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E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression ☆

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ABSTRACT

E-cadherin, a well-characterized cell–cell adhesion molecule, executes multifunction roles on cell behaviors. However, its effect on chemo-resistance remains controversial. In this study, we found that E-cadherin positive breast cell lines were less sensitive to staurosporine compared to E-cadherin negative ones. Next, we substantiated that the expression of E-cadherin in MDA-MB-435 cells could partly counteract the cytotoxic effect induced by staurosporine through a series of apoptosis assay. The resistance of E-cadherin over-expressing cells to staurosporine may due to the up-regulation of Bcl-2/Bax ratio. When E-cadherin interference plasmids were transfected into MCF-7 cells, Bcl-2 expression was down-regulated. Moreover, perturbation of E-cadherin function by blocking the cell–cell contact resulted in decreased cellular levels of Bcl-2 protein expression. All these results demonstrated the chemo-resistance function of E-cadherin in the condition of staurosporine treatment, therefore, might contribute effective therapeutic strategies in breast carcinoma.

Introduction

Apoptosis, a type of programmed cell death, is an evolutionarily conserved biochemical pathway in controlling cell suicide that plays an essential role in regulating normal development and homoeostasis in multicellular organisms [1]. Induction of apoptosis is considered to be the underlying mechanism that accounts for the efficiency of most chemotherapy drugs. Staurosporine (STS)[2], a potent protein kinase C inhibitor with a broad spectrum of activity, is used *in vitro* as an initiator of apoptosis in a wide variety of cell types. Nevertheless, STS has not been used as a clinical chemotherapy drugs until now. The potential factors that influence the STS-induced apoptosis remain largely unknown.

Generally, it is believed that a mitochondrial pathway plays a critical role in STS-induced apoptosis [2]. This involves release of mitochondrial apoptotic proteins such as cytochrome *c*, apoptosis-inducing factor (AIF) and second mitochondrial-derived activator of caspase (SMAC) [3–5]. On release, cytochrome *c* interacts with apoptotic proteinase-activating factor-1 and pro-caspase-9 to form apoptosomes. The latter activates caspase-9 and downstream effector caspases such as caspase-3 that are responsible for apoptotic destruction of the cells [6]. The anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-X_L and MCL1 appear to preserve the integrity of outer mitochondrial membrane [7,8]. Over-expressions of Bcl-2 and Bcl-X_L inhibit mitochondria-dependent pathway to apoptosis in different kinds of cells, thus protecting those cells from killing by chemotherapeutic agents [9,10]. And reduction of Bcl-2 levels increases the activity of chemotherapy against many tumor types *in vitro* and *in vivo* [10–14]. As such, the proto-oncogene Bcl-2 is thought to be directly associated with cellular transformation and resistance chemotherapy [9,15].

E-cadherin, a well-characterized cell adhesion molecule, is essential for cell–cell adhesion, which in turn regulates various aspects of cell fate including developmental decisions, cellular differentiation and possibly cell survival [16,17]. Just as integrins' function to mediate cell–extracellular matrix interactions in anchorage-dependent survival, cadherins may also act in such a capacity, possessing a functional role in the regulation of intercellular adhesion-dependent survival. Several studies have reported the association between E-cadherin-mediated aggregation and survival of carcinoma cells [18]. However, the actual mechanism by which cadherins mediate these signals is not known. As a hallmark of tumor progression, E-cadherin expression varies in a larger range in different types and stages of tumor [19,20]. It would be much helpful for the applications

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E-mail address: xlzha@shmu.edu.cn (X. Zha).
[1] These authors are equally contributed to this work.
[2] *Abbreviations used:* STS, staurosporine; AIF, apoptosis-inducing factor; SMAC, second mitochondrial-derived activator of caspase; MTT, methyl thiazol tetrazolium; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride.

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of chemotherapy if the relationship between the E-cadherin expression level and the susceptibility of tumor cells to chemotherapy drugs be clarified.

We investigated in this report that E-cadherin over-expressing breast cancer cells were less sensitive to STS, thereby reducing the effectiveness of chemotherapy. The mechanism by which E-cadherin protected cells from STS-induced apoptosis was due to the up-regulation of Bcl-2.

Materials and methods

Cell culture

The human breast carcinoma cell lines MDA-MB-435, MDA-MB-231, T47D and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new-born bovine serum at 37 °C, at 5% CO_2.

MTT cell viability assay

Cell proliferation and viability were quantified using the methyl thiazol tetrazolium (MTT) cell proliferation reagent (Roche Diagnostics) according to the manufacturer's protocol. Cells were seeded at a density of 2×10^5 cells/ml into 96-well plates and incubated for 12 or 24 h in completed medium with DMSO (control) or supplemented with various dose of STS; then 20 μl MTT labeling solution (5 mg/ml) per well was added. After incubation at 37 °C for 4 h, culture medium was discarded and 150 μl DMSO was added to each well. The samples were quantified spectrophotometrically by measuring the absorbance of the formazan product at 490 nm with an ELISA plate reader.

Stable transfection

The MDA-MB-435 cells (1×10^5/well) were transfected with pcDNA3-E-cadherin or pcDNA3 empty vector plasmids (4 μg/well) in 6-well plates using Lipofectamine™ 2000 (Invitrogen Inc., Carlsbad, CA), according to manufacturer's guidelines. Twenty-four hours after transfection, the cells were seeded into 100 mm dishes with a dilution of 1:20 and cultured in DMEM with 800 μg/mL of G418 (Promega, Pittsburgh, PA) for selection. The anti-G418 clones were further screened by Western blot (see [21]), and then one of the E-cadherin expressing stable clones named Ecad6-435 and an empty vector pcDNA3 stable clone named Neo1-435 were selected for next investigation. The stable clones, which were maintained in DMEM with 200 μg/mL of G418, were validated for their E-cadherin expression every three month.

Immunofluorescent staining

The human breast carcinoma cell lines MDA-MB-435 cells were plated at densities of 2×10^4 cells/cm^2 onto glass coverslips and grown for overnight. Cells were washed twice in 1× PBS, and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. Then the fixed cells were incubated with primary antibody against E-cadherin (diluted 1:500) for 6 h at 37 °C, washed three times with 1× PBS followed by treatment with FITC-conjugated secondary antibody (diluted 1:200) for 1 h at 37 °C. Negative control was produced as mentioned above except for omitting the primary antibody. The immunofluorescently stained cells were examined on the OLYMPUS™ microscope.

Cell aggregation assay

Cells were detached with HCMF buffer (150 mM NaCl, 0.6 mM Na_2HPO_4, 10 mM glucose and 10 mM Hepes, pH 7.4) containing 0.02% trypsin and 2 mM $CaCl_2$. Single cell suspensions were re-suspended in HCMF buffer containing 2 mM $CaCl_2$ at a concentration of 10^5 cells/ml. Aliquots of cell suspension (300 ml) were added to each well of 24-well plates pre-coated with 1% BSA and incubated for different times at 37 °C with 80 rpm agitation. The aggregation index was calculated by $(N_0 - N_t)/N_0$, where N_0 was the number of particles before the experiment started, and N_t was the number at final time.

DAPI staining

To visualize DNA, cells were washed twice with 1×PBS, fixed for 30 min in 4% paraformaldehyde, incubated with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) for 10 min, and then analyzed under a fluorescence microscope (OLYMPUS).

Annexin V apoptosis assay

For apoptosis quantification by annexin V, cells were scraped and stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit, Beyotime institute of biotechnology, China). In brief, cells after treatment with STS were washed with PBS/BSA. After the addition of 195 μl binding buffer, 5 μl FITC-labeled annexin V were added and incubated for 10 min at room temperature. Followed by incubation with 10 μl propidium iodide for 10 min on ice in the dark, apoptotic cells were measured by FACS analysis.

Immunoblotting assay

Immunoblotting analysis was carried out as described previously [21]. In brief, the separated proteins were transferred onto a PVDF membrane and probed with the appropriate primary antibodies and horseradish peroxidase labeled-secondary antibodies, and then detected using the ECL kit (Pierce).

E-cadherin SiRNA plasmid construct

SiRNA specific for E-cadherin (NM_004360) was designed according to a reported DNA sequence of the type AA(N19) (GCAGAATTGCTCACATTTC). According to Ambion company's guideline, a pair of oligonucleotides (sense: 5′-GATCCGCAGAATTGCTCA CATTTCTTCAAGAGAGAAATGTGAGCAATTCTGCTT TTTTGGAAA-3′ antisense: 5′-AGCTTTTCCAAAA AAGCAGAATTGCTCACATTTCTCTC TTGAAGAAATGTGAGCAATTCTGCG-3′) was synthesized, annealed and ligated to the BamHI and HindIII sites of pSilencer 2.0 (Ambion Co., USA) to get plasmid pSilencer 2.0-E-cadherin. The inserts were confirmed by sequencing. The negative control vector (control) was purchased from Ambion.

E-cadherin functional blocking experiments

Incubation was conducted with either monoclonal anti-E-cadherin antibody DECMA-1 (Sigma, U3254) or calcium chelator EGTA as described previously [21]. In brief, 0, 5, 10 and 20 μg/ml DECMA-1 or 0, 0.01, 0.1, 0.5, 1 and 2 mM EGTA was added into the ~95% confluent Ecad6-435 cells, separately, and incubated for 12 h. Then these treated cells were collected and lysed for Western blot analysis.

Statistical analysis

The Western blotting experiment was performed at least three times. For the MTT cell proliferation assay, three experiments with seven parallels ($n = 21$) were tested for every concentration.

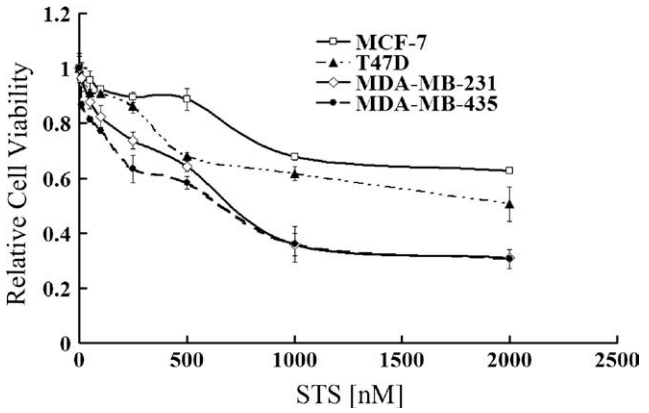

Fig. 1. Effect of STS on the cell growth inhibition of E-cadherin null and E-cadherin wild-type breast cancer cells. MCF-7, T47D, MDA-MB-435 and MDA-MB-231 cells were incubated with various dose of STS for 24 h, rinsed, and then detected by MTT assay. Data represent means ± SD obtained from three independent experiments with seven parallels (n = 21) for every concentration and normalized to the value of the control. IC_{50} values were extracted from assay data using SigmaPlot 8.0 software.

Results

Effect of STS on the growth inhibition of E-cadherin negative and positive breast cancer cells

To study the effect of STS on human breast cancer cells, E-cadherin negative cell lines (MDA-MB-435 and MDA-MB-231) and E-cadherin positive cell lines (MCF-7 and T47D) were treated with various doses of STS for 24 h, and assayed for growth inhibition. As shown in Fig. 1, STS treatment resulted in a loss of cell viability in a dose-dependent manner in each cell line. Among four breast carcinoma cell lines, MDA-MB-435 was the most sensitive cell line towards STS with IC_{50} value of 0.56 μmol/L, whereas MCF-7 was the least sensitive cell line with IC_{50} value of 8.3 μmol/L. The cytotoxicity of STS to E-cadherin null cells were markedly greater (IC_{50} of 0.56 and 0.69 μmol/L) compared with that of E-cadherin positive cells (IC_{50} 8.3 and 2.0 μmol/L). Thus, it seemed that the expression of E-cadherin counteracted the cytotoxic effects induced by STS in breast cancer cells.

Characterization of positive expression of E-cadherin transfectants in breast cancer cells MDA-MB-435

To determine whether E-cadherin regulated STS-induced breast cancer cells apoptosis, MDA-MB-435 cells (E-cadherin null) were transfected with pcDNA3-E-cadherin expression plasmid or pcDNA3 empty vector to build their stable transfectants, which were designated as Ecad6-435 and Neo1-435, respectively. Then, the E-cadherin expression of every transfectant was validated. As shown in Fig. 2A, E-cadherin protein level was significantly increased in Ecad6-435 cells, whereas hardly detected in Neo1-435 cells. Next, we tested the function of the exogenous E-cadherin in Ecad6-435 cells. Using immunofluorescent staining, we found that exogenous E-cadherin could locate to the membrane surface in Ecad6-435 cells, suggesting the build-up of E-cadherin-mediated cell–cell adhesion (Fig. 2B). Cell aggregation assay, which further confirmed this assume, showed that positive expression of E-cadherin significantly increased the cell–cell adhesion in Ecad6-435 cells. (Fig. 2C) These results demonstrated that the E-cadherin-mediated cell–cell adhesion had been built up in Ecad6-435 cells.

E-cadherin expression repressed STS-induced apoptosis

To test the effect of E-cadherin on protecting breast carcinoma cells from STS-induced apoptosis, STS stimulation of Ecad6-435 and Neo1-435 cells was conducted and the percentage of viable cells was determined by the MTT assay, firstly. At each concentration level of STS, Neo1-435 cells were induced to death with

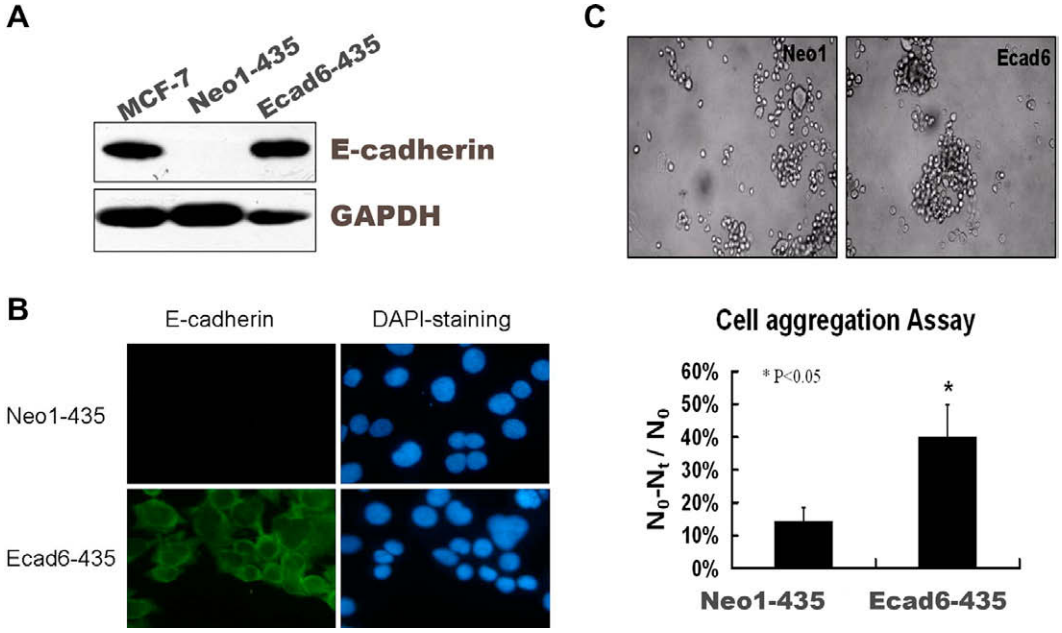

Fig. 2. Characterization of positive expression of E-cadherin transfectants in breast cancer cells MDA-MB-435. (A) Relative changes in E-cadherin protein levels in the human breast cancer cell line MCF-7 and in stable transfectant clones Neo1-435 and Ecad6-435, by Western blot analysis. Levels of GAPDH were used as a normalizing factor for total amount of protein loaded. (B) Immunofluorescent staining for E-cadherin. The nuclei were counterstained with DAPI, and second antibody used for E-cadherin was labeled by FITC (green). E-cadherin was positively expressed on the surface of Ecad6-435 cells. Conversely, the membrane staining of E-cadherin was hardly detected in Neo1-435 cells. (C) Cell aggregation analysis of Neo1-435 and Ecad6-435 cells. The cell suspension was seeded in each of quadruplicate wells of 24-well plates precoated with BSA. The aggregation index was calculated by $(N_0 - N_t)/N_0$, where N_0 is the number of particles before the experiment started, and N_t is the number at final time. The assay was repeated three times $^{*}p < 0.05$.(For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

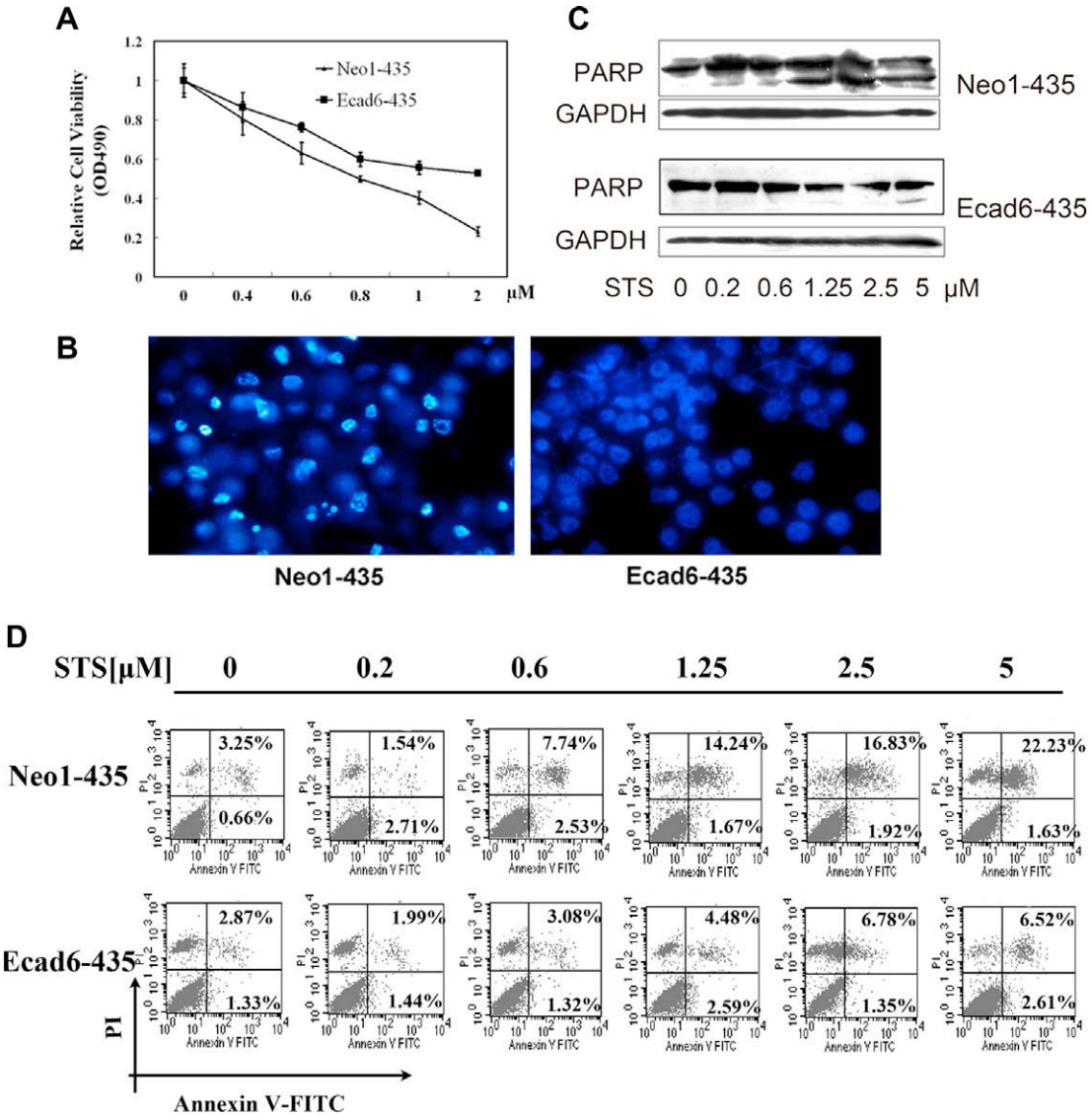

Fig. 3. Changes in chemosensitivity of MDA-MB-435 cells to STS after transfection with E-cadherin gene. (A) Quantitation of viability in Ecad6-435 and Neo1-435 cells treated with indicated concentration of STS for 12h (B) After incubation with 5 μM STS for 5 h, Neo1-435 and Ecad6-435 cells were stained by DAPI for the assessment of nuclear fragmentation. As indicated, Neo1-435 cells showed more aggregated and condensed nuclear DNA. (C) PARP cleavage analysis by Western blotting with anti-PARP. Neo1-435 and Ecad6-435 cells were treated with 0, 0.2, 0.6, 1.25, 2.5, or 5 μM STS for 8 h. After this treatment, cells were collected, lysed and the cleavage of PARP were determined. (D) Evaluation of Apoptosis of Neo1-435 and Ecad6-435 cells treated with different concentrations of STS by using annexin V/PI staining and flow cytometry. All those cells were treated with various dose of STS for 5 h. Cells were double stained with annexin-V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Annexin-V−, PI− cells are live cells, annexin-V+, PI− cells are early apoptotic cells and annexin-V+, PI+ cells are late apoptotic or necrotic cells. The fraction of annexin V-positive Neo1-435 cells was 3.91% before treatment and 4.25%, 10.27%, 15.91%, 18.75% and 23.86% after treatment with STS at concentrations of 0.2, 0.6, 1.25, 2.5 and 5 μM, respectively. The fraction of annexin V-positive Ecad6-435 cells was 4.20% before treatment and 3.43%, 4.40%, 7.07%, 8.13% and 9.13% after treatment with STS at concentrations of 0.2, 0.6, 1.25, 2.5 and 5 μM, respectively.

a larger proportion than that of Ecad6-435 cells. The extreme difference of cell viability between Neo1-435 and Ecad6-435 cells occurred at a 2 μM STS treatment, where cell viability in Ecad6-435 cells was about 55% of its untreated control level in comparison to that in Neo1-435 cells which was only 23% of its control level (Fig. 3A). Then, we examined the apoptosis by testing DNA fragmentation and poly(ADP-ribose) polymerase (PARP) cleavage. As shown in Fig. 3B, treatment of Neo1-435 cells with STS resulted in PARP significant cleavage, whereas Ecad6-435 cells had minimal amount of cleaved PARP. We also found that DNA fragmentation was more easily induced in Neo1-435 than in Ecad6-435 cells upon a 5 μM STS stimulation using DAPI staining (Fig. 3C).

One of the membrane changes in apoptosis or necrosis is the translocation of phosphatidylserine (PS) from the inner of the cell membrane to the outside. To further evaluate the efficiency of STS-induced apoptosis in those stable clones, Annexin V/PI staining assay was carried. In Neo1-435 cells, within 5 h after various doses of STS treatment, annexin V labeling was significantly increased from approximately 4% to 24% (Fig. 3D, upper), suggesting a rapid induction of apoptosis. Whereas, in Ecad6-435 cells, there appeared to be a slight increase in annexin V staining which was from approximately 4% to 9% (Fig. 3D, lower). This further substantiated E-cadherin expression might impair STS-induced apoptosis in MDA-MB-435 cells.

E-cadherin expression up-regulated Bcl-2 protein level

Given that the interplay between prosurvival and pro-apoptotic Bcl-2 family proteins is capable of controlling anticancer drug-induced apoptosis, we examined the expression of Bcl-2 and Bax in Ecad6-435 and Neo1-435 cells. Using quantitative Western blot-

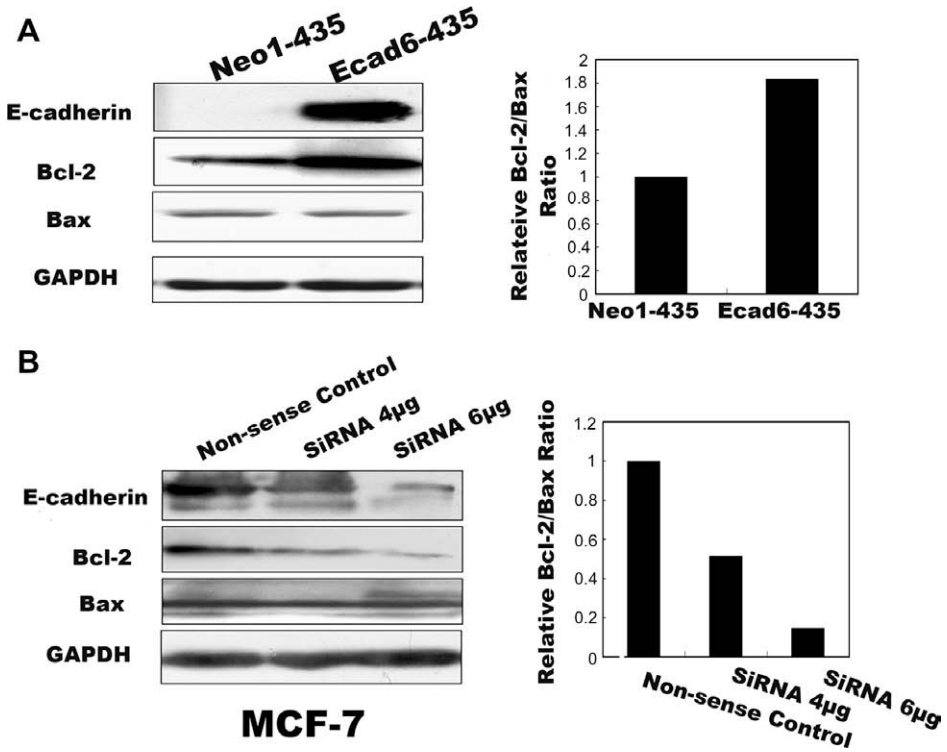

Fig. 4. E-cadherin expression up-regulated Bcl-2 protein level. (A) Western blot analysis of Bcl-2 and Bax expression in the Neo1-435 and Ecad6-435 cells. The right chart is the quantification of Bcl-2/Bax expression ratio by densitometric analysis. (B) Western blot analysis of Bcl-2 and Bax expression in the MCF-7 cells, transfected with 4, or 6 μg E-cadherin siRNA plasmid, or non-sense control siRNA as indicated. The right chart is the quantification of Bcl-2/Bax expression ratio by densitometric analysis.

ting, Bcl-2 levels were found to be significantly higher in Ecad6-435 as compared to Neo1-435. In contrast, the levels of pro-apoptotic Bax remained constant. Thus, the Bcl-2/Bax ratio increased after E-cadherin-mediated adhesion (Fig. 4A). To further validate the relationship between E-cadherin and Bcl-2, another breast cancer cell line MCF-7 with well-structured E-cadherin-mediated cell–cell adhesion was used. After transiently transfected with E-cadherin SiRNA plasmids, the amount of prosurvival Bcl-2 protein was markedly decreased in MCF-7 cells in a dose-dependent manner (Fig. 4B).The data suggested the Bcl-2 protein might be a determinant factor that regulated the chemosensitivity of E-cadherin over-expressing MDA-MB-435 cells.

Disruption of homophilic binding of the E-cadherin extracellular domain by DECMA-1 or EGTA correlated with decreased cellular levels of Bcl-2 protein expression

Since the calcium-dependent interactions among E-cadherin molecules are critical for the formation and maintenance of cell–cell adhesion, we utilized calcium chelator EGTA and a neutralizing antibody against E-cadherin (DECMA-1) to specifically block E-cadherin-mediated adherens junction formation. After those treatments, the morphology and cell–cell adhesion of Ecad6-435 cells were largely changed (Fig. 5A). At the same time, the disruption of cell-cell adhesion led to a decrease of Bcl-2 protein level, as well as Bcl-2/Bax ratio, which was consistent with the decrease of its aggregation extent (Fig. 5B and C). These observations suggested that the maintenance of E-cadherin-mediated cell-cell adhesion was necessary for the up-regulation of Bcl-2.

Discussion

The current work was done to evaluate that E-cadherin homophilic adhesion could initiate anti-apoptotic signaling by enhancing the Bcl-2 expression. Inhibition of homophilic binding of the E-cadherin extracellular domain correlated with decreased cellular levels of Bcl-2 protein expression. This work confirmed the chemo-resistance function of E-cadherin, therefore, might contribute effective therapeutic strategies in breast carcinoma.

As a critical epithelial cell adhesion molecule, E-cadherin not only mediates cell–cell adhesion, but also plays important roles in cell behavior such as cell invasion, growth and survival [22,23]. Down-regulation of E-cadherin expression is commonly seen in different tumor types and experimental evidences support its role in suppressing cells growth and invasion. However, tumor suppression is achieved not only by cell cycle arrest but by the initiation of cell death programs as well. The effect of E-cadherin-mediated cell–cell adhesion on cell survival and apoptosis remains controversial. On one side, many of the cells, especially tumors, which lack direct interaction with surrounding extracellular matrix depend on survival signaling generated by cell–cell contacts. Maintenance of E-cadherin-mediated cell–cell interaction has been reported to prevents cells from apoptosis in different kinds of cells, which directly or indirectly antagonizing the tumors sensitivity to chemotherapy drug [24,25]. On the other side, Loss of functional E-cadherin was also reported to render cells more resistant to the apoptotic agent taxol in hereditary diffuse gastric cancer [26]. Therefore, understanding the potentially important role of E-cadherin survival signaling on the tumor progression may contribute effective therapeutic strategies in breast carcinoma. These findings present evidence that E-cadherin-mediated aggregation results in Bcl-2 up-regulation which is critical for survival of breast tumor cells.

The signaling induced by E-cadherin-mediated cell–cell adhesion is complex. We here showed that E-cadherin up-regulated Bcl-2 expression. E-cadherin engagement induces several signaling pathways including β-catenin/wnt, Akt and EGFR signaling [27–30]. Future studies will be needed to elucidate the complicated crosstalk

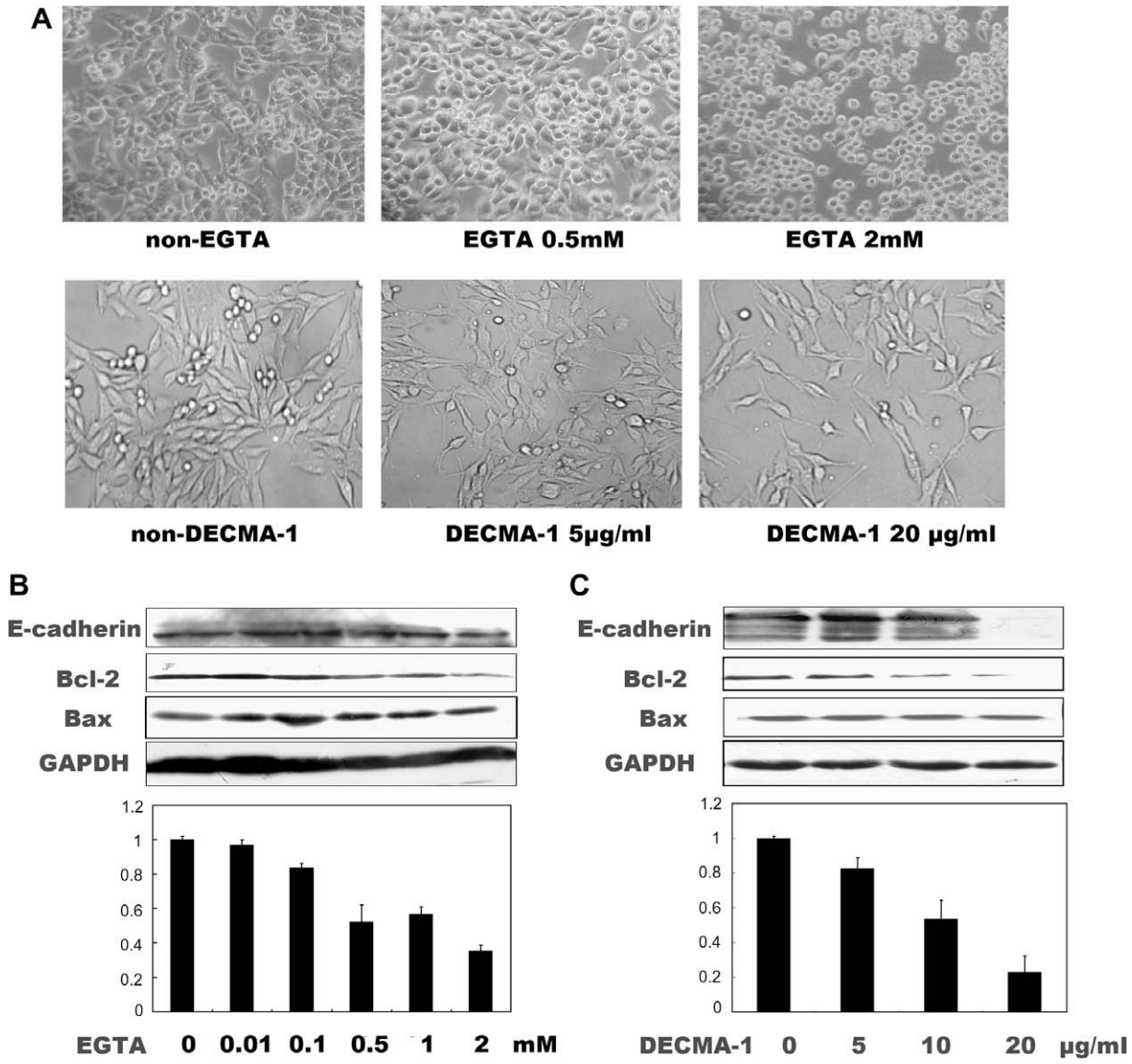

Fig. 5. The effect of EGTA or DECMA-1 on the protein expression of Bcl-2 and Bax. (A) morphology changes of Ecad6-435 cells with treatment of 0, 0.5 or 2 mM EGTA for 12 h (upper panel) or 0, 5 or 20 μg/ml DECMA-1 for 12 h (lower panel) (B, C) Ecad6-435 cells, treated with indicated concentration of EGTA (B) or DECMA-1 (C) for 12 h. Whole cell lysates were then extracted and subjected to Western blotting using E-cadherin, Bcl-2, Bax and GAPDH Antibody. The data presented are from three independent experiments.

of cell surface adhesion molecules with apoptosis-related signaling pathways. Studies have shown that ERK/MAPK can increase the level of Bcl-2 through the phosphorylation of Bad in SCC cells [31]. On the premise of this, an appealing hypothesis was to test the possibility that E-cadherin engagement is the trigger for anti-apoptotic signaling via ERK/MAPK signaling.

In conclusion, we showed that in this *in vitro* model, upon transfection of wild-type E-cadherin, cells become less sensitive to apoptotic stimuli. This could be of relevance for the understanding of the tumorigenic process in the E-cadherin wild-type breast cells. We also observed an increase in the level of the anti-apoptotic Bcl-2 in E-cadherin over-expressing cells, suggesting the existence of an interplay between E-cadherin and Bcl-2 regulation. Because of the potential therapeutic relevance of this finding, further studies aiming at elucidating its molecular mechanisms are warranted.

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