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C-FLIP(L) contributes to TRAIL resistance in HER2-positive breast cancer

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ABSTRACT

Breast cancers with HER2 amplification have a poorer prognosis than the luminal phenotypes. TRAIL activates apoptosis upon binding its receptors in some but not all breast cancer cell lines. Herein, we investigated the expression pattern of c-FLIP(L) in a cohort of 251 invasive breast cancer tissues and explored its potential role in TRAIL resistance. C-FLIP(L) was relatively high-expressed in HER2-positive breast cancer in comparison with other molecular subtypes, co-expressed with TRAIL death receptors, and inversely correlated with the apoptosis index. Downregulation of c-FLIP(L) sensitized SKBR3 cells to TRAIL-induced apoptosis in a concentration- and time-dependent manner and enhanced the activities and cleavages of caspase-8 and caspase-3, without altering the surface expression of death receptors. Together, our results indicate that c-FLIP(L) promotes TRAIL resistance and inhibits caspase-3 and caspase-8 activation in HER2-positive breast cancer.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and one of the leading causes of cancer death for women in the developed countries. Recent cDNA microarray studies have allowed breast cancer to be divided into four molecular subtypes: luminal A, luminal B, HER2-positive, and basal-like [1]. HER2-positive breast cancer, featured by amplification of HER2/neu and negative expression of ER and PR, has a poorer prognosis than the luminal subtypes and requires treatment with trastuzumab and chemotherapy [2]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been suggested as an anti-neoplasia agent for its ability to selectively kill tumor cells over nontransformed cells [3]. Phase I clinical trials demonstrated that both recombinant TRAIL and agonistic receptor antibodies are well tolerated at doses in serum levels above the therapeutic concentrations [4]. However, the majority of breast cancer cell lines are highly resistant to TRAIL treatment, which poses a limitation to its use in clinic [5]. Recent study showed that a subset of basal-like breast cancer cells is sensitive to TRAIL when given as a single agent, indicating that molecular phenotype of breast cancer cells may influence their response to TRAIL [6].

Cellular FLICE-inhibitory protein (c-FLIP) belongs to the death effector domain (DED) family and is a catalytically inactive homologue of caspase-8/10 [7]. Three c-FLIP splice variants have been identified as cellular proteins: the 55 kDa long form c-FLIP(L), the 26 kDa short form c-FLIP(S), and the 24 kDa form c-FLIP(R). When expressed at high level, all forms of c-FLIP are widely accepted as anti-apoptotic proteins and compete with caspase-8/10 for recruitment to the death-inducing signaling complex (DISC) [8]. At physiological low expression level, c-FLIP(L) has been shown to promote proteolytic processing of caspase-8 and death receptor-mediated apoptosis [8]. Moreover, c-FLIP is also involved in the regulation of several signaling pathways that promote the survival of cancer cells, including the nuclear factor (NF)-κB signaling cascade [9]. These findings suggest that c-FLIP(L) may play an important role in the apoptosis and survival of cancer cells.

To address the question of whether c-FLIP(L) is a potential target in TRAIL treatment in HER2-positive breast cancer, we first systematically examined c-FLIP(L) expression in a total of 251 breast cancer tissues and found that c-FLIP(L) was relatively high-expressed in HER2-positive tumors. Then, cell based studies revealed that downregulation of c-FLIP(L) sensitized SKBR3 cells to TRAIL-induced apoptosis in a dose- and time-dependent manner. Furthermore, c-FLIP(L) inhibition enhanced cleavage and activation of caspase-8 and caspase-3, which may at least in part attenuate TRAIL resistance in HER2-positive breast cancer.

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2. Materials and methods

2.1. Antibodies and reagents

The primary antibodies used in this study were antibodies against c-FLIP(L) (Rockland, PA, USA), death receptors DR4 and DR5 (Santa Cruz Biotechnology, CA, USA), caspase-3/8 and processed subunits (Cell Signaling Technology, MA, USA), HER2 (Neomarker, WA, USA), ER, PR and Ki67 (Zymed Laboratories, Beijing, China). Recombinant human TRAIL was purchased from PeproTech, Inc. (NJ, USA).

2.2. Tissue collection and cell culture

A total of 251 breast cancer patients who underwent surgery in our hospital between 1996 and 1999 were enrolled in this study. Breast cancer cell line SKBR3 was purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Hyclone, Beijing, China) and were incubated in a humidified 5% CO_2 incubator at 37 °C.

2.3. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH)

After deparaffinization and rehydration, 4 μm tissue sections were incubated with various primary antibodies and stained using a PV6000 Kit (Zymed Laboratories, Beijing, China). C-FLIP(L) expression was evaluated as the staining index (SI) by multiplying the proportion of positive tumor cells and product of staining intensity. High level of c-FLIP(L) expression was recorded when SI $\geqslant$6. DR4 and DR5 immunoreactivities were evaluated by the percentage of tumor cells with cytoplasm or membrane reactivity counted across five representative fields. The proliferation index (PI) of cancer cells was assessed by the percentage of Ki-67 positive nuclei. HER2/neu FISH assay was performed with the PathVysion DNA Probe Kit (Abbott Molecular, IL, USA).

2.4. Real-time RT-PCR

Total RNA was extracted with the TRIzol reagent (Invitrogen, MA, USA). Single strand cDNA was synthesized using the M-MLV reverse transcriptase (TaKaRa, Dalian, China) and amplified with SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China). The specific primers of *c-FLIP(L)* were: 5′-ACCGAGACTACGACAGCTTTGTG-3′ and 5′-CAATGTGAAGATCCAGGAGTGGG-3′.

2.5. Western blotting

Briefly, cells were lysed and protein amounts were quantified using a Micro BCA Protein Assay Kit (Pierce, IL, USA). Equal amounts of protein were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, MA, USA). The membranes were then incubated overnight at 4 °C with the appropriate primary antibodies and were visualized using the enhanced chemiluminescence reagents (Pierce, IL, USA).

2.6. Flow cytometric assays of DR4 and DR5

A total of 5×10^5 SKBR3 cells were incubated with anti-DR4/DR5 antibody and PE-conjugated secondary antibody (Zymed Laboratories, Beijing, China). Immunofluorescence was measured by using a FACScan flow cytometry. At least $1 \times 10{,}000$ events were examined for each sample, and data were expressed as the percentage of specific positive cells. Isotype-matched antibodies were carried out as controls.

2.7. Cell transfection and viability assay

SKBR3 cells were transfected with small interfering RNAs (siRNAs) against c-FLIP(L) (Invitrogen, Shanghai, China) or a scrambled nonsense control using the Lipofectamine 2000 reagent (Invitrogen, Shanghai, China). The sequence of the c-FLIP(L) siRNA-1 was 5′-CCAUCAGGUUGAAGAAGCACUUGAU-3′ and the sequence of the c-FLIP(L) siRNA-2 was 5′-GUAACUUGUCCCUGCUCCUUG-3′. Western blot and real-time RT-PCR assays were performed to determine the knockdown efficiency.

After 48 h of transfection, SKBR3 cells were treated with various concentrations of TRAIL for further 4–48 h. Cell viability was analyzed in 96-well plates by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) (Sigma, MO, USA) assay.

2.8. Analysis of apoptosis

The TdT-mediated dUTP nick end-labeling (TUNEL) assay was carried out to detect the apoptosis index (AI) of breast cancer tissues by using an *in situ* Cell Death Detection Kit (Roche Diagnostics, Germany). AI was expressed as the percentage of positive nuclei, including apoptotic bodies, among 1000 tumor cell nuclei. After TRAIL treatment, SKBR3 cells were stained using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, NJ, USA) and measured by flow cytometry. Furthermore, the DNA fragmentation analysis of SKBR3 cells was performed using an Apoptotic DNA Ladder Kit (Roche Diagnostics, Germany). After binding with isopropanol, equal amounts of DNA samples were checked in 1% agarose gel.

2.9. Caspase activity assays

Approximately 2×10^5 cells were resuspended in lysis buffer and centrifuged in a microcentrifuge. The supernatants were subsequently added into the ApoAlert Caspase Profiling Plate (TaKaRa, Dalian, China) and fluorescence intensities were measured in a fluorescence microplate reader (emission at 460 nm, ThermoFisher, MA, USA). Fold increase in protease activity was determined by comparing the levels of caspase activity in cells transfected with c-FLIP(L) siRNA with those transfected with control siRNA.

2.10. Statistical analysis

All statistical analyses were performed by using the SPSS 17.0 Package (Chicago, IL, USA), including the Chi-square test, student's *T*-test (two-sided), one-way analysis of variance (ANOVA) and Spearman's rank correlation. A value of P <0.05 was considered statistically significant.

3. Results

3.1. C-FLIP(L) was relatively high-expressed in HER2-positive breast cancers as compared to other subtypes

C-FLIP(L) protein expression was localized in the cytoplasm from light yellow to brown color (Fig. 1A and B, Fig. 2A and B), and strong staining (SI $\geqslant$ 6) was observed in 140 cases (55.78%) of breast cancer. Based on the staining results of ER, PR, and HER2/neu (Fig. 1C–F), our invasive breast cancer cohort was classified into four molecular subtypes, including luminal A (121 cases, 48.2%), luminal B (47 cases, 18.7%), HER2-positive (40 cases,

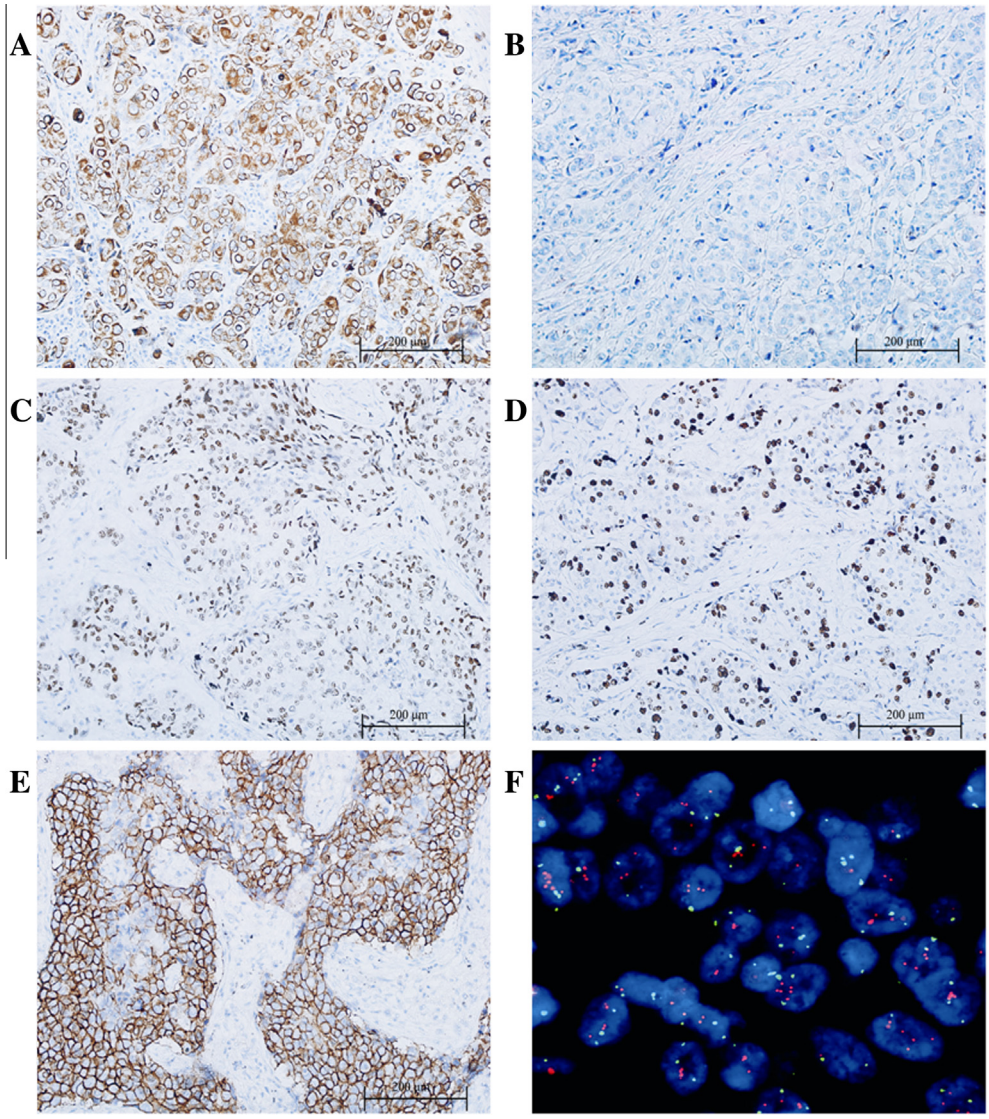

Fig. 1. IHC staining and FISH of invasive breast tumors. (A) C-FLIP(L) was positively expressed in the cytoplasm of breast cancer cells. (B) Negative control of breast cancer tissues using PBS instead of c-FLIP(L) primary antibody. (C–E) Positive expression of ER, PR and HER2/neu were shown in breast cancer cells. (F) FISH indicated HER2/neu gene amplification (original magnification A, B, C, D, and E, ×200; F, ×1000).

15.9%), and basal-like (43 cases, 17.2%). C-FLIP(L) expression was different in the four subtypes of breast cancer samples ($P = 0.013$) and high expression rate was 72.5% (29 cases) in HER2-positive, 63.83% (30 cases) in luminal B, 52.89% (64 cases) in luminal A, and 39.53% (17 cases) in basal-like breast cancer tissues, respectively (Table 1). The highest level of c-FLIP(L) expression was identified in the HER2-positive subtype as compared with the basal-like ($P = 0.003$) or luminal A subtype ($P = 0.030$).

3.2. Increased expression of c-FLIP(L) was inversely correlated with AI and co-expressed with TRAIL receptors in HER2-positive breast cancer

The AI was evaluated in all HER2 amplified cases and the mean value was 3.2% with a range of 0–5.5. TUNEL-positive cancer cells displayed the characteristic features of cytoplasm shrinkage, chromatin condensation and apoptotic bodies (Fig. 2C). The Spearman's test revealed a significantly inverse relationship between c-FLIP(L) expression with AI in HER2-positive breast cancer tissues (Supplementary Table 1, $P < 0.01$). The Ki67 staining was performed to evaluate the PI and the percentage of proliferative cells ranged from 1.0% to 60.2% with a mean of 45.7% (Fig. 2D). However, there was no significant correlation between PI and c-FLIP(L) protein (Supplementary Table 1).

DR4 and DR5 were found cytoplasmic/membranous staining in our breast tumor series (Fig. 2E and F). In HER2 amplified breast cancer, the rates of DR4 and DR5 expression were 57.5% (23 cases), and 82.5% (33 cases), respectively. Strikingly, 78.9% of the tumors expressing both DR4 and DR5 also exhibited increased distribution of c-FLIP(L), and there was a positive association between c-FLIP(L) protein and DR4 or DR5 expression (Supplementary Table 2, $P < 0.05$). These results suggest that c-FLIP(L) may play a critical role in TRAIL-mediated pathway in HER2-positive breast cancer.

3.3. Inhibition of c-FLIP(L) expression enhanced TRAIL-induced apoptosis in HER2 amplified SKBR3 cells

SKBR3 cells were demonstrated as a HER2 amplified breast cancer cell line by western blotting (Supplementary Fig. 1). As shown in Fig. 3A and B, c-FLIP(L) was substantially decreased at both mRNA and protein levels in SKBR3 cells after specific siRNAs targeting. MTT assay revealed that the number of dead cells was significantly increased in cells transfected with c-FLIP(L) siRNAs

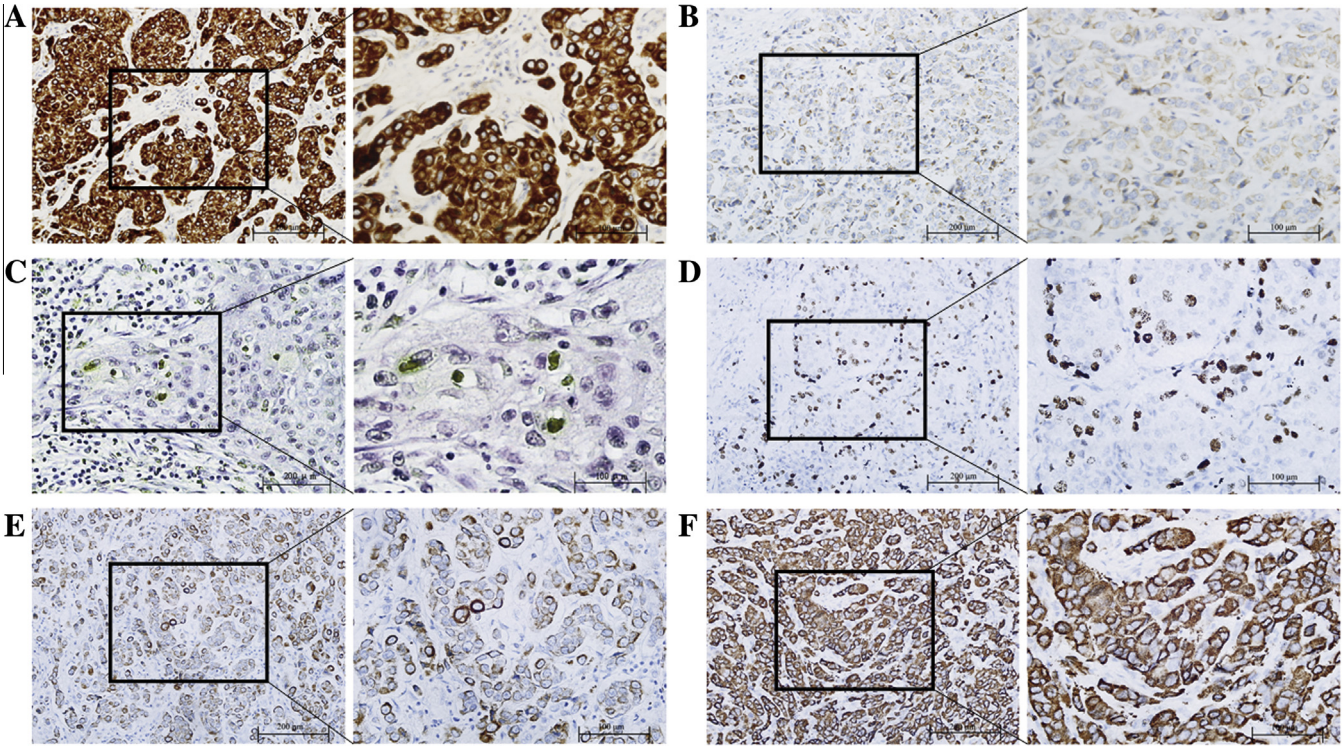

Fig. 2. Expression of c-FLIP(L) and its relevant apoptotic factors were analyzed in HER2-positive breast cancers. (A) C-FLIP(L) is strong staining (brown) in the cytoplasm of breast cancer cells. (B) Weak staining (light yellow) of c-FLIP(L) was shown in breast cancer. (C) AI was measured by TUNEL assay and apoptosis positive nuclei were identified by dark brown bodies in intact cells. (D) PI was measured by Ki-67 staining and the positive staining was located in the nuclei of cancer cells. (E) DR4 was identified in the cytoplasm or on the membrane of breast cancer cells. (D) DR5 expression was positive in breast cancer tissues (×200 and ×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Expression of c-FLIP(L) in different molecular subtypes of invasive breast tumors.

Subtype	Molecular phenotype	No. of cases	C-FLIP(L) expression (%)		χ^2	*P*-Value
			High level	Low level		
Luminal A[†]	ER/PR+, HER2−	121	64 (52.89)	57 (47.11)	10.78	0.013
Luminal B[¶]	ER/PR+, HER2+	47	30 (63.83)	17 (36.17)		
HER2-positive[§,†]	ER/PR−, HER2+	40	29 (72.50)	11 (27.50)		
Basal-like[§,¶]	ER/PR−, HER2−	43	17 (39.53)	26 (60.47)		

[§] $P = 0.003$.
[†] $P = 0.030$.
[¶] $P = 0.021$.

and antitumor effect of TRAIL was raised in a time- and dose-dependent manner. Compared with control and parental cells, the death rates were nearly 35% and 55% for c-FLIP(L) siRNA-1 and c-FLIP(L) siRNA-2, after 24 h and 48 h treatment with 500 ng/ml TRAIL ($P < 0.05$, Fig. 3C). In addition, approximately 45% and 55% of c-FLIP(L) knockdown cells were killed following exposure to 500 ng/ml and 1000 ng/ml TRAIL for 24 h (*v.s.* control siRNA, $P < 0.05$, Fig. 3D). Apoptotic levels were subsequently measured by flow cytometry with Annexin V-FITC and PI staining. The percentages of apoptotic cells were significantly higher in SKBR3 cells transfected with c-FLIP(L) siRNA-1 (21.43%) and c-FLIP(L) siRNA-2 (23.92%) than control (2.33%) and parental cells (2.01%) after treatment with 500 ng/ml TRAIL for 24 h (Fig. 3E and F, $P < 0.05$). Incubation of SKBR3 cells with c-FLIP(L) siRNAs and TRAIL elicited a characteristic DNA "ladder" bands indicative of apoptotic internucleosomal DNA fragmentation (Fig. 3G). These results indicated that c-FLIP(L) downregulation eliminated TRAIL resistance in HER2-positive breast cancer cells.

3.4. Downregulation of c-FLIP(L) selectively promoted the activities of caspase-8 and caspase-3, but did not alter surface expression of TRAIL death receptors in SKBR3 cells

To explore the molecular mechanism underlying TRAIL resistance in HER2-positive breast cancers, we examined the effects of c-FLIP(L) on the surface expression of DR4 and DR5 as well as on the caspase activity in SKBR3 cells. As depicted in Fig. 4A and B, DR5 was the predominant receptor expressed at the membrane of SKBR3 cells and both DR4 and DR5 expression were not significantly affected by c-FLIP(L) siRNAs. Hence, that c-FLIP(L) did not appear to inhibit SKBR3 cells to TRAIL-induced apoptosis by modulating the surface expression of death receptors. Western blotting revealed that procaspase-8 and procaspase-3 were reduced and processed subunits caspase-8-p43/41 and caspase-3-p20/17 were increased in c-FLIP(L)-knockdown SKBR3 cells following exposure to TRAIL, when compared with parental cells and control cells (Fig. 4C). Determination of enzymatic activation using fluorogenic

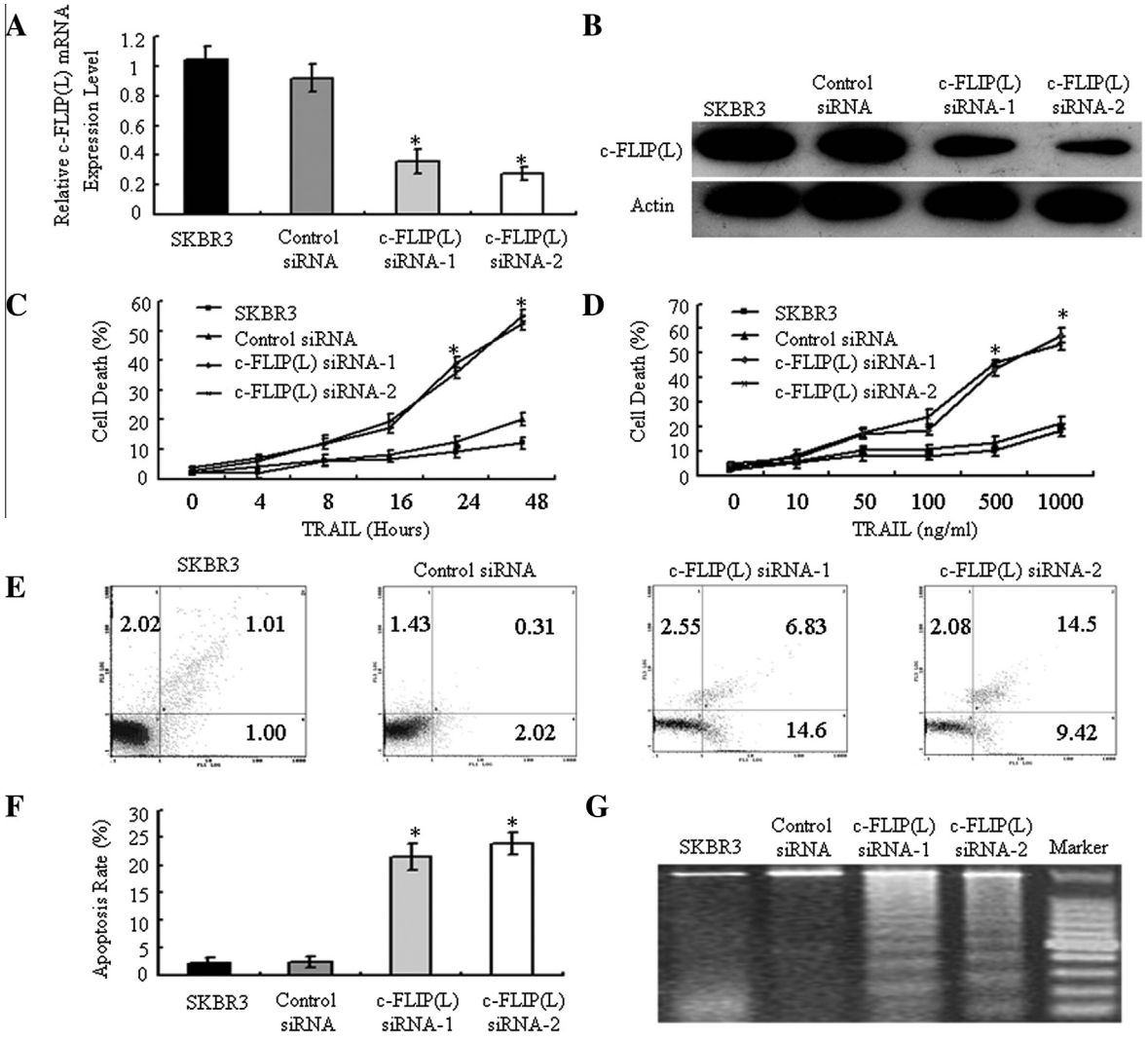

Fig. 3. Knockdown of c-FLIP(L) expression increased sensitivity of SKBR3 cells to TRAIL-induced apoptosis. (A and B) Confirmation of c-FLIP(L) reduced expression by real-time RT-PCR and Western blotting in SKBR3 cells transfected with c-FLIP(L) siRNAs. β-Actin was used as an internal control. (C and D) Downregulation of c-FLIP(L) expression increased death of SKBR3 cells as revealed by a MTT assay. The time- and dose-dependent curves were plotted with TRAIL treatments. (E) As measured by flow cytometry, reduced expression of c-FLIP(L) increased apoptotic percentage in SKBR3 cells treated with TRAIL (500 ng/ml, 24 h). (F) The flow cytometry data were shown in histograms. (G) A typical DNA ladder in SKBR3 cells was observed after being cultured with c-FLIP(L) siRNAs and 500 ng/ml TRAIL for 24 h. All the values represent the mean from at least three independent experiments. (*$P < 0.05$, c-FLIP(L) siRNAs *v.s.* control siRNA).

substrates showed that knockdown of c-FLIP(L) led to the apparent increase of caspase-3 and caspase-8 proteolytic activities in SKBR3 cells after TRAIL treatment. The caspase-8 activity began to increase at 500 ng/ml TRAIL treatment for 4 h ($P < 0.05$, Fig. 4D and F) and increased pronouncedly nearly 6-folds after TRAIL treatment for 24 h ($P < 0.01$, Fig. 4E and G). In addition, the caspase-3 activity increased significantly beyond 5-folds at 500 ng/ml TRAIL treatment for 24 h ($P < 0.01$, Fig. 4E and G). However, both caspase-2 and caspase-9 activities were only slightly altered by c-FLIP(L) siRNA-1 and c-FLIP(L) siRNA-2 transfection (Fig. 4D–G). These results illustrated that targeting c-FLIP(L) effectively mediated caspase-8 and caspase-3 cleavage and enhanced proteolytic activities in HER2-positive breast cancer cells.

4. Discussion

Breast cancer is a heterogeneous disease with different prognosis and may respond differently to therapy. Patients with HER2-positive breast cancer often experience an adverse outcome and resistance to hormonal treatment. The use of trastuzumab has been shown to greatly improve survival in HER2-positive patients [10]. However, approximately 15% of patients treated with trastuzumab will relapse and primary resistance to trastuzumab can occur in both adjuvant as well as metastatic settings [11]. These limitations of anti-HER2 targeted therapy have led to a rapid development of newly efficient biological agents. TRAIL has been described as a promising tumor-targeting cytokine that induces apoptosis in a variety of cancer cell types but not in normal cells [3]. Nevertheless, it should be noted that a few breast cancer cell lines, including HER2-positive cancer cells, are relatively resistant to TRAIL-mediated apoptosis [5]. Although the underlying mechanisms of TRAIL resistance have not been clearly characterized, the receptors and the downstream caspases are two predominant regulators involved in TRAIL pathway. Receptor-related causes include the mutations and deficient redistribution of the death receptors DR4 and DR5, and overexpression of the decoy receptors DcR1 and DcR2 [12,13]. Caspase-related causes include gene methylation and degradation of caspase-8, alteration in expression of the Bcl-2 family proteins and upregulation of c-FLIP [14–16]. In this study, we showed that c-FLIP(L) may act as an important contributor to TRAIL resistance in HER2 amplified breast cancer, especially in those with positive expression of death receptors.

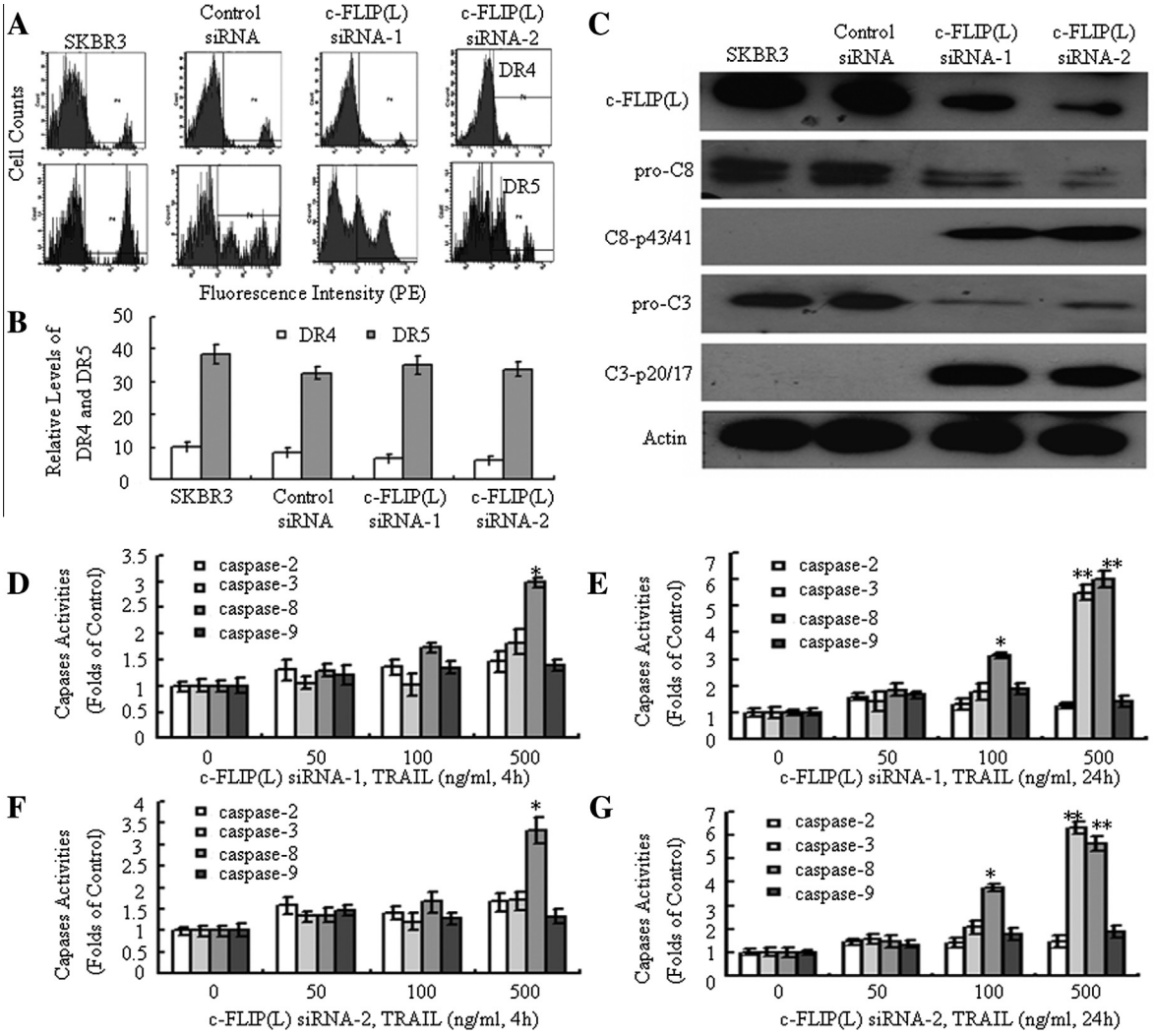

Fig. 4. C-FLIP(L) siRNAs treatment specifically enhanced the activities of caspase-8 and caspase-3. (A) The cell surface expression of DR4 and DR5 were examined by flow cytometry. (B) Quantification analysis revealed the relative level of cell surface DR4 and DR5. (C) The cells were treated with 500 ng/ml TRAIL for 24 h and harvested. Western blotting was performed using antibodies against procaspase-3/8 and the processed subunits. β-Actin was used as the protein loading control. (D–G) Effects of c-FLIP(L) knockdown on the activities of caspase-2, 3, 8 and 9 in SKBR3 cells. Results were presented by fold increase in cells transfected with c-FLIP(L) siRNAs rather than cells transfected with control siRNA. The values represent the mean of three individual experiments ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

C-FLIP is believed as an inhibitor of apoptotic signaling due to its elevated expression in multiple cancers [16,17], while it has been reported that c-FLIP was absent in Burkitt lymphomas and primary T-cell lymphomas [18]. These inconsistent findings imply that c-FLIP expression pattern varies in a tissue-type-dependent manner. In the present study, c-FLIP(L) protein was found to be over-expressed in breast cancer, indicating it may be involved in tumorigenesis, metastasis or other malignant phenotypes. Our investigation also showed that c-FLIP(L) was relatively high-expressed in HER2-positive breast cancer as compared to other subtypes, indicating the role of c-FLIP(L) may be more important in HER2-positive breast cancer. HER2 is a member of the epidermal growth factor receptor family with the tyrosine kinase activities. The mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways are frequently activated in cells overexpressing HER2 [19,20]. Upon activation, ERK and Akt translocate to the nucleus, which in turn upregulates several genes expression at the transcriptional level, including *c-FLIP* [7]. C-FLIP(L) is a catalytically inactive caspase-8/10 homologue that interferes with efficient DISC formation and prevents the processing and relapse of active caspase-8/10 in the extrinsic pathway [8]. Here we showed that c-FLIP(L) was co-expressed with death receptors in HER2-positive breast cancer tissues, and that SKBR3 cells transfected with c-FLIP(L) siRNAs had increased sensitivity to TRAIL independent of the expression of death receptors. These findings are in an agreement with a recent investigation reporting that c-FLIP(L) knockdown sensitized breast cancer stem cells to TRAIL and a combined TRAIL/c-FLIP(L) siRNA therapy could prevent tumor initiation and metastatic progression [21]. In addition to its anti-apoptotic function, c-FLIP(L) also acts as a pro-apoptotic molecule by heterodimerizing with caspase-8, when expressed at moderate amounts in combination with strong receptor stimulation or in the presence of high amounts of c-FLIP(S) or c-FLIP(R) [22]. Moreover, c-FLIP(L) N-terminal cleavage products have been reported to induce the NF-κB pathway rather than apoptotic pathways. P43-c-FLIP(L) was described to recruit tumor necrosis factor receptor-associated factors 1 (TRAF1), TRAF2 and receptor-interacting protein (RIP), which together promote NF-κB activation [23]. P22-c-FLIP(L) directly interacted with the IκB kinase (IKK) complex via the NF-κB essential modulator (NEMO)/IKKγ subunit independent of DR stimulation [24]. The mechanisms that account for the

dual regulation of c-FLIP(L) between life and death are still needed to be well elucidated.

Trastuzumab has been approved for use in the treatment of metastatic HER2-positive breast cancer when combined with chemotherapy, and as a single agent in the first-line therapy of HER2 amplified advanced breast cancer [10]. Trastuzumab treatment was demonstrated to enhance TRAIL-induced apoptosis in breast and ovarian cancer cells [25]. Caspase-8-dependent HER2 cleavage was detected in response to tumor necrosis factor α (TNF-α) and c-FLIP(L) overexpression was shown to inhibit TNF-α-triggered cleavage of the HER2 survival factor [26]. These findings proposed that there may be a certain molecular cross-talk between EGFR survival pathways and death receptor-mediated apoptotic pathways. Chemotherapeutic agents, including cisplatin and bortezomib, were reported to restore susceptibility to TRAIL by downregulation of c-FLIP(L) expression [27,28], opening new ways to develop combined anticancer therapies for HER2-positive cancers. In this study, we present evidence that c-FLIP overexpression may contribute to TRAIL resistance, suggesting that TRAIL could be manipulated for the management of primary or acquired resistance to trastuzumab in HER2-positive patients, and that the combination of trastuzumab with chemotherapy and TRAIL may allow enhance therapeutic efficacy and specificity in the treatment of HER2-overexpression tumors. These data render c-FLIP(L) an attractive candidate to predict the effectiveness of future immune therapies and TRAIL-targeted therapies in HER2-positive breast cancer.

In conclusion, our data demonstrate that c-FLIP(L) was overexpressed in HER2-positive breast cancer and may promote TRAIL resistance. Downregulation of c-FLIP(L) expression causes the cleavage and activation of caspase-8 and caspase-3. Our findings emphasize the crucial role played by c-FLIP(L) in regulating TRAIL resistance, and suggest that targeting c-FLIP(L) expression may restore TRAIL sensitivity and complement trastuzumab resistance in HER2-positive breast cancer.

Conflict of interest

The authors declare that they have no potential conflicts of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.106.

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