malignant biological activities of CCCs. *(Afr J Reprod Health 2024; 28 [11]: 46-55).*

Keywords: CC; ZA, EMT, AKT/GSK3β/β-cat pathway

Résumé

Le cancer du col de l'utérus (CC) est une tumeur maligne chez la femme caractérisée par des taux d'incidence et de mortalité élevés, entraînant souvent un mauvais pronostic pour les patientes. L'acide zolédronique (ZA), un bisphosphonate de troisième génération, présente des propriétés antitumorales sur divers types de tumeurs. Pour mieux comprendre l’effet du ZA dans le traitement du CC, cet article a inclus deux types de cellules CC humaines (CCC) comme objet de recherche, examinant l’impact de différents niveaux de ZA sur les propriétés biologiques des cellules. Hela et Siha ont été cultivés et exposés à ZA à 0, 50, 100 et 200 μM, et les changements dans les caractéristiques de prolifération cellulaire, de formation de clones, de migration et d'invasion ont été détectés. L'ARN cellulaire a été extrait pour détecter la transition épithéliale-mésenchymateuse (EMT) et l'expression relative (RE) des protéines liées à la voie AKT/GSK3β/β-caténine (β-cat). Les résultats montrent que par rapport à 0 μM, le taux de prolifération, le nombre de cellules de formation de clones, la distance de migration et le nombre de cellules invasives de Hela et Siha ont été nettement réduits, tandis que la E-cadhérine (E-cad) a été nettement améliorée. La N-cadhérine (N-cad), la vimentine (Vim), le p-AKT, le p-GSK3β et le β-cat étaient nettement diminués à 50, 100 et 200 μM de ZA ; Avec l'augmentation de la concentration de ZA, les caractéristiques biologiques et les niveaux d'expression des protéines de Hela et Siha ont changé de manière plus marquée, montrant des caractéristiques dépendantes de la concentration ($P < 0,05$). Il a été conclu que ZA peut influencer les activités biologiques malignes des CCC. *(Afr J Reprod Health 2024; 28 [11]:46-55).*

Mots-clés: CC, ZA; ambulancier, Voie AKT/GSK3β/β-cat

Introduction

The incidence and mortality of cervical cancer (CC) ranks fourth among all female tumors in the world, which greatly threatens the life and health of women[1]. Persistent infection by high-risk HPV is identified as the principal cause of CC[2]. As therapeutic approaches continue to evolve, there has been an enhancement in the survival rates and prognosis for most CC patients after treatment. However, the prognosis for patients with CC at stages III/IV, those in advanced stages, or those with drug resistance remains grim[3,4].

Consequently, identifying potential therapeutic targets for CC and the pursuit of new drug development is deemed highly meaningful. V-akt murine thymoma viral oncogene homolog (AKT) and Glycogen synthase kinase 3 beta

(GSK3β) belong to serine/threonine kinases. AKT can activate the downstream factor GSK3β and participate in the regulation of cytoskeleton stability after activation[5]. Beta-catenin (β-cat) is a key molecule of Wnt signaling pathway (Wnt), involving in cell-cell adhesion and gene related regulation[6]. AKT is known to phosphorylate GSK3β, which results in the suppression of GSK3β's activity. This suppression subsequently leads to the stabilization of β-cat, which is then facilitated to enter the nucleus where it can initiate the transcription of downstream target genes, thus playing a role in the regulation of various biological processes[7]. In a variety of cancers, the AKT/GSK3β/β-cat pathway is abnormally activated, which in turn enhances cell proliferation and migration[8-10].

Zoledronic acid (ZA) is a third-generation bisphosphonate that offers advantages such as rapid onset, high remission rates, and prolonged duration of action compared to the first two generations. ZA binds to hydroxyl groups in hydroxyapatite crystals, forming soluble compounds that induce osteoclast apoptosis *in vitro*[11]. In the context of cancer bone metastasis, increased osteoclast activity leads to significant bone destruction, resulting in pain, fractures, hypercalcemia, and other associated symptoms. ZA can directly or indirectly inhibit bone metastasis of tumor cells (TCs) by suppressing osteoclast activity, and effectively enhance the well-being of people with diseases[12]. In addition to its effect on bone metabolism, ZA also has the impact of directly suppressing the multiplication of TCs, which can inhibit the growth and spread of TCs by interfering with their living environment[13]. ZA can inhibit the multiplication of TCs, slow down the growth rate of tumour, and ultimately improve the therapeutic effect. At present, ZA has been used to treat varieties of malignant tumors, for example, multiple myeloma[14-15]. Nevertheless, the therapeutic mechanism of ZA in combating CC still requires further investigation. This article explored whether ZA could mediate AKT/GSK3β/β-cat pathway to participate in the progression of CC. It offers references for understanding the pathogenesis and development mechanism of CC, as well as the selection of therapeutic targets and drugs

Methods

Materials

The following materials were used[16]: Hela and Siha (American Type Culture Collection); Fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco's modified eagle medium (DMEM) (Gibco, USA); ZA (Qilu Pharmaceutical Co., Ltd., China); Methyl Thiazolyl Tetrazolium(MTT) cell proliferation and cytotoxicity detection kit, bicinchoninic acid assay (BCA) protein quantification kit, and enhanced chemiluminescence (ECL) solution (Shanghai Beyotime Biotechnology Co., Ltd., China); Paraformaldehyde (Shandong Chengtai Chemical Co., Ltd., China); Crystal violet (Jinan Mingbang Chemical Co., Ltd., China); Matrigel and radio-immunoprecipitation assay (RIPA) (Sigma-Aldrich, USA); E-Cadherin(E-cad), N-Cadherin(N-cad), Vimentin(Vim), AKT, Phosphorylated v-akt murine thymoma viral oncogene homolog(p-AKT), GSK3β, Phosphorylated Glycogen Synthase Kinase 3 Beta(p-GSK3β), β-cat, Glyceraldehyde-3-Phosphate Dehydrogenase(GAPDH) protein first antibody (Ab) and horseradish peroxidase labeled Rabbit anti human IgG secondary Ab (Abcam, UK).

Experimental method

Grouping culture

HeLa and SiHa were cultured in DMEM that was enriched with 10% FBS and fortified with 1% penicillin-streptomycin (PS) to prevent microbial contamination. They were then placed in a controlled environment incubator set at 37°C, within an atmosphere composed of 5% carbon dioxide (CO_2) in air, to simulate the physiological conditions. This environment facilitated the cells' adherence and maintenance of their phenotypic characteristics. Upon reaching a confluence of about 90%, the cells underwent digestion with trypsin followed by passaging. The concentration was adjusted, and they were seeded at 5×10^3 per well in a 96-well plate, divided into groups with ZA (0, 50, 100, and 200 μM), with three replicate wells for each [17].

MTT cell proliferation assay

At 12, 24, 48, 72, and 96 h, 10 μL of MTT reagent was applied, and incubated or 4 h. Subsequently, the absorbance A value at 490 nm was measured to determine the proliferation rate and to chart the proliferation-time curve. Measurements for each group were conducted thrice, with the mean value being recorded[18].

Clone formation assay

Cells in a 6-well plate at 1×10^5 were cultured with ZA. At the initial time point and 48 h, they underwent a rinsing process using phosphate-buffered saline (PBS) to remove debris. Following the rinse, the samples were fixed in a 4% paraformaldehyde solution to preserve their morphology and structure for subsequent analysis. This step was essential for maintaining cell integrity. Following a 10-min staining period with 0.1% crystal violet, observations were made regarding the number of clones formed. Measurements were conducted three times, subsequently the mean value was determined[19].

Scratch healing assay

Cells in a 6-well plate at 1×10^5 were cultured with ZA until full confluence, until 100%, was achieved. A scratch was made at the bottom of the culture dish with a sterile 10 μL pipette tip, and the non-adherent were removed by rinsing with PBS. DMEM medium without serum was applied. At 0 h and 48 h, observation of the cells, capturing the images, the scratch healing was calculated. The average value was calculated based on three measurements[20].

Invasion assay

Cells were seeded at 1×10^5 in the upper compartment of a Transwell chamber pre-coated with Matrigel. In the lower compartment, 600 μL of DMEM medium having 10% FBS and 1% PS was added, culture for 24 h. Once fixed using 4% paraformaldehyde and stained with 0.1% crystal violet for 10 min, the stained state was subsequently observed. Triplicate measurements were conducted, and the mean was then derived from these values[21].

Western blotting assay

Cells were fully lysed with radioimmunoprecipitation assay buffer (RIPA) lysis buffer, and the protein concentration was quantitatively detected according to the bicinchoninic acid assay (BCA) protein concentration assay kit. A quantity of 30 μg of protein underwent boiling for 10 min to achieve complete denaturation, followed by undergoing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. Post-separation, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. Following the initial procedures, the membrane was subjected to a blocking step, which involved incubation with skim milk as a blocking agent. This step was conducted at a temperature of 25°C for a duration of 2 h, ensuring that the membrane was adequately covered to prevent non-specific binding of Ab in subsequent steps. Incubation was carried out overnight at 4°C with first Ab diluted at 1:1000 for E-cad, N-cad, Vim, AKT, p-AKT, GSK3β, p-GSK3β, β-cat, and diluted at 1:2000 for GAPDH. After the membrane was rinsed with TBST, it was incubated with a 1:5000 diluted horseradish peroxidase-labeled rabbit anti-human immunoglobulin G (IgG) secondary Ab at 25°C for 2 h. Following rinsing the membrane with Tris Buffered Saline with Tween (TBST), the enhanced chemiluminescence (ECL) solution was used for staining, and the membrane was photographed and developed under a gel imaging system. The relative expression (RE) of the target proteins were calculated using GAPDH as the reference[22].

Statistical analysis

GraphPad Prism 9.0 and *SPSS 23.0* were used to statistically evaluate the data. The representation of quantitative data was $\bar{x} \pm s$. For contrast, independent sample *t*-test and one-way ANOVA were adopted. The difference was statistically considerable with $P < 0.05$.

Ethical consideration

This study received ethical approval from Wuxi Second People's Hospital, with approval number [insert number].

Results

Impact of ZA on multiplication of cervical cancer cells

The multiplication-time curve of cervical cancer cells (CCCs) was plotted (Figure 1). As the culture time extended, the multiplication rate of Hela and Siha gradually enhanced. As against 0 μM, the multiplication rates of Hela and Siha treated with 50, 100, and 200 μM ZA were markedly reduced; With the escalation of ZA levels, the rates of cell multiplication for Hela and Siha progressively reduced, presenting visible distinctions observed among the groups ($P < 0.05$).

Impact of ZA on CC cell clone formation

Figure 2 illustrates the impact of ZA on the clone formation ability of CCCs. As against 0 μM, the number of Hela and Siha clone formation was markedly reduced at 50, 100, and 200 μM; As the concentration of ZA increased, the number gradually decreased, showing notable disparities ($P < 0.05$).

Impact of ZA on migration of CCCs

As against 0 μM, the migration distance of Hela and Siha at 50, 100, and 200 μM was markedly reduced; With the escalation of ZA levels, the migration distances of Hela and Siha progressively diminished, exhibiting discernible variations ($P < 0.05$) (Figure 3).

Impact of ZA on invasion of CCCs

As against 0 μM, the number of Hela and Siha invasion was markedly reduced at 50, 100, and 200 μM; With the escalation of ZA levels, the number gradually decreased, exhibiting discernible variations ($P < 0.05$) (Figure 4).

Impact of ZA on epithelial-mesenchymal transition of CCCs

As against 0 μM, the RE of E-cad in Hela and Siha was markedly raised, while the RE of N-cad and Vim was markedly decreased at 50, 100, and 200 μM; With the escalation of ZA levels, the RE of E-cad in Hela and Siha gradually raised, and the RE of N-cad and Vim gradually decreased, exhibiting discernible variations ($P < 0.05$) (Figure 5).

Impact of ZA on AKT/GSK3β/β-cat signaling pathway in CCCs

As against 0 μM, the RE of p-AKT, p-GSK3β, and β-cat in Hela and Siha were markedly decreased at 50, 100, and 200 μM; With the escalation of ZA levels, the RE gradually decreased, with visible distinctions observed ($P < 0.05$) (Figure 6)

Discussion

CC is a female reproductive tract malignant tumour that occurs in the cervix. At present, the treatment methods of CC include surgery, radiotherapy, chemotherapy, immunity, and targeted therapy. In addition to suppressing bone resorption, ZA can also exert antitumour effects *in vivo*. Zheng *et al*[23] earlier reported that ZA combined with immune checkpoint inhibitors can markedly suppress the tumor growth of non-small cell lung cancer transplanted mice, and promote the increase of anti-tumor cytokines interferon (IFN)-γ and interleukin-18 (IL-18) levels in serum. Lin *et al*[24] found that ZA can accelerate apoptosis by regulating autophagy. Polyploid cancer giant cells are the key cause of treatment failure. Adibi *et al*[25] found that ZA at various concentrations could markedly clear polyploid cancer giant cells and change the metabolism of the cells. ZA showed the impact of anti-multiplication of many kinds of tumor cells (TCs). This article further explored the mechanism of ZA against CC.

Abnormal and uncontrolled multiplication of cells is one of the main characteristics of cancer. The continuous multiplication of cancer cells makes the tumour volume constantly increase, causing compression and destruction of surrounding tissues and organs. Therefore, suppressing the multiplication of cancer cells can effectively control the progression of cancer[26]. Clone formation experiment is an important method to evaluate cell multiplication ability and population dependence. The stronger the clone formation ability of cancer cells *in vitro*, the stronger their tumorigenicity *in vivo*[27]. The transfer and attack of cancer cells are important steps in tumor metastasis. Cancer cells can reach sites far away from the primary lesion by transfer, forming new tumor lesions[28].

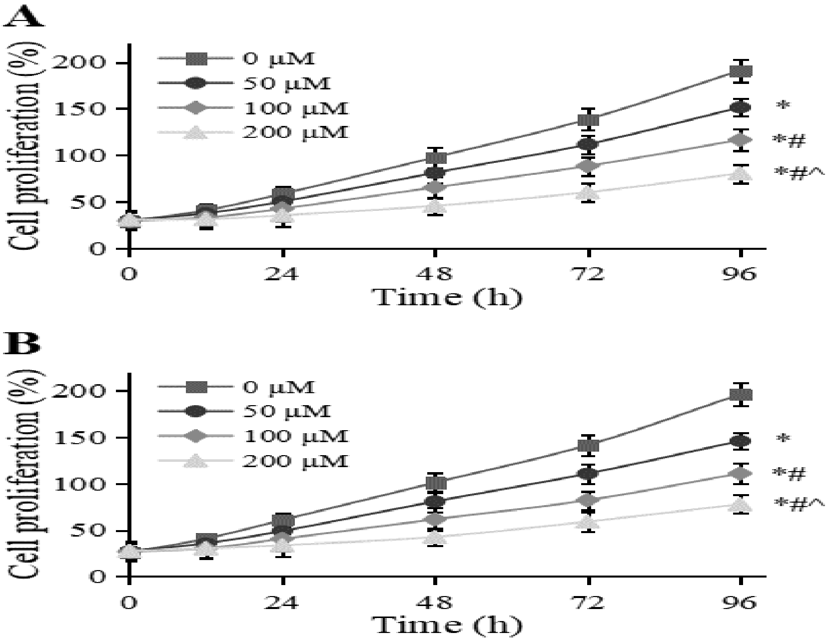

Figure 1: Contrast of multiplication-time curve of Hela and Siha. Note: A: Hela; B: Siha; as against *0, #50, ^100 μM, $P < 0.05$

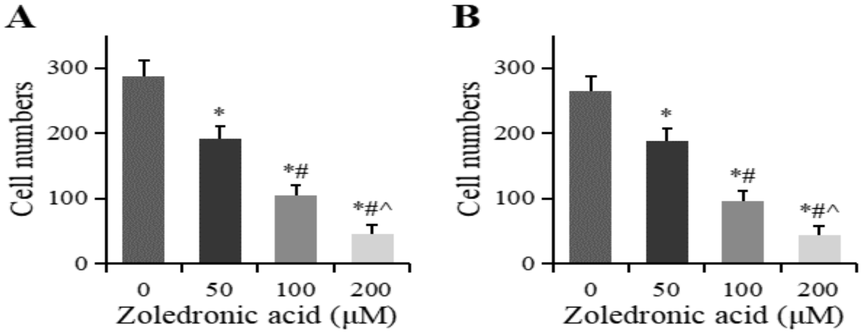

Figure 2: Contrast of clone formation numbers of Hela and Siha. Note: A: Hela; B: Siha; * as against *0, #50, ^100 μM, $P < 0.05$

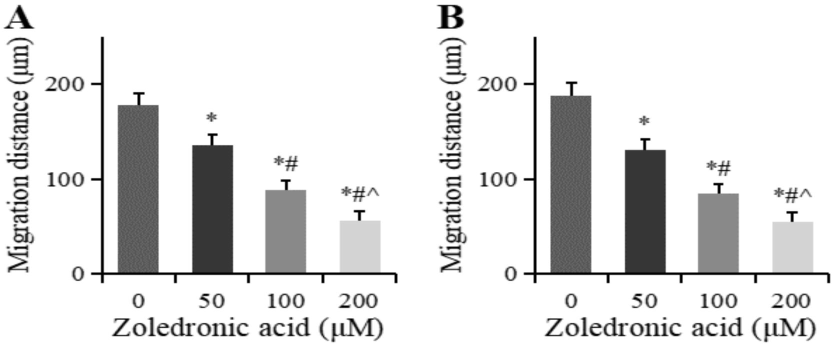

Figure 3: Contrast of migration distance of Hela and Siha CC. Note: A: Hela; B: Siha; as against *0, #50, ^100 μM, $P < 0.05$

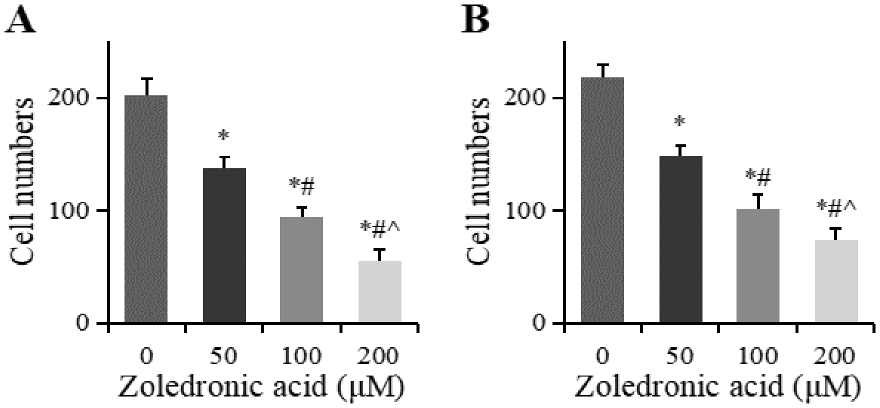

Figure 4: Contrast of invasion numbers of Hela and Siha. Note: A: Hela; B: Siha; as against *0, #50, ^100 μM, $P <$ 0.05

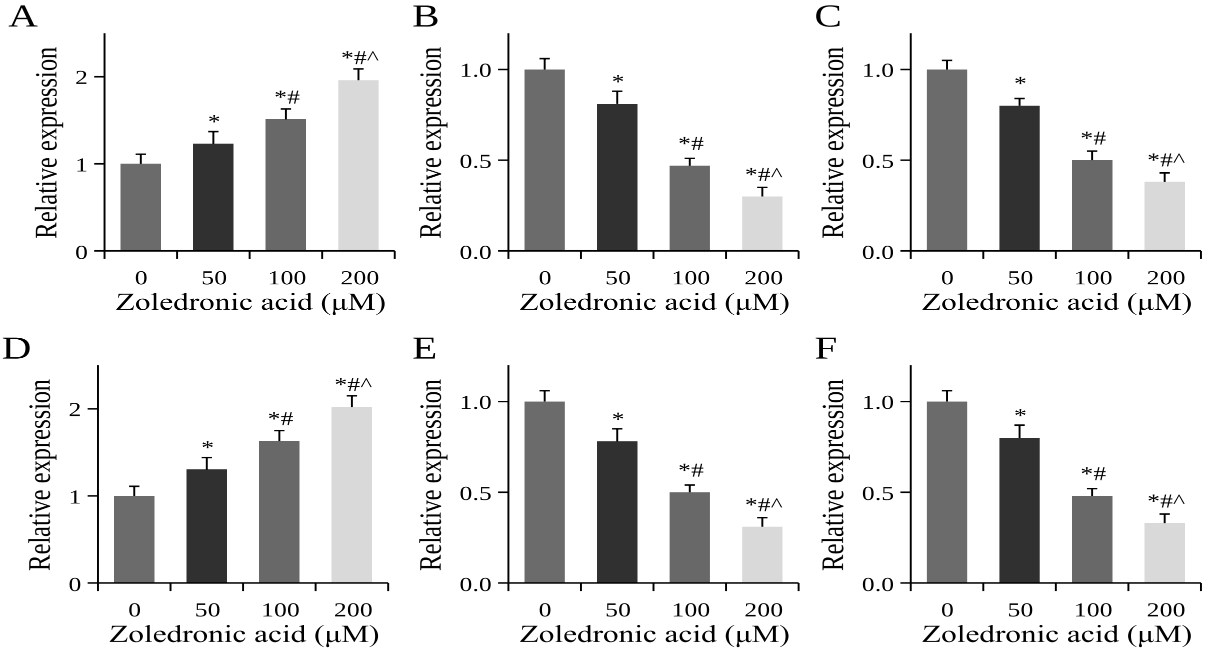

Figure 5: Contrast of Epithelial-Mesenchymal Transition (EMT) related protein expression levels in Hela and Siha. Note: A: E-cad in Hela; B: N-cad in Hela; C: Vim in Hela; D: E-cad in Siha; E: N-cad in Siha; F: Vim in Siha; as against *0, #50, ^100 μM, $P < 0.05$

In the process of cancer cell attack, it needs to break through the tissue barrier such as basement membrane, so as to enter the circulatory system such as blood vessels or lymphatic vessels, finally achieving distant metastasis[29]. The invasive ability of cancer cells can evaluate the degree of tumor malignancy[30-31]. It was found that ZA could effectively inhibit the multiplication, clone formation, transfer, and attack of Hela and Siha in a concentration dependent manner. This is in line with the results of Xu *et al*. (2019)[32], who found that ZA can inhibit the multiplication of multiple CC cell lines, and they also found that the impact of ZA combined with paclitaxel or doxorubicin in the treatment of CC is better than that of single drug. Wang and colleagues[33] pointed out that ZA can inhibit the viability of Hela, Siha, and Caski, and play a synergistic role by inducing apoptosis and autophagy activation.

Therefore, ZA acts in CC by suppressing the multiplication, clone formation, transfer, and attack of CCCs.

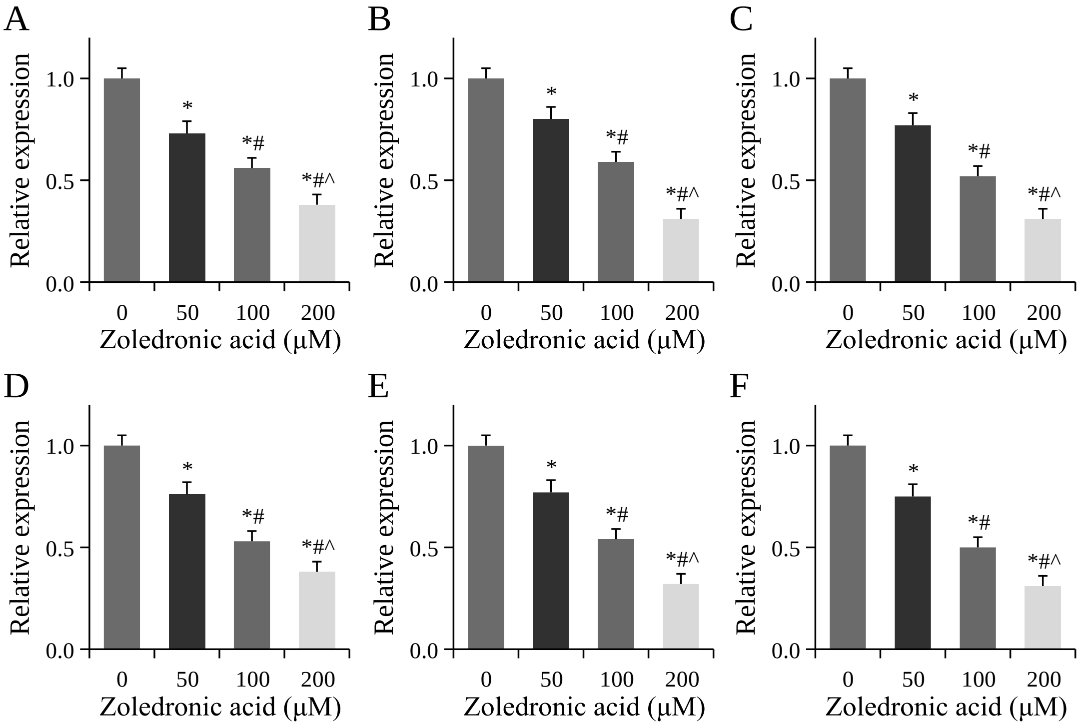

Figure 6: Contrast of AKT/GSK3β/β-cat signaling pathway related protein expression levels in Hela and Siha. Note: A: p-AKT in Hela; B: p-GSK3β in Hela; C: β-cat in Hela; D: p-AKT in Siha; E: p-GSK3β in Siha; F: β-cat in Siha; as against *0, #50, ^100 μM, $P < 0.05$

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells acquire metastatic ability after gradually transforming into mesenchymal phenotype. E-cad is an adhesion molecule that mainly exists in human and animal epithelia. Its main function is to maintain the morphology and structural integrity of normal epithelial cells. In cancer cells, the expression of E-cad is often downregulated, resulting in the decline of cell-cell adhesion ability, thus promoting the attack and metastasis of cancer cells[34]. N-cad is an epithelial cell adhesion junction related protein, which is involved in the process of cell separation and motility. Vim is an important type III intermediate filament protein, which widely exists in interstitial cells and mesoderm derived cells[35]. In CC, the expression of N-cad and Vim is frequently upregulated, which correlates with the attachment and invasiveness of cancer cells[36]. In the malignant process of tumor, EMT enables cancer cells to acquire transfer force, invade surrounding tissues through local infiltration, and eventually spreading to distant places through lymph nodes and blood circulation. Because the occurrence of EMT often means that CCCs have stronger transfer and attack ability and are more prone to attack and metastasis, this process often predicts a poor prognosis of CC patients[37]. In this article, it was found that ZA was able to suppress N-cad and Vim in Hela and Siha of CC. Therefore, ZA can inhibit the EMT of CCCs, and then suppress cell transfer and attack.

AKT/GSK3β/β-cat acts in carcinogenesis, and its abnormal trigger can lead to uncontrolled cell multiplication, blocked apoptosis, and enhanced transfer ability, promoting cancer cell metastasis and attack[38-39]. In the canonical Wnt signaling pathway, the accumulation of downstream target β-cat is mainly achieved by degrading GSK3β/APC/Axin complex. This article suggested that ZA could effectively inhibit AKT, GSK3β phosphorylation, and β-cat in Hela and Siha. Kim[40] found that ZA as a radiosensitizer can effectively promote deoxyribonucleic acid (DNA) damage and inhibit the phosphorylation of related proteins in the phosphoinositide 3-kinase (PI3k)/AKT pathway. Peng[41] found that ZA derivatives can inhibit the activation of PI3k/AKT pathway, affect the phosphorylation level of GSK3β, and then exert antitumor effects. Wang[42] pointed out that ZA could induce apoptosis and cell cycle arrest of CC stem cells in a concentration dependent manner, and this effect was mainly involved in this process by regulating AKT pathway activation. It is confirmed that AKT/GSK3β/β-cat pathway is involved in the progression of CC, and ZA can inhibit the progression of CC by suppressing the activation of this signaling pathway.

Limitations

The strengths of this study lie in its detailed mechanistic exploration, which reveals the potential of ZA to inhibit the malignant biological characteristics of cervical cancer cells. Additionally, the reliability and scientific rigor of the conclusions are reinforced through multiparametric assessments and statistical analyses. However, the study has certain limitations, primarily related to its reliance on *in vitro* cell models, which do not fully replicate the *in vivo* environment, and the use of a limited number of cell lines, necessitating further validation with a broader range of cell lines and clinical samples to ensure the generalizability and consistency of the results. Moreover, the research is confined to short-term treatment effects, lacking long-term observational data that would fully capture the role of ZA in extended therapy.

Nevertheless, this study provides a significant theoretical foundation for the use of zoledronic acid as a potential therapeutic approach for cervical cancer.

Future investigations, including *in vivo* experiments, preclinical studies, and clinical trials, are needed to confirm its safety and efficacy, potentially offering valuable insights for the development of novel anticancer agents and influencing relevant policy-making while supporting the conduct of additional clinical trials.

Conclusion

ZA inhibits the multiplication, clonal formation, transfer, and attack of CCCs in a concentration-dependent manner. It exerts its effects by suppressing the aberrant activation of the AKT/GSK3β/β-catenin pathway. However, the specific effect of ZA in the treatment of CC needs to be further confirmed by constructing animal models. In conclusion, the results of this article can provide references for further understanding the mechanism of ZA in the treatment of CC.

Contribution of authors

LQ and XQD conceptualised this study. LQ worked on the literature review. LQ and XQD worked on the data analysis and interpretation of results. All authors worked on the discussion of the findings. All the authors read and approved the final manuscript.

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Conflicting interests

The authors declare no competing interests.

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