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Sinomenine inhibits breast cancer cell invasion and migration by suppressing NF-κB activation mediated by IL-4/miR-324-5p/CUEDC2 axis

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ABSTRACT

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a vital transcription factor that regulates multiple important biological processes, including the epithelial–mesenchymal transition (EMT) and metastasis of breast cancer. Sinomenine is an isoquinoline well known for its remarkable curative effect on rheumatic and arthritic diseases and can induce apoptosis of several cancer cell types. Recently, sinomenine was reported as a tumor suppressor via inhibiting cell proliferation and inducing apoptosis. However, the role and mechanism of sinomenine in invasion and metastasis of breast cancer are largely unknown. Here, we report that sinomenine suppressed the invasion and migration of MDA-MB-231 and 4T1 breast cancer cells in a dose-dependent manner. We detected binding of NF-κB to the inhibitor of NF-κB (IκB) after the MDA-MB-231 cells were treated with 0.25, 0.5, and 1 mM sinomenine. Co-IP analysis revealed that sinomenine enhanced the binding of NF-κB and IκB in a dose-dependent manner, suggesting that sinomenine had an effect on inactivation of NF-κB. Western blotting and ELISA approaches indicated that the suppression effect was closely associated with the phosphorylation of IκB kinase (IKK) and its negative regulator CUEDC2. Sinomenine treatment decreased miR-324-5p expression, thus increased the level of its target gene CUEDC2, and then blocked the phosphorylation of IKK through altering the upstream axis. Finally, transfection of a miR-324-5p mimic inhibited the suppression of invasion and metastasis of MDA-MB-231 and 4T1 cell by sinomenine, providing evidence that sinomenine treatment suppressed breast cancer cell invasion and metastasis via regulation of the IL4/miR-324-5p/CUEDC2 axis. Our findings reveal a novel mechanism by which sinomenine suppresses cancer cell invasion and metastasis, *i.e.*, blocking NF-κB activation.

1. Introduction

Breast cancer is a type of malignant tumor that occurs in the mammary gland epithelial tissue and is the greatest cancerous threat to women's health [1]. The incidence rate of breast cancer has exhibited a global upward trend since 1980s [2–6]. Breast cancer is occasionally invasive or metastatic, and causes a high fatality rate once it spreads. In 2013, the Chinese Tumor Registry Annual Reports showed that the incidence and mortality of breast cancer ranked second among all the malignant tumors [7,8]. Exploring the mechanism of metastasis and developing drugs to inhibit metastasis are important to reduce the mortality caused by breast cancer.

Abbreviations: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; EMT, epithelial–mesenchymal transition; IκB, inhibitor of NF-κB; IKK, IκB kinase; pIKK, phosphorylated IKK; IL-4, interleukin-4; CUEDC2, CUE domain-containing 2; TBS-T, t-butyldimethylsilyl-Triton X-100; HRP, horseradish peroxidase; CCK, cholecystokinin; MCP-1, monocyte chemoattractant protein-1; IL-11, interleukin-11; TRAF2, TNF receptor-associated factor 2; RIP, repeat-induced point mutation; NIK, mitogen-activated protein kinase kinase kinase 14.

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Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a vital transcription factor with a variety of target genes that are involved in the regulation of multiple important biological processes, including cell proliferation and apoptosis, enzyme synthesis, and cell cycle control, as well as cell adhesion and secretion [9,10]. Angiogenesis, reduction of cell adhesion, and a change in the extracellular matrix components are the preferred microenvironment for tumor metastasis [11–14]. In the last decade, studies revealed that NF-κB is essential for invasion and metastasis of some cancers [15,16]. In breast cancer, NF-κB is required for the epithelial–mesenchymal transition (EMT), which is considered to be closely identified with invasion and metastasis [17,18].

NF-κB is present in cells in an inactive state in which it is sequestered in dimers by IκB (inhibitors of κB) in the cytoplasm until cellular stimuli trigger its inducers [19]. The induction of NF-κB activation consists of a series of signal transductions, which are usually summarized as being canonical and non-canonical pathways [20]. The final effects of these pathways are IκB degradation and NF-κB release, which then translocates into the nuclear. During these processes, activation of a kinase called IκB kinase (IKK) is required [21]. Therefore, a key mechanism of keeping NF-κB inactive is to block the activation of IKK. CUE domain-containing 2 (CUEDC2) was recently proven to be an IKK activation inhibitor through recruitment of the phosphatase PP1 and by blocking phosphorylation of IKK [22]. This function of dephosphorylating IKK highlighted CUEDC2 as an important suppressor of NF-κB-mediated tumorigenesis and progression. Moreover, upstream regulators of CUEDC2, such as miR-324-5p [23], have also attracted attentions.

Sinomenine is an isoquinoline named after its extraction from the Chinese medicinal plant *Sinomenium acutum*. Sinomenine is well known for its remarkable curative effect on rheumatic and arthritic diseases [24]. In recent years, many reports have suggested that sinomenine has an anti-cancer function in breast and many other cancers due to its role in cell proliferation suppression and apoptosis promotion [25,26]. Furthermore, sinomenine was found to reduce invasion and migration ability of several types of cells [27,28]. However, the mechanism is still not fully clarified.

In this study, MDA-MB-231 and 4T1 breast cancer cell lines were incubated with sinomenine, and their invasion and migration capacity were examined. Sinomenine significantly suppressed the invasion and migration capacity of MDA-MB-231 and 4T1 cell lines. Sinomenine inhibited phosphorylation of IKK and activation of NF-κB through mediating the upstream IL4/miR-324-5p/CUEDC2 axis.

2. Materials and methods

2.1. Cell culture

The MDA-MB-231 human triple negative and 4T1 mouse breast cancer cell lines were cultured in RPMI-1640 medium supplemented with (Invitrogen) 10% FBS. Stock cultures were maintained in 25-cm^2 Corning flasks (Lowell, MA, USA). The cells were subcultured when they reached ~80% confluence.

2.2. Measurement of cell proliferation

Sinomenine (Sigma–Aldrich) was dissolved in DMSO and diluted to different concentrations in the medium. For the experiments, the cells were grown in 24-well plates at 3.5×10^4/well. Following incubation for 24 or 48 h, proliferation of the cells were detected with Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

2.3. In vitro invasion and migration assays

A wound-healing assay was conducted to assess cell migration. An artificial wound was created 24 h after treatment using a 10-μL pipette tip. To visualize migrated cells and wound healing, images were taken at 0, 6, 12, and 24 h.

For the Transwell invasion assay, 5×10^4 cells were plated in serum-free medium in the top chamber with a non-coated membrane (24-well insert; 8 μm; Corning). Medium containing 10% serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h. After that, a cotton swab was used to remove the non-migrated cells in the upper chamber, and the filters were individually stained with 2% crystal violet. The invasive cells adhering to the underside of the filter were examined and counted, and images were captured under a light microscope (×100; Olympus IX70; Olympus Corporation, Osaka, Japan).

2.4. Quantitative polymerase chain reaction (qPCR)

Total RNA of each clone was extracted using TRIzol; reverse transcription and cDNA amplification were performed according to the manufacturer's instructions (Invitrogen). The stem-loop and quantitative primers for miR-324-5p and primers for the U6 RNA (reference) were designed and synthesized by Ribobio Inc. (Guangzhou, China). Data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method. Three separate experiments were performed for each clone.

2.5. Western blot analysis

A total of 50 μg of protein from each sample was separated on a 12% SDS-PAGE. Afterwards, proteins were transferred to PVDF membranes at 80 V for 2 h. After the transfer, the membranes were blocked using 5% non-fat dried milk in TBS-T. The membranes were then incubated with primary antibodies (Abcam) respectively: anti–NF–κB p65 antibody (1:500 dilution), anti-CUEDC2 (1:500), anti-IL4 (1:500), anti-p-IKKβ (Ser 181) (1:300), Vimentin (1:500), anti-Tenascin-C (1:300) and anti-IKKβ(1:500). For the loading control, the membranes were probed with anti-β-actin antibody (1:800) in TBS-T for 1 h. The membranes were then incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000 dilution) in TBS-T for 1 h. Finally, the membranes were incubated with ECL chemical luminescence solution (Boehringer Mannheim, Mannheim, Germany), then exposed in a ChemiDoc XRS imaging system and analyzed with software Quantity One (Bio-Rad).

2.6. Co-immunoprecipitation (Co-IP)

The cells were washed with PBS, pelleted by centrifugation, and resuspended in lysis buffer supplemented with a protease inhibitor cocktail. The cell lysate was collected by centrifugation, pre-cleared by incubation with protein A-Sepharose Fast Flow (Sigma), and then pre-equilibrated with lysis buffer on a rotating platform. Centrifuged supernatants were collected and incubated with the anti-IκB primary antibody or IgG overnight. These supernatants were further mixed with protein G-Sepharose Fast Flow beads, which were pre-equilibrated in lysis buffer. Beads collected by centrifugation were washed and resuspended in an equal volume of 5 × SDS loading buffer. Immunoprecipitated proteins were separated by 10% SDS-PAGE. Then Western blot was performed as described above.

2.7. Transfection of miR-324-5p mimic

After complete adherence, the cells were incubated with mixtures of 6 pmol NC or miR-324-5p mimic, and 1 mL

Lipofectamine™ RNAi-MAX (Invitrogen) in 100 mL serum-free medium at 37 °C with 5% CO_2. Five hours later, the supernatant was removed and fresh serum-containing medium was added.

2.8. NF-κB nuclear translocation

NF-κB location in the cell was detected using Laser scanning confocal microscope (Olympus Corporation, Osaka, Japan). NF-κB was stained into green and the nuclear was stained with fluorescent dye DAPI (blue). The location of NF-κB was analyzed by OLympus FV-1000.

2.9. Statistical analysis

All measured values are expressed as mean ± SEM. Student's t test was used to determine the significance of differences in multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. Sinomenine suppressed the proliferation, invasion, and migration of MDA-MB-231 and 4T1 breast cancer cells in a dose-dependent manner

MDA-MB-231 and 4T1 breast cancer cells were both treated with sinomenine at concentrations of 0, 0.0625, 0.25, 0.5, 0.75, 1, 2, 4, 8, and 16 mM. Viability of the cells was detected with Cell Counting Kit-8 at 24 or 48 h after treatment. Cell viability gradually decreased with increasing sinomenine concentration (Fig. 1A and Figure S1A). We used concentrations of 0.25 and 0.5 mM sinomenine in the following wound-healing assay as these dosages of sinomenine caused only a minor decrease in cell viability. In the transwell migration assay, the migration ability of MDA-MB-231 cells was significantly weakened by 0.25, 0.5, and 1 mM of sinomenine treatment. Furthermore, the weakening effect increased with increasing sinomenine concentration (Fig. 1B and Figure S1B). The wound-healing assay revealed that 0.25 and 0.5 mM sinomenine significantly suppressed the healing of the wound (all $P < 0.05$, Fig. 1C and Figure S1C). In particular, when the MDA-MB-231 cells were treated with 0.5 mM sinomenine, the healing progress was about 50%, but in the group treated with 0.25 mM sinomenine and the untreated control, the healing was about 80% and nearly 95%, respectively (Fig. 1C). Similarly, These results indicate that sinomenine suppressed the proliferation, invasion, and migration of breast cancer cells in a dose-dependent manner.

3.2. Sinomenine suppressed activation of NF-κB in MDA-MB-231 cells

NF-κB was demonstrated to be required for the EMT and metastasis in breast cancer, and so the effect of sinomenine treatment on NF-κB activity was investigated. The inactive form of NF-κB is usually in the NF-κB/IκB complex. A co-IP assay was performed to detect binding of NF-κB to IκB after the MDA-MB-231 cells treated with sinomenine at concentrations of 0, 0.25, 0.5, and 1 mM. The IB assay following IκB antibody IP showed that the binding of NF-κB to IκB was inhibited by sinomenine treatment in a dose-dependent manner (Fig. 2A). Laser scanning confocal

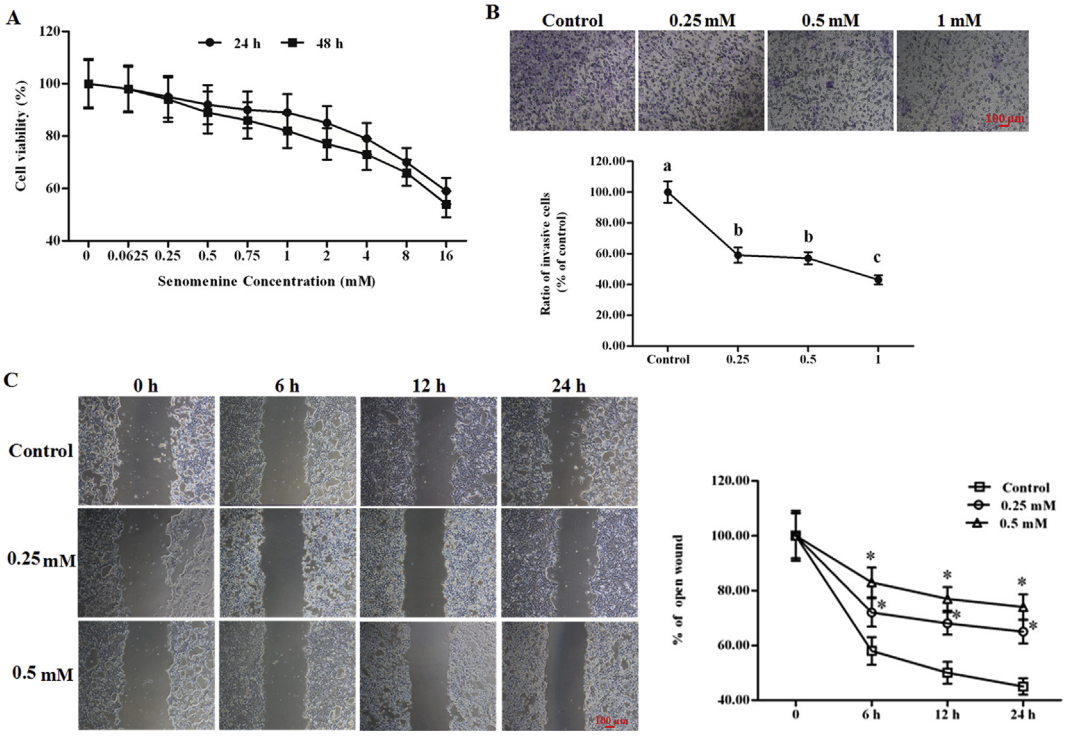

Fig. 1. Invasion and metastasis of MDA-MB-231 breast cancer cells were suppressed by sinomenine treatment. A. Proliferation of MDA-MB-231 cells was suppressed by sinomenine in a dose-dependent manner. MDA-MB-231 breast cancer cells were grown in 24-well Corning plates (3.5×10^4 cells/well). The cells were treated with sinomenine at the concentrations of 0, 0.0625, 0.25, 0.5, 0.75, 1, 2, 4, 8, and 16 mM for 24 or 48 h. B. Sinomenine suppressed the invasion of MDA-MB-231 cells. Following treatment, 5×10^4 cells were plated in the transwell plates for the invasion assay and stained with 2% crystal violet after another 24 h. The migrated cells were examined and counted, and images were captured under a light microscope (× 100). C. Sinomenine suppressed the wound-healing migration of MDA-MB-231 cells. A wound-healing assay was conducted to assess cell migration. An artificial wound was created after treatment with 0.5 or 0.25 mM sinomenine for 24 h using a 10-μL pipette tip. To visualize migrated cells and wound healing, images were taken at 0, 6, 12, and 24 h *$P < 0.05$; different letters indicates that there were statistically significant differences between the groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

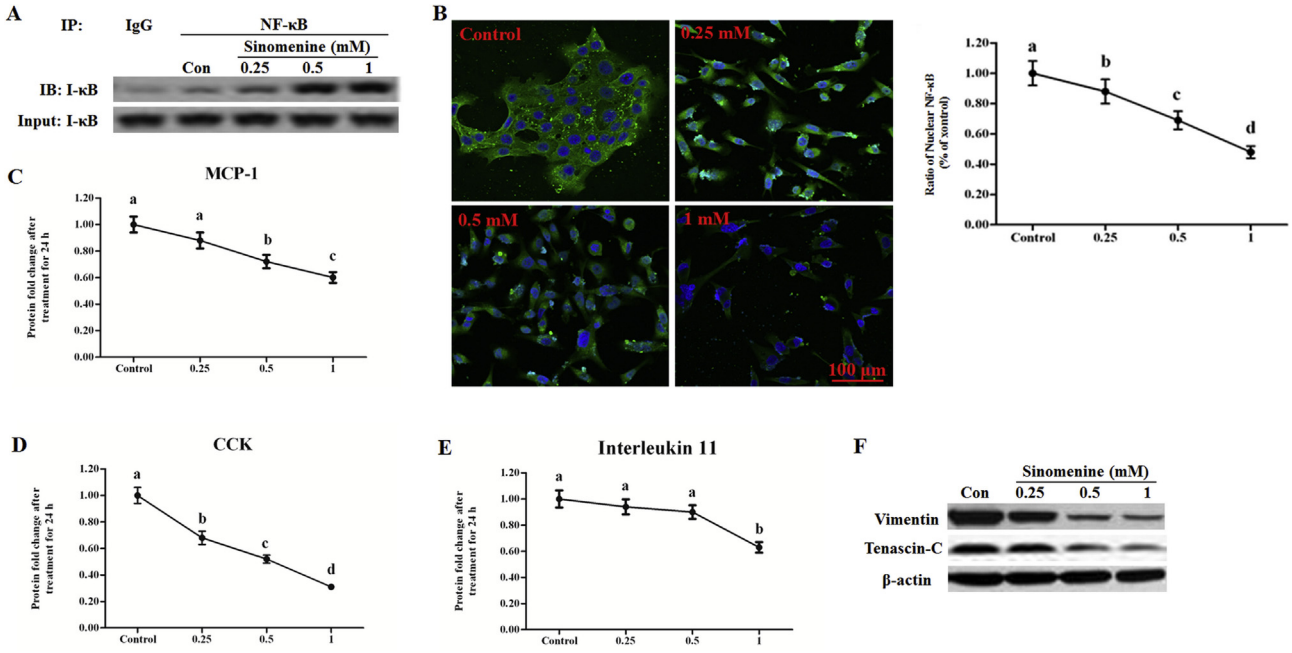

Fig. 2. Activation of NF-κB in the cells was blocked by sinomenine treatment. A. Co-IP analysis for the binding of NF-κB and IκB. The cells were with 0.25, 0.5, or 1 mM sinomenine for 24 h. IκB antibody was applied in the IP experiment, and NF-κB antibody was applied in the following IB analysis; IgG antibody was used as the negative control. 50% of the total input was used to detect the level of total NF-κB. B. NF-κB location was detected using Laser scanning confocal microscopy combined with immunofluorescence staining (×1000). NF-κB was stained into green and the nuclear was stained with fluorescent dye DAPI (blue). The ratio of nuclear NF-κB was analyzed by OLympus FV-1000 software. C, D, and E. Detection of cytokines cholecystokinin, MCP-1, and interleukin-11 by ELISA after the cells were treated with 0.25, 0.5, or 1 mM sinomenine for 24 h. E. Western blotting analysis for the protein levels of mesenchymal markers vimentin and tenascin-C. F. Western blotting analysis for mesenchymal markers vimentin and tenascin-C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microscopy also revealed that nuclear translocation of NF-κB was suppressed in a dose-dependent manner (Fig. 2B). Protein levels of mesenchymal markers vimentin and tenascin-C, as well as cytokines cholecystokinin (CCK), monocyte chemoattractant protein-1 (MCP-1), and interleukin-11 (IL-11), were determined. These genes contain functional NF-κB—binding sites in their promoter/enhancer regions and are directly regulated by NF-κB. Western blotting and ELISA analyses showed that protein levels of these genes were all decreased by sinomenine treatment (Fig. 2C, D, E and F). These data suggest that sinomenine treatment suppressed activation of NF-κB.

3.3. *Sinomenine treatment elevated the expression of CUEDC2 via a decrease in IL4/miR-324-5p*

NF-κB activation may be caused by changes in expression of upstream genes, protein modification, or cell components. To explore the reason why sinomenine treatment suppressed activation of NF-κB, protein levels of TRAF2, RIP, and NIK were examined. These three genes are the intracellular initial transducers of the canonical and non-canonical NF-κB pathways. Western blotting analysis showed that sinomenine treatment did not influence the levels of TRAF2, RIP, and NIK (Fig. 3A). Interestingly, we found that

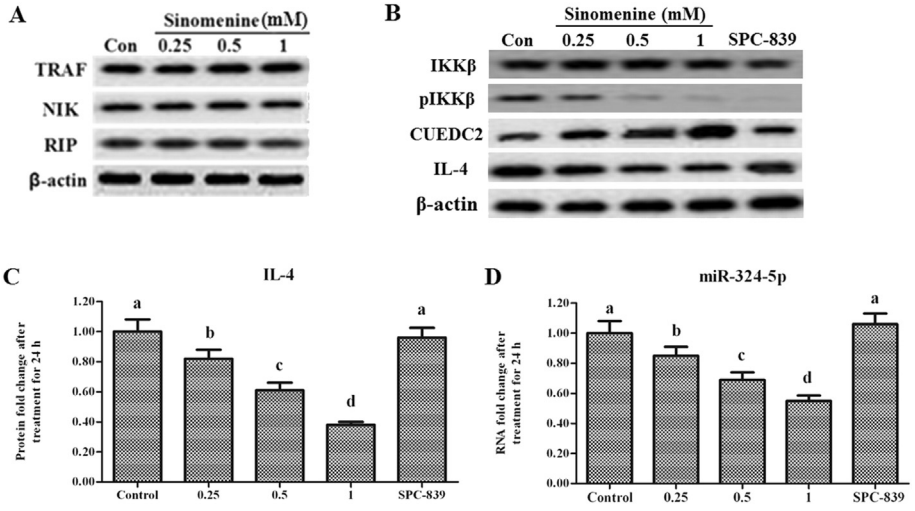

Fig. 3. Phosphorylation of IKK was blocked by sinomenine through the IL4/miR-324-5p CUEDC2 axis. A. Sinomenine treatment did not alter the expression of TRAF2, RIP, and NIK. After treatment for 24 h, the protein levels of TRAF2, RIP, and NIK were detected by Western blotting. B. Sinomenine treatment elevated the expression of CUEDC2 and reduced phosphorylation of IKKβ. C and D. Sinomenine treatment reduced the secretion of IL-4 and expression of miR-324-5p.

the phosphorylated form of IKKβ, the key subunit of the IKK complex and of which phosphorylation is essential for NF-κB activation, was decreased by sinomenine in a dose-dependent manner (Fig. 3B). An inhibitor of pIKKβ named SPC-839 was applied as a negative control. Moreover, expression of CUEDC2, a newly identified IKK phosphorylation inhibitor, was elevated by sinomenine (Fig. 3B).

However, there is a problem regarding how sinomenine influenced the expression of CUEDC2. Western blotting and ELISA analyses both revealed that the level of IL-4 was down-regulated by sinomenine treatment (Fig. 3B and C). IL-4 is a negative regulator of CUEDC2 via induction of miR-324-5p, a CUEDC2-targeting miRNA. Consistent with this, the level of miR-324-5p was also decreased by sinomenine in a dose-dependent manner (Fig. 3D).

3.4. The miR-324-5p mimic decreased the inhibition of cell invasion and migration caused by sinomenine treatment

The above data suggests that sinomenine treatment caused down-regulation of IL-4, and via miR-324-5p/CUEDC2/IKK eventually inhibited NF-κB activation. To further test this hypothesis, the miR-324-5p mimic was applied to co-treat MDA-MB-231 or 4T1 cells with sinomenine. At the time point of 12 and 24 h, the wound-healing assay revealed that the healing of the co-treatment group exhibited no significant difference to the control, but was significantly faster than the sinomenine treatment group ($P < 0.05$, Fig. 4A). Similarly in the invasion assay, the proportion of migrated cells in the co-treatment group was much higher than that of the sinomenine treatment group ($P < 0.05$, Fig. 4B). In consistent with the phenomenon in MDA-MB-231 cells miR-324-5p mimic also decreased the inhibition of cell invasion and migration caused by sinomenine treatment (Figure S2 A and B).

4. Discussion

In this study, we report that invasion and metastasis of MDA-MB-231 and 4T1 breast cancer cells were suppressed by sinomenine in a dose-dependent manner. Our data indicates that the suppressive effect was closely associated with blocking of phosphorylation of IKK and with activation of NF-κB. Finally, sinomenine caused IL-4 down-regulation, and this may be the reason for the suppression of IKK phosphorylation and NF-κB activation.

For a long time, owing to its anti-inflammatory and immunosuppressive effects, sinomenine was regarded as being specific for rheumatic and arthritic diseases [29]. About a decade ago, sinomenine was discovered to have an inhibitory impact on cell proliferation [30], and the anti-cancer effect of sinomenine gradually gained attention. It has been reported that sinomenine inhibits cancer cell proliferation and induces cancer cell apoptosis in multiple tissues, including synovia, esophagus, colon, gastric, liver, and lung, as well as breast tissue [31–33]. However, the effect of sinomenine on tumor invasion and metastasis has been little reported. Sinomenine has been reported to reduce invasion and migration ability of fibroblast-like synoviocytes and human monocytic THP-1 cells [27]. Sinomenine could inhibit maturation of monocyte-derived dendritic cells through blocking activation of NF-κB, which was proven to be required for EMT and metastasis of breast cancer [26,34]. This evidence hinted that sinomenine has a potential application in the suppression of breast cancer invasion and metastasis. Here, we show that sinomenine suppressed invasion and migration of breast cancer cells through blocking activation of NF-κB.

There are multiple pathways to activation of NF-κB. We initially suspected that sinomenine suppressed NF-κB activation through hindering a particular extracellular inducer or by down-regulating a certain transmembrane receptor. Surprisingly, we did not find any change in the levels of the intracellular initial transductants TRAF2, RIP, and NIK after sinomenine treatment. However, we found that the level of phosphorylated IKKβ was decreased by sinomenine in a dose-dependent manner. These findings suggest that sinomenine influenced phosphorylation of IKKβ in some way. There have been some reports that showed that sinomenine induced apoptosis of several types of cells [30,33]. However, it wasn't hardly reported that sinomenine could inhibit any one of these kinases and then inhibit IKK phosphorylation and NF-κB activation. These clues hinted that its inhibitory effect on IKK phosphorylation might be associated with PP1. Therefore, we determined the levels of

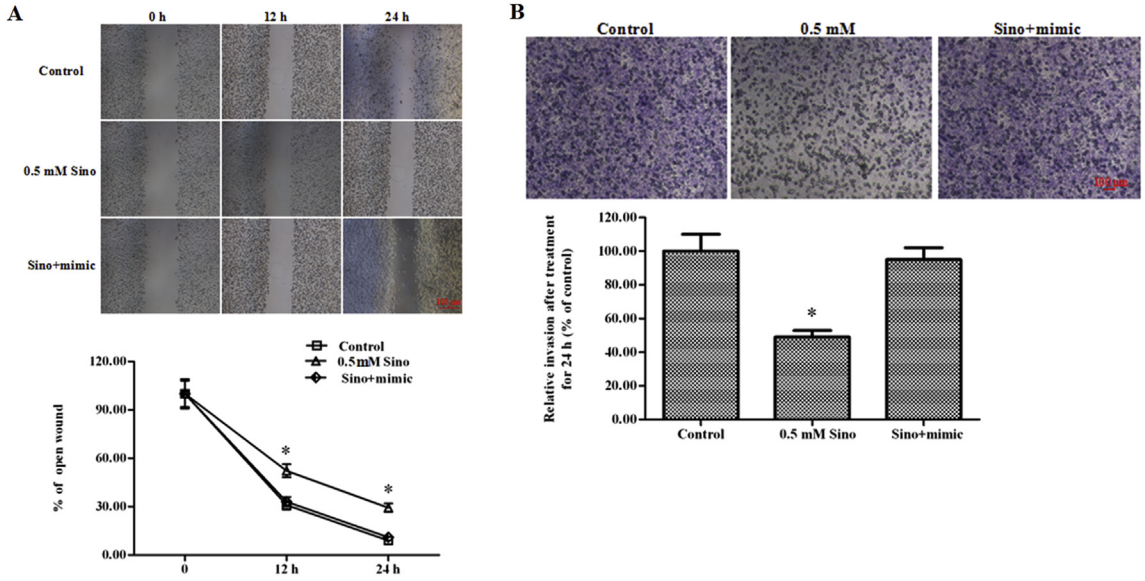

Fig. 4. The miR-324-5p mimic inhibited the suppression of MDA-MB-231 cell invasion and metastasis caused by sinomenine. A. The miR-324-5p mimic inhibited the suppression of wound healing caused by sinomenine. B. The miR-324-5p mimic inhibited the suppression of invasion caused by sinomenine. MDA-MB-231 cells were incubated with a mixture of 6 pmol miR-324-5p mimic or NC miRNA mimic (0.5 mM DMSO as control). After 24 h, the cells were incubated with 0.5 mM sinomenine for another 24 h.

CUEDC2, which can recruit PP1 and deactivate IKK. CUEDC2 was up-regulated by sinomenine treatment, when CUEDC2-targeting miR-324-5p was decreased when sinomenine diminished the level of IL-4.

There have been many studies that focused on inhibition of IKK activation. Many commercial IKK inhibitors, such as IKK-16, TPCA-1 and Bay 11-7085, have been applied in scientific and even clinical studies [35,36]. However, because the roles of the NF-κB pathway vary in different tumors, the anti-cancer efficacy of IKK inhibitors were limited to a portion of tumors [37]. Moreover, IκB is not the only substrate of IKK, and so the clinical side effects of the IKK inhibitors require further research. In this paper, the data revealed that CUEDC2 was an IKK inhibitor in cancer invasion and metastasis. Sinomenine could induce this IKK inhibitor through abating expression of IL-4 and CUEDC2-targeting miR-324-5p.

Therefore, sinomenine treatment suppressed phosphorylation of IKK and NF-κB activation and then exhibited a suppressive effect on invasion and metastasis of breast cancer cells. This suppressive effect was achieved by regulating the IL4/miR-324-5p/CUEDC2 axis.

Declare of conflicts

No.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.004.

Transparency document

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