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Original Article

CecropinXJ, a silkworm antimicrobial peptide, induces cytoskeleton disruption in esophageal carcinoma cells

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Antimicrobial peptides exist in the non-specific immune system of organism and participate in the innate host defense of each species. CecropinXJ, a cationic antimicrobial peptide, possesses potent anticancer activity and acts preferentially on cancer cells instead of normal cells, but the mechanism of cancer cell death induced by cecropinXJ remains largely unknown. This study was performed to investigate the cytoskeleton-disrupting effects of cecropinXJ on human esophageal carcinoma cell line Eca109 using scanning electron microscopy observation, fluorescence imaging, cell migration and invasion assays, western blotting, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. The electronic microscope and fluorescence imaging observation suggested that cecropinXJ could result in morphological changes and induce damage to microtubules and actin of Eca109 cells in a dose-dependent manner. The cell migration and invasion assays demonstrated that cecropinXJ could inhibit migration and invasion of tumor cells. Western blot and qRT-PCR analysis showed that there was obvious correlation between microtubule depolymerization and actin polymerization induced by cecropinXJ. Moreover, cecropinXJ might also cause decreased expression of α-actin, β-actin, γ-actin, α-tubulin, and β-tubulin genes in concentration- and time-dependent manners. In summary, this study indicates that cecropinXJ triggers cytotoxicity in Eca109 cells through inducing the cytoskeleton destruction and regulating the expression of cytoskeleton proteins. This cecropinXJ-mediated cytoskeleton-destruction effect is instrumental in our understanding of the detailed action of antimicrobial peptides in human cancer cells and cecropinXJ might be a potential therapeutic agent for the treatment of cancer in the future.

Keywords antimicrobial peptide; cytoskeleton; microtubule; actin

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Introduction

Esophageal carcinoma is one of the most common malignant tumors worldwide with marked regional variations in incidence [1]. In China, the incidence and mortality of esophageal cancer are very high, especially in Xinjiang Province [2], due to relatively late diagnosis and inefficient treatment. The pathogenesis and development of esophageal carcinoma are very complicated, and in most cases, esophageal carcinoma shows no symptoms until the cancer is in an advanced stage, thus making a cure less likely [3]. The overall survival rate of esophageal cancer patients still remains low. Chemotherapy is the standard treatment option; however, the chemo-resistance, potential toxicity, and various side effects of chemotherapeutics accelerate the progression of disease. Although a lot of esophageal cancer patients can survive >5 years after initial surgical treatment, over 60% die of metastasis and local recurrence [4,5]. Hence, it is imperative to design new therapeutic strategies for the treatment of esophageal cancer.

Antimicrobial peptide is an important component of the host innate immune system, which could prevent bacterial infections and is particularly critical for invertebrates lacking lymphocytes and antibodies, such as insects [6]. It can activate the immune system by participating in cell-signaling pathways involved in processes of anti-microbial [7], antiviral [8], and anti-parasitic activities [9], and in the maintenance of cell steady-state and various immune responses such as inflammation, wound healing and acquired immune system regulation [10–12]. These peptides have minimal toxicities and low sensitivities to the host [13], offering many advantages over other therapies, and emerged as potential alternative anticancer therapeutics.

Antimicrobial peptides can trigger necrosis via cell membrane lysis or apoptosis via mitochondrial lytic effect in cancer cells [14–16] in the presence of anionic lipids [17–19]. Thus, they are expected to cause little side effects,

as chemo-resistance and cytotoxicity will be less likely to occur to normal cells due to the action mode and specificity.

Cytoskeletal structure change is one of the important features of tumor cell proliferation and metastasis. Interestingly, it was reported that antibacterial peptides can specifically destroy prokaryotic cells and problematic eukaryotic cells with incomplete cellular skeleton, but leave the normal eukaryotic cells intact [20,21]. However, the effects of antibacterial peptides on tumor cell microtubule and actin cytoskeleton as well as cytoskeletal gene expression remain unclear.

CecropinXJ belongs to cecropin-B family and is a cationic antimicrobial peptide isolated from the larvae of *Bombyx mori*. It is positively charged and can form specific amphipathic α-helices to interact with the non-polar lipid cell membranes [12,22]. CecropinXJ could kill cancer cells but have no hemolytic activity against human erythrocytes and no toxicity to some normal cells [23]. In this study, we investigated the effects of cecropinXJ on cytoskeleton in human esophageal carcinoma Eca109 cells. Our results suggested that cecropinXJ could induce damage to microtubules and actin of Eca109 cells, and inhibit migration and invasion of tumor cells. Additionally, cecropinXJ could inhibit the expression of cytoskeletal genes. These findings suggest that cecropinXJ may be a promising candidate as a new anticancer agent.

Materials and Methods

Preparation of antimicrobial peptide cecropinXJ

CecropinXJ of *B. mori* was prepared through the *Saccharomyces cerevisiae* eukaryotic expression system and purified with Ni-NTA agarose column as reported previously [12]. The concentration of purified recombinant cecropinXJ protein was measured using a Bradford protein assay kit (Bioteke, Beijing, China). It has the amino acid sequence of MNFAKILSFVFALVLALSMTSAAPEPRWKI FKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK. For experiments, cecropinXJ was dissolved in Dulbecco's modified Eagle's medium (DMEM; Gibco, Cortland, USA) at 50 mM stock concentration and diluted using culture medium to the desired concentration just before use. For control, cells were incubated in cecropinXJ-free medium.

Cell culture and reagents

Eca109 cells were obtained from the Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 μg/ml penicillin, and grown at 37°C in an incubator (Thermo,Waltham, USA) with humidified atmosphere of 5% CO_2 in air. The cells were subcultured three times weekly.

Scanning electron microscopy

Totally, 1×10^4 cells were grown on poly-L-lysine-coated coverslips for 24 h and treated with 10 μM cecropinXJ for 30 min. After removal of the supernatant, 4% formaldehyde (Sigma, St Louis, USA) was added to each well and cells were fixed for 20 min. The fixed cells were then washed three times with 0.1 M tris-buffered saline (TBS) (pH 7.4) and permeabilized using 0.5% Triton X-100 (Sigma) for 10 min. The samples were postfixed with 1% (v/v) osmium tetroxide at 4°C for 2 h, dehydrated in ethanol and dried with liquid carbon dioxide in a critical point dryer (BAL-TEC, Balzers, Switzerland) for 30 min. Cells on coverslips were coated with gold and visualized by a LEO1430VP scanning electron microscope (LEO, Germany) [24].

Wound healing assay

A total of 5×10^4 cells were seeded into six-well plate and grown to confluence at 37°C for 18 h. Wound was created by scraping confluent cell monolayers with pipette tips. The cells were allowed to migrate for 48 h. At 0 and 48 h after scratching, images were taken under the inverted microscope (Leica, Nussloch, Germany) to assess the ability of the cells to migrate into the wound area.

Cell migration and invasion assays

For the cell migration assay, 5×10^4 cells were suspended in 200 μl of serum-free DMEM medium and seeded into the upper chamber of a 24-well plate (BD Bioscience, Bedford, USA). Then, 800 μl of DMEM containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation at 37°C, the migrated cells were fixed and stained for 30 min in a dye solution containing 0.2% crystal violet (Sigma) and 20% methanol (Sigma).

For the cell invasion assay, chambers were covered with 40–80 μl of Matrigel (BD Bioscience), which was diluted with DMEM to a certain percentage, and incubated at 37°C for 2–4 h. Then, 5×10^4 cells were suspended in 200 μl of DMEM and seeded into the upper chamber, and 800 μl of DMEM containing 10% FBS was added to the lower chamber. After incubation at 37°C, the cells were fixed and stained, and the number of invading cells was counted under fluorescence microscope in five random high power fields.

Immunofluorescence and confocal microscopy

Cells were cultured at low density to ensure log phase growth. Totally, 1×10^4 cells were grown on coverslips for 24 h and treated with 1, 5, 10, 50 μM cecropinXJ for 24 h and fixed with 4% formaldehyde in CBS buffer containing 10 mM MES (pH 6.1), 138 mM KCl, 3 mM $MgCl_2$, and 2 mM EGTA. Cells were washed three times with TBS buffer, permeabilized with ice-cold 0.5% Triton X-100 for 10 min and then blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. Next, cells were

incubated for 1 h in the presence of fluorescein isothiocyanate tagged DM 1-α antibody (Abcam, San Francisco, USA) and phalloidin-TRITC (Sigma). Nuclei were stained using 4′,6-diamidino-2-phenylindole (Sigma). The fluorescence images were visualized using the TCS SP5 confocal laser-scanning microscope (Leica).

Soluble tubulin and actin extraction assay

Samples for soluble tubulin and actin extraction were prepared according to Kim *et al.* [25]. Cells were collected and incubated with tubulin extraction buffer containing 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 0.5% Triton X-100, 300 nM paclitaxel, and protease inhibitor (Sigma) for 5 min. The supernatant was collected for the soluble fraction and the remaining cells were harvested for the polymerized fraction. Both fractions were lysed using protein sample buffer (Invitrogen, Grand Island, USA) and each fraction was analyzed by western blotting.

Western blotting analysis

Cells were lysed using protein sample buffer (Invitrogen) and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene fluoride membrane (Pall, Cortland, USA). After being washed with tris-buffered saline with Tween (TBST) (20 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20), the membrane was blocked with 3% (v/v) BSA/TBST buffer for 2 h at 37°C. The membrane was washed three times with TBST buffer and incubated with anti-α-tubulin and anti-β-actin primary antibodies (1 : 1000) in 1% (v/v) BSA/TBS buffer overnight at 4°C. Then, the membrane was washed three times with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) at 37°C for 1 h. After washing, the membrane was analyzed using the ECL chemiluminescent kit (Invitrogen).

Quantitative reverse transcription polymerase chain reaction assay of α-actin, β-actin, γ-actin, α-tubulin, β-tubulin expression

Total RNA was extracted from cells using TRIzol reagent (Sigma). For quantitative polymerase chain reaction (PCR), 3 μg of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, USA) in a reaction mixture (60 μl) containing 30 μM dNTP mixture, 6 μl oligo$(dT)_{12-18}$ primer, 12 μl 5× M-MLV buffer, 1.5 μl RNase inhibitor (40 U/μl), and 1.5 μl RTase M-MLV (200 U/μl) (TaKaRa, Dalian, China). The total RNA and oligo$(dT)_{12-18}$ primer were incubated at 70°C for 10 min, and added into the reverse transcription reaction mixture at 42°C for 1 h and at 70°C for 15 min. The PCRs were performed in a 25 μl reaction mixture containing the SYBR Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa) and 0.5 mM of each primer with the 7500 Real-Time PCR System (Applied Biosystems, Foster, USA). Primer sets used are listed in **Table 1**. Cycling conditions were 3 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 58°C, and 20 s at 72°C. *GAPDH* was used as an internal control. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that a single PCR product was detected, and PCRs without the addition of the template were used as blanks. Each experiment was repeated for three times. After the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, USA). The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method [26].

Table 1. Nucleotide sequence of primers used in the study

Gene	Sequence of primer
α-actin	F: 5′-AGCCCTCTTTCATTGGTAT-3′
	R: 5′-CAAGTCCTGGTCTGGTTTAT-3′
β-actin	F: 5′-CGGTCAGGTCATCACTATCGG-3′
	R: 5′-CACAGGATTCCATACCCAGGA-3′
γ-actin	F: 5′-CTGCTGCATGAGCTGATTCTG-3′
	R: 5′-CAAAGGCTTATTCCAGTTTCATGAG-3′
α-tubulin	F: 5′-CTCGCATCCACTTCCCTCTG-3′
	R: 5′-GCTGTTCATGGTAGGCTTTCTC-3′
β-tubulin	F: 5′-TTCCATTGACAATGAGGCTCTGT-3′
	R: 5′-TGGTTGAGATCGCCATAGGTA-3′
GAPDH	F: 5′-ATCAAATGGGGTGATGCTGGTGCTG-3′
	R: 5′-CAGGTTTCTCCAGGCGGCATGTCAG-3′

Statistical analysis

Data were presented as the mean ± SD. Student's *t*-test was used to determine differences between two populations. One-way analysis of variance was used to determine differences between more than two populations. Statistical differences were determined at the $P < 0.05$ or < 0.01 level for all analyses, respectively.

Results

Morphological observations under scanning electron microscope

To test directly whether cecropinXJ affects cytoskeleton network in Eca109 cells, we examined its effects on cytoskeleton by scanning electron microscope. The morphology of control Eca109 cells at 0 μM cecropinXJ was provided as control, showing complete cytoskeleton network, and cellular microtubules can also be seen clearly (**Fig. 1A,C**). However, cytoskeleton disruption was induced when tumor cells were treated with cecropinXJ at 10 μM for 30 min, showing that the cytoskeleton of cancer cells began to break, scatter, and disintegrate into pieces (**Fig. 1B,D**).

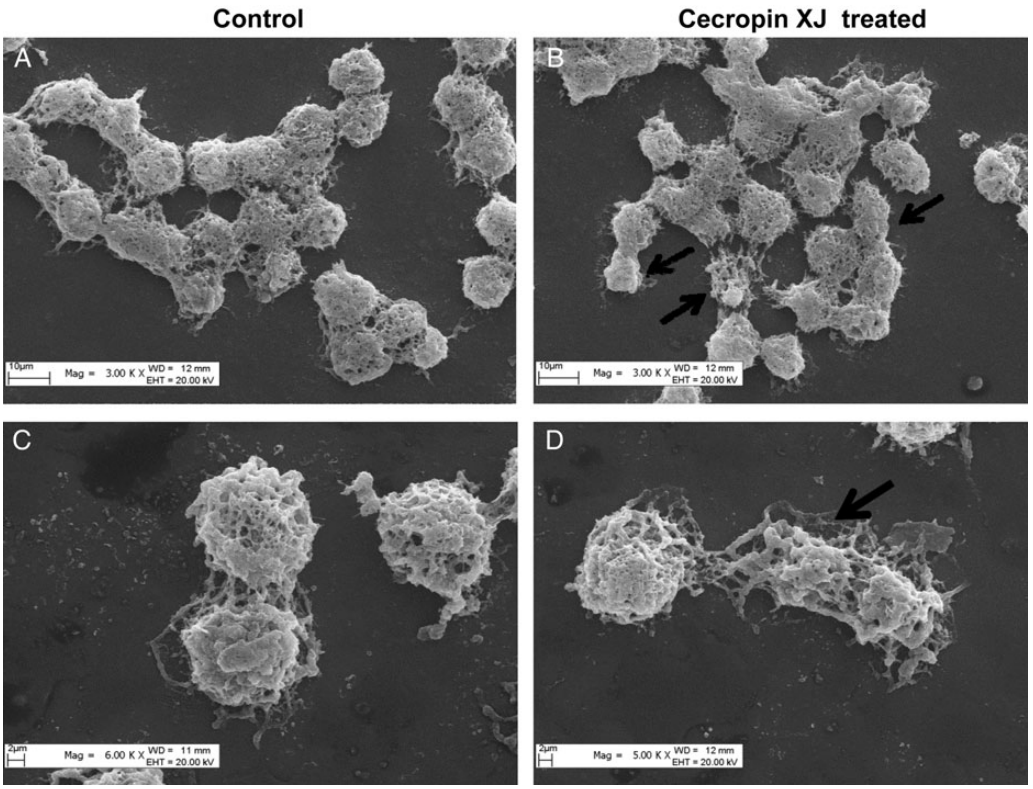

Figure 1. Morphological changes of cytoskeleton were observed in cecropinXJ-treated Eca109 cells Control (A,C) and cecropinXJ-induced (B,D) changes of cell morphology were observed by scanning electron microscopy. When Eca109 cells were treated with 10 μM cecropinXJ for 30 min, broken cytoskeleton pieces were observed. The pictures were selected from at least three independent experiments with similar results.

CecropinXJ affects the microtubule and actin cytoskeleton

To evaluate whether cecropinXJ interferes with the microtubule and actin cytoskeleton in Eca109 cells, we examined its effects on microtubule and actin by laser scanning confocal microscopy (**Fig. 2**). In control cells, microtubule structures radiated out from the centrosomes and thin actin bundles were visible throughout the cells. CecropinXJ induced dose-dependent microtubule disruption. In cells treated with cecropinXJ at minimal concentration, the remaining microtubules lost their radial structure, and actin bundles were tangled up. At 10 μM cecropinXJ, microtubules were completely depolymerized and actin bundles appeared surrounding the nucleus. Paclitaxel was used as positive control, with an increased density of cellular microtubules and formation of long thick microtubule bundles. These results indicate that the growth inhibition of Eca109 cells induced by cecropinXJ may also be associated with the cytoskeleton disruption.

CecropinXJ inhibits wound healing ability of Eca109 cells

Cell migration is an essential step in metastasis. Therefore, we next examined the ability of cecropinXJ to affect cell motility using the scratch wound healing assays in human esophageal cancer cell line Eca109. It was shown that untreated Eca109 cells covered almost the entire damaged area by 24 h. In contrast, the wound area was only partially covered by cecropinXJ-treated cells after 24 h of incubation (**Fig. 3A**) in a dose-dependent manner, and the distance of the scratch wound in cecropinXJ group is significantly larger compared with control groups, suggesting that cecropinXJ inhibits the wound healing ability of Eca109 cells.

CecropinXJ inhibits migration and invasion of Eca109 cells

To further detect the influence of cecropinXJ on migration and invasion, we performed transwell migration and invasion assay. It was shown that there were significant differences between the untreated group and the groups treated with cecropinXJ or DOX in transwell migration assay. The number of cells migrating through the chamber in 10 μM cecropinXJ was significantly decreased compared with the untreated group. Quantification of cells which had invaded through Matrigel 24 h later showed that cecropinXJ reduced esophageal cancer cell migration by ~90% ($P < 0.01$, **Fig. 3B**). The same result was observed in the invasion assay. The invasive cell number in the group treated with 10 μM cecropinXJ alone was remarkably lower than that in the untreated group ($P < 0.01$, **Fig. 3C**). Together, these results indicate that

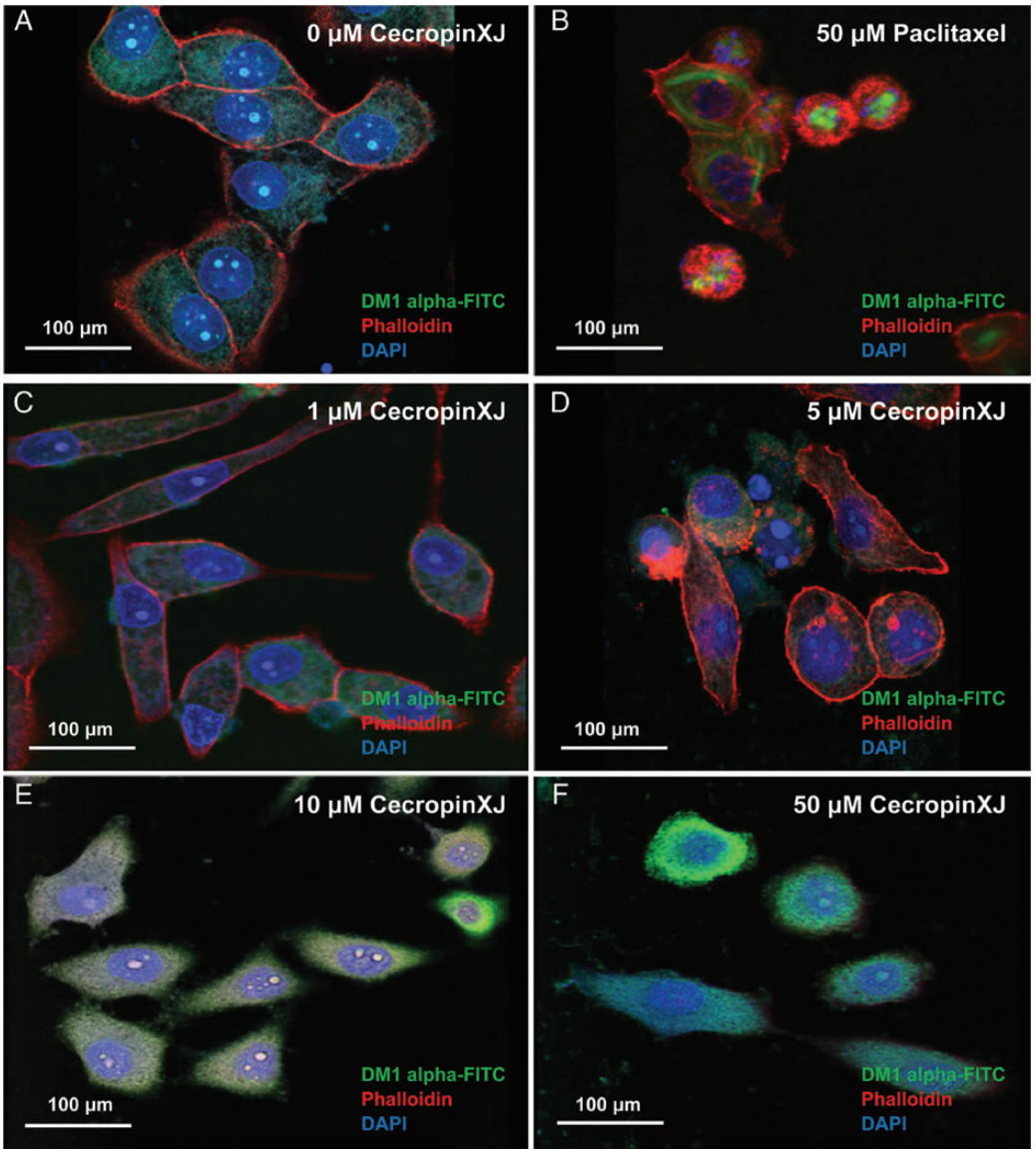

Figure 2. CecropinXJ disturbs the structure of microtubule and actin Eca109 cells were cultured with 0 (A), 1 (C), 5 (D), 10 (E), 50 μM (F) cecropinXJ and 50 μM paclitaxel (B) for 24 h, then the cells were fixed and stained with fluorescein isothiocyanate-DM 1-α antibody, phalloidin-TRITC to visualize microtubule (green), and actin (red), respectively, and nuclei were stained using 4′,6-diamidino-2-phenylindole (blue) as described in section. Paclitaxel was used as positive control. The white bar indicated 100 μm.

cecropinXJ inhibits the migration and invasion of human esophageal cancer Eca109 cells.

CecropinXJ causes microtubule depolymerization

Microtubule and actin are the major proteins of cytoskeleton and both regulate cell shape. To obtain more quantitative data on possible cytoskeleton effects of cecropinXJ, we assayed the fractions of tubulin and actin in the monomer and polymer pools with a cell permeabilization assay. Eca109 cells were treated with cecropinXJ at different concentrations for 24 h. Tubulin and actin in both fractions were quantified by western blotting. The result shows that cecropinXJ caused dose-dependent microtubule depolymerization (**Fig. 4A**). The fraction of actin in polymerized form was reproducibly increased following cecropinXJ treatment. The actin polymerization was higher than tubulin depolymerization.

To test whether microtubule depolymerization for cecropinXJ is related to actin polymerization, we pretreated Eca109 cells with paclitaxel to prevent microtubule depolymerization (**Fig. 4B**). The response of actin to cecropinXJ was decreased by paclitaxel pretreatment, suggesting that microtubule disruption is closely related to actin polymerization after being treated with cecropinXJ.

Changes of α-actin, β-actin, γ-actin, α-tubulin, and β-tubulin expression during cell disruption process

The standard curves were obtained with serial dilution of constructed recombinant plasmids. Direct effects of cecropinXJ

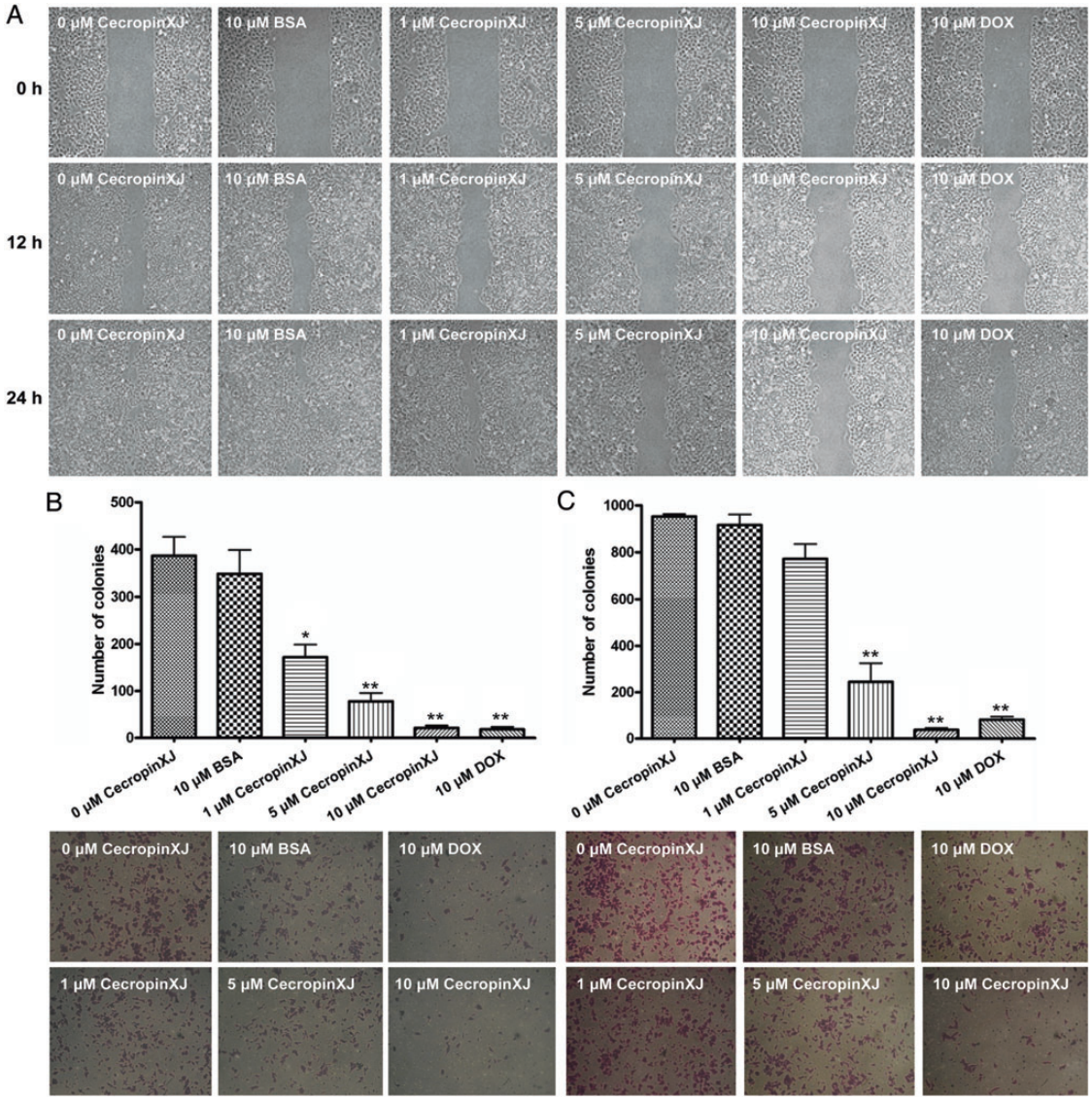

Figure 3. CecropinXJ inhibits wound healing ability (A), migration (B), and invasion (C) of Eca109 cells (A) Eca109 cells were grown on 12-well plate. A uniform cell free zone was made in the center of each well and different concentrations of cecropinXJ (1, 5, 10 μM) were added into each well. Migration was monitored from 0 to 24 h. The same fields were photographed under a phase-contrast microscope and the wound margins were depicted with black lines. The widths of the gaps from three independent experiments were measured and representative images were presented. (B) Transwell assay on the migration of Eca109 cells treated with cecropinXJ or control DOX, together with BSA as control. Cell numbers were counted from five random fields at 40× magnification, and the average numbers of migrated cells were shown as histogram. *$P < 0.05$, **$P < 0.01$ compared with control (0 μM cecropinXJ). (C) Transwell assay on the invasion of Eca109 cells treated with different concentrations of cecropinXJ (1, 5, 10 μM). Representative photographs of invasive Eca109 cells on the membranes were shown. The cell numbers per field were counted and the results are summarized in a bar graph. **$P < 0.01$ compared with control (0 μM cecropinXJ).

on α-actin, β-actin, γ-actin, α-tubulin, and β-tubulin expression were examined at various concentrations and incubating times (**Fig. 5**). Nevertheless, the relative expression levels of cytoskeletal genes were decreased in cecropinXJ treated cells, and especially, α-actin expression was decreased to minimum at 12 h or 5 μM cecropinXJ. CecropinXJ tended to cause the decrease of tubulin expression level, while α-tubulin was more susceptible to cecropinXJ treatment. The levels of β- and γ-actin expression were also down-regulated, although not as drastic as that of α-actin.

Discussion

Despite tremendous advances in treatment modalities, cancers remain a major cause of mortalities worldwide. Chemotherapy is currently one of the principal strategies for cancer treatment,

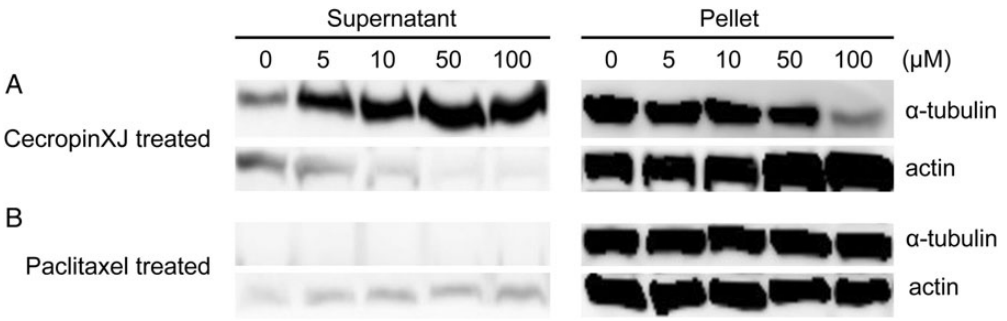

Figure 4. CecropinXJ caused microtubule depolymerization (A) Soluble tubulin and actin were extracted after treatment with 5, 10, 50, and 100 μM of cecropinXJ for 24 h. The monomers of tubulin and actin were extracted from supernatant, while the polymerized tubulin and actin remained in the pellets. Proteins were collected and subject to western blot analysis. (B) Eca109 cells were pretreated with 300 nM paclitaxel for 30 min before cecropinXJ treatment, and the extraction and analysis for tubulin and actin in both supernatant and pellet were performed as in A.

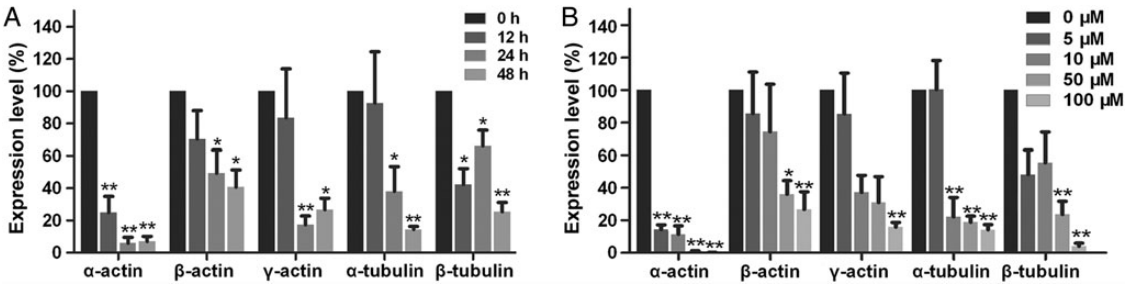

Figure 5. Changes of five kinds of cytoskeletal gene expression during cell disruption process After Eca109 cells were treated without (control) and with 5, 10, 50, and 100 μM cecropinXJ for 24 h or with 10 μM cecropinXJ for 12, 24, and 48 h, the relative amounts of α-actin, β-actin, γ-actin, α-tubulin, and β-tubulin gene expression were determined by quantitative reverse transcription polymerase chain reaction, *GAPDH* was used as reference. The expression levels of the five cytoskeletal genes were decreased in the treated cells. Significantly, the amounts of α-actin gene expression were significantly down-regulated. Data are presented as the mean ± SD from three independent tests. Statistical significances compared with control were calculated using Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

especially for those at advanced or metastatic stages [27]. However, severe side effects on normal cells and tissues and the easy acquisition of multi-drug resistances by cancer cells are often intimately associated with conventional chemotherapeutical agents [28]. Thus, new anticancer drugs with high potency, low toxicity/side effects to normal host cells and a distinct mode of action that disfavors drug resistance represent the new directions in the development of more effective anticancer drugs.

Cecropins show selective cytotoxicity and antitumor capabilities against a variety of cancer cell types, including bladder cancer [29], hepatocellular carcinoma [18], leukemia [30], colon cancer [31], breast cancer [32], small cell lung cancer [33], and gastric carcinoma [34]. Previous studies have provided the modes of action for antimicrobial peptides, which involve the disruption of the plasma and mitochondrial membranes, alternative pathways such as mediated immunity [35], hormonal receptors [36], DNA synthesis inhibition [37,38], and anti-angiogenic effects [39].

However, there have been very few measurements of the disruption of the cytoskeleton by cationic peptides. In this study, we show that cecropinXJ causes damage to microtubule and actin of Eca109 cells in a dose-dependent manner, and inhibits migration and invasion of tumor cells. Using scanning electron microscope, we confirm that cecropinXJ affects cytoskeleton network in Eca109 cells and causes the cytoskeleton of cancer cells disrupted, scattered, and disintegrated into pieces (**Fig. 1**).

In eukaryotic cells, the cytoskeleton supports many fundamental cellular processes, including morphogenesis, cell division, and vesicle trafficking [40–42]. Actin cytoskeleton remodeling, which is tightly controlled by intracellular signaling complexes, does affect not only cell motility, hereby supporting migration, invasion, and metastatic spreading, but also other vital cellular processes associated with cancer cell survival and aggressiveness, and therefore constitutes a crucial aspect of tumor progression [43].

Since it is well known that the cytoskeleton is one of the major inducers of cell shape, we decided to test directly whether cecropinXJ affects the microtubule or actin cytoskeleton in endothelial cells, and we imaged microtubule and actin in fixed cecropinXJ-treated Eca109 cells by laser scanning confocal microscopy (**Fig. 2**). In control cells, microtubule structures radiated out from the centrosomes and thin actin bundles were visible throughout the cells. CecropinXJ induced dose-dependent microtubule

disruption, starting at 1 μM. In cells treated with this minimal concentration, the remaining microtubules lost their radial structure and were tangled up.

Dynamic cytoskeletal changes are required for cell motility and invasion by cancer cells. Besides the effects of morphological changes in cells, we also analyzed the cell migratory and invasive inhibition effect of cecropinXJ on esophageal cancer cells. Our results showed that cecropinXJ significantly suppressed the migratory and invasive ability of Eca109 cells in a dose-dependent manner (**Fig. 3**). Cofilin is hypothesized to drive migration by depolymerizing F-actin [44]. It was reported earlier that LIMK phosphorylation of cofilin might inactivate cofilin by preventing its binding to F-actin filaments and this inactivation is predicted to inhibit cell migration [45], but recent data indicated that phosphorylation of cofilin is essential for establishing directed cell protrusion [46] and cell migration also requires cofilin phosphorylation step which is mediated by PAK4 [47]. Further studies are needed to elucidate the mechanism by which cecropinXJ affects the migration and invasion of esophageal cancer cells.

Paclitaxel is a widely used chemotherapeutic drug for various types of human cancers. It works by stabilizing microtubules to induce cell cycle arrest and prevent cell proliferation [48]. **Figure 4A** shows that cecropinXJ caused dose-dependent microtubule depolymerization and fraction of actin in polymerized form was increased following cecropinXJ treatment, suggesting that microtubules might be depolymerized for the actin cytoskeleton to respond. To test whether microtubule depolymerization is necessary for cecropinXJ to induce actin polymerization, we pretreated cells with paclitaxel to prevent microtubule depolymerization (**Fig. 4B**). Our results indicate that the response of actin to cecropinXJ is decreased by paclitaxel pretreatment, suggesting that microtubule disruption is required for actin polymerization induced by cecropinXJ.

The most relevant data to date revealed that tubulin synthesis in several cell types decreases in response to increasing levels of depolymerized tubulin subunits [49]. It is conceivable that a similar mechanism may exist for the other cytoskeletal genes. In this regard, the dramatic changes in cell configuration during suspension may trigger subtle mechanisms involving a reorganization of microfilaments and consequently cause an inhibition of actin synthesis. Reattachment may then initiate processes to restore the normal cytoskeleton and therefore require an enhanced actin protein production [50].

The morphological state of a cell is closely related to the regulation of cytoskeletal gene expression. Spiegelman and Farmer [51] observed a down-regulation of actin, tubulin, and vimentin synthesis before the dramatic morphological changes accompanying 3T3 preadipocyte differentiation. From our results, direct effects of cecropinXJ on α-actin, β-actin, γ-actin, α-tubulin, and β-tubulin expression were examined by a dose- and time-dependent manner (**Fig. 5**). It is possible, therefore, that regulation of cytoskeletal genes plays an important role during both growth and differentiation of many cells.

In summary, cecropinXJ could induce damage to microtubule and actin of Eca109 cells in a dose-dependent manner, and inhibit migration and invasion of tumor cells. There was an obvious correlation between microtubule depolymerization and actin polymerization induced by cecropinXJ. Meanwhile, cecropinXJ could decrease the expression of cytoskeletal genes in concentration- and time-dependent manners. Despite the promise that cationic cecropins could be used as potential anticancer agents, a number of issues and obstacles must be addressed and overcome before they can be effectively employed in a clinical setting.

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