Inhibition of p38 mitogen-activated protein kinase potentiates the apoptotic effect of berberine/tumor necrosis factor-related apoptosis-inducing ligand combination therapy

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Abstract. It was previously reported that berberine (BBR) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) exhibited a synergistic apoptotic effect on triple negative breast cancer (TNBC) cells. In addition, the BBR/TRAIL combination treatment sensitized TRAIL-resistant TNBC cells to TRAIL. The aim of the present study was to investigate a novel pathway for enhancing the apoptotic effect of BBR/TRAIL through mitogen-activated protein kinases (MAPKs). Selective inhibitors and small interfering RNAs were utilized to understand the role of p38 MAPK in this pathway. The results demonstrated that p38 MAPK was activated in response to the combination therapy in TRAIL-resistant TNBC cells. In addition, it was revealed that the inhibition of p38 enhanced apoptosis in epidermal growth factor receptor (EGFR)-overexpressing MDA-MB-468 TNBC cells and EGFR-mutant PC-9 non-small-cell lung carcinoma cells, which was associated with the downregulation of EGFR serine phosphorylation. Viability assays for these two cell lines also confirmed the significant reduction of cell viability following p38 inhibition in BBR/TRAIL-treated cells. In conclusion, the present study provided novel evidence for the role of p38 in suppressing BBR/TRAIL-mediated apoptosis and its association with EGFR, which may explain the mechanism of treatment resistance in certain types of cancer.

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Introduction

Berberine (BBR) is an isoquinoline alkaloid found in the rhizome of numerous valuable medicinal plants (1). BBR has been reported to exhibit anti-inflammatory activity via the inhibition of transforming growth factor-β-activated kinase 1 (TAK1) (2). TAK1 is an important mediator of nuclear factor-κB activation, which in turn has important roles in cell survival and inflammation (3).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has potential antitumor activity, due to its high selectivity for various types of cancer cells over normal cells (4). However, the clinical efficacy of TRAIL has been limited due to primary or acquired resistance (5,6). TRAIL-based combination therapy approaches have therefore been introduced as a novel strategy against resistance (1,7,8). Of note, several studies have reported the contribution of the activated epidermal growth factor (EGF) receptor (EGFR) pathway in TRAIL-resistance (9,10). TRAIL activates TAK1 in tumor cells (11), which may result in the phosphorylation of downstream mitogen-activated protein kinases (MAPKs), most importantly p38 and extracellular signal-regulated kinase (ERK), and the subsequent regulation of the EGFR pathway (12,13).

EGFR (ErbB-1) is a member of the ErbB family of receptors, which is activated through the binding of its specific ligands (14). In canonical EGFR activation, EGF binds to EGFR to induce the preferential phosphorylation of tyrosine residues, which subsequently results in the internalization of EGFR into the cytoplasm to enhance the survival and proliferation of cells (14). By contrast, the TNF-α ligand, a member of the TNF family, binds to its relevant receptor to induce the TAK1-mediated activation of MAPKs and the subsequent phosphorylation of serine/threonine residues of EGFR. This non-canonical pathway of MAPKs/EGFR may eventually result in the p38/serine-dependent internalization of EGFR (14). However, the functional role of the p38/serine-dependent internalization remains to be fully elucidated (12,15).

A previous study demonstrated the efficiency of a BBR/TRAIL combination therapy against triple negative breast cancer (TNBC) cells as well as its effect on the sensitization of TRAIL-resistant TNBC cells to TRAIL (11). The

aim of the present study was to investigate a novel pathway for potentiating the apoptotic effect of BBR/TRAIL combination therapy through p38 MAPKs.

Materials and methods

Antibodies and reagents. Phospho-specific antibodies against p38 (Thr-180, Tyr-182; rabbit anti-human; cat. no., 9211), ERK (Thr-202, Tyr-204; rabbit anti-human; cat. no. 9101), and EGFR (Tyr-1068, Thr-669 and Ser-1046/1047; mouse anti-human; cat. nos. 2236, 3056 and 2238, respectively) as well as antibodies against poly-(adenosine diphosphate-ribose) polymerase 1 (PARP-1; rabbit anti-human; cat. no. 9542) and caspase-3 (rabbit anti-human; cat. no. 9662) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against EGFR (1005; rabbit anti-human; cat. no. sc-03) and β-actin (C-11; goat anti-human; cat. no. sc-1615) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All antibodies were polyclonal, with the exception of EGFR (Tyr-1068) that was monoclonal. Primary antibodies were used at 1:1,000 dilution. Recombinant human TRAIL Apo II ligand was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). SB203580, U0126 and PD153035, which are inhibitors of p38, ERK and EGFR tyrosine kinase, respectively, were obtained from Merck Biosciences (Danvers, MA, USA), while 5Z-7-oxozeaenol, a selective TAK1 inhibitor, was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). BBR chloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemical inhibitors, in addition to, BBR chloride were dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd.).

Cell cultures. MDA-MB-468 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen Life Technologies), 2 mM glutamine (Life Technologies, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) at 37˚C in a humidified atmosphere of 5% CO_2. PC-9 cells were provided by Dr Kiura (Okayama University, Okayama, Japan) and were maintained in RPMI-1640 medium (Invitrogen Life Technologies), with identical supplements and under identical conditions.

Cell viability assay. A WST-1 Cell Counting kit (Dojindo, Kumamoto, Japan) was used to detect cell viability. In brief, cell suspensions in DMEM supplemented with 10% fetal calf serum were seeded into a 96-well plate (5-8x10^3/70 μl/well) and incubated at 37˚C in a humidified atmosphere of 5% CO_2. Following 24 h of incubation, 10 μl FBS-supplemented DMEM containing the inhibitors at specified concentrations were added to the wells. After 30 min, 10 μl medium containing BBR (Wako Pure Chemical Industries, Ltd.) was added to the respective wells and incubated at 37˚C for 90 min, followed by 10 μl medium containing TRAIL (PeproTech, Inc., Rocky Hill, NJ, USA). Plates were then incubated for 24 h at 37˚C. WST-1 solution (10 μl) was added to each well 2 h prior to the end of the experiment. Absorbance was measured at 450 nm using a microplate reader (Multiskan Plus Model 355 Microplate Reader; Thermo Labsystems, Helsinki, Finland). Cell viability
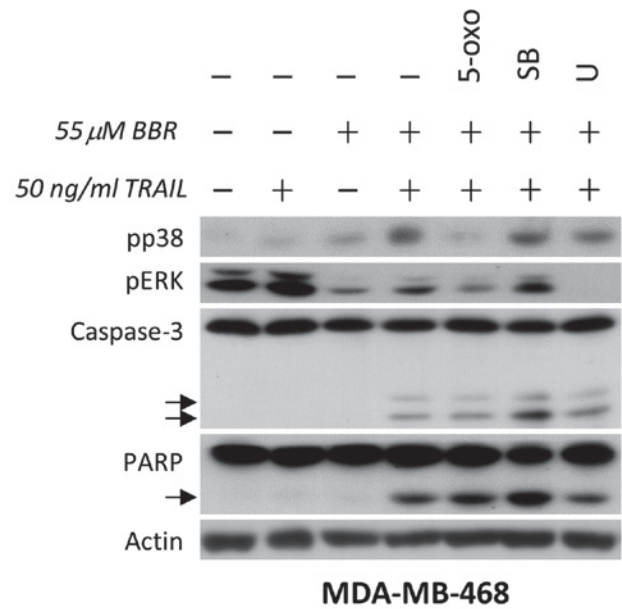

Figure 1. p38 counteracts the apoptotic effect mediated by BBR/TRAIL combination therapy in human breast cancer cells. TRAIL-resistant MDA-MB-468 cells were pretreated with dimethyl sulfoxide, 0.3 μM 5-oxo (transforming growth factor-β-activated kinase 1), 10 μM SB (p38 inhibitor) or 5 μM U (ERK inhibitor) for 30 min, followed by incubation with 55 μM BBR for 90 min. Cells were then further incubated in the presence or absence of recombinant human TRAIL (50 ng/ml) for 3 h. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; BBR, berberine; 5-oxo, 5Z-7-oxozeaenol; SB, SB203580; U, U0126; ERK, extracellular signal-regulated kinase; PARP, poly-(adenosine diphosphate-ribose) polymerase 1.

was determined from the absorbance of soluble formazan dye generated by living cells.

Transfection of small interfering RNAs (siRNAs). Duplex siRNAs were synthesized at Hokkaido System Science Co.,Ltd. (Sapporo, Japan). The target sequences were as follows: p38α, 5'-GCAUUACAACCAGACAGUUGAUAUU-3'; and firefly luciferase (GL2), 5'-CGUACGCGGAAUACUUCGA-3'. MDA-MB-468 cells were transfected with siRNAs in a final concentration of 50 nM using lipofectamine (Life Technologies). At 72 h post-transfection, the cells were treated.

Immunoblotting. Following stimulation, whole-cell lysates were prepared as described previously (16). Cell lysates were resolved by 7.5, 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P nylon membranes (EMD Millipore, Billerica, MA, USA). The membranes were treated with BlockAce (Dainippon Pharmaceutical Co., Ltd., Suita, Japan) and probed with primary antibodies (dilution, 1:1,000). The antibodies were then detected using polyclonal goat anti-rabbit (cat. no. P0448), polyclonal rabbit anti-mouse (cat. no. P0260) or polyclonal rabbit anti-goat (cat. no. P0449) horseradish peroxidase-conjugated immunoglobulin G antibody (dilution, 1:2,000; Dako, Carpinteria, CA, USA) and visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). Certain antibody reactions were performed using in Can Get Signal® immunostain solution (Toyobo Co., Ltd, Osaka, Japan).
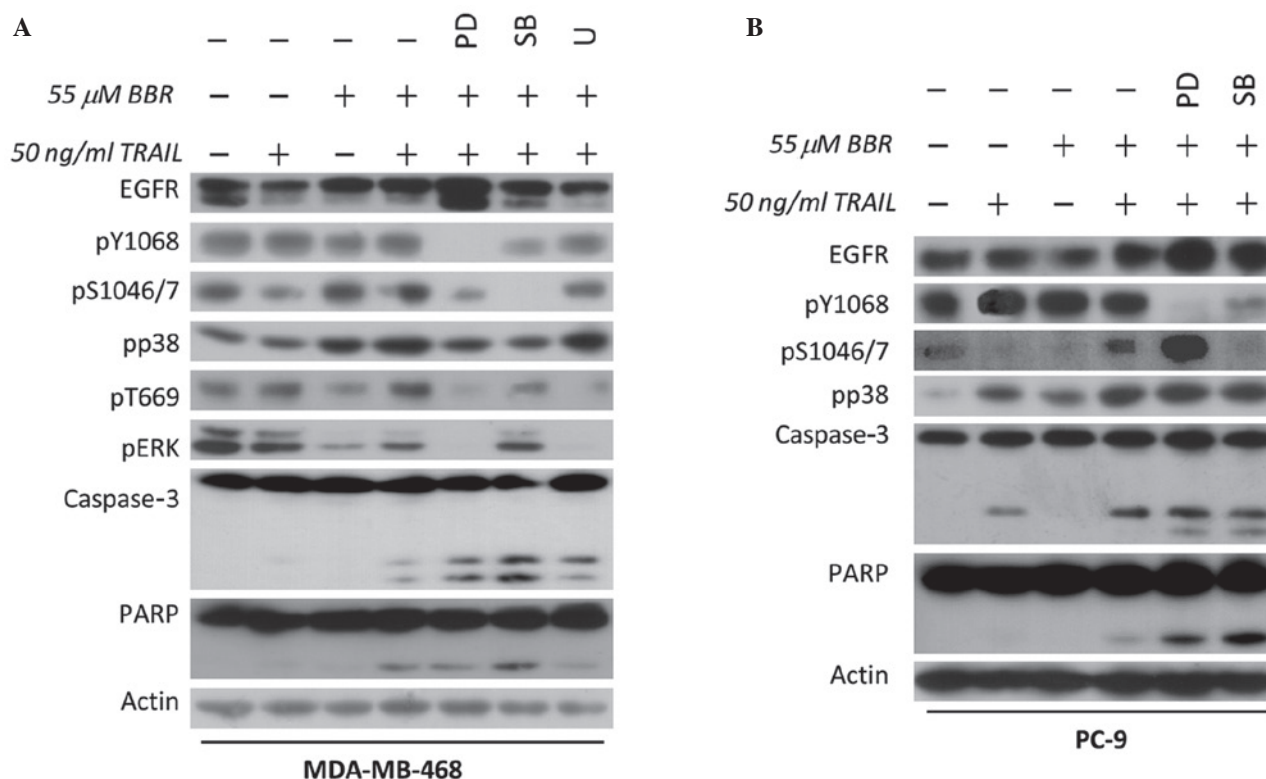
Figure 2. Crosstalk between EGFR and the apoptotic effect mediated by BBR/TRAIL combination therapy in EGFR-expressing breast cancer cells. (A) EGFR-overexpressing MDA-MB-468 and (B) mutant EGFR-overexpressing PC-9 cells were pretreated with dimethyl sulfoxide, 1 μM PD (EGFR tyrosine kinase inhibitor), 10 μM SB (p38 inhibitor) or 5 μM U (ERK inhibitor) for 30 min, followed by incubation with 55 μM BBR for 90 min. Cells were then further incubated in the presence or absence of 50 ng/ml recombinant human TRAIL for 3 h. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; BBR, berberine; EGFR, epidermal growth factor receptor; PD, PD153035; SB, SB203580; U, U0126; ERK, extracellular signal-regulated kinase; PARP, poly-(adenosine diphosphate-ribose) polymerase 1.

Statistical analysis. Data are presented as the mean ± standard deviation of 3 experiments. The statistical analysis was performed using JMP software (version 10; SAS Institute Inc., Cary, NC, USA). The statistical significance was determined by applying the Bonferroni method (compared to BBR/TRAIL). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TRAIL enhances BBR-mediated p38 activation and counteracts BBR-mediated pERK downregulation in a TAK1-dependent manner. In light of the previous findings of BBR and TRAIL studies (1,17), the present study aimed to investigate the effect of the BBR/TRAIL combination therapy on MAPK activation in EGFR-overexpressing breast cancer cells. The results demonstrated that TRAIL alone markedly upregulated ERK phosphorylation and slightly upregulated p38 phosphorylation in MDA-MB-468 cells (Fig. 1). This effect was not associated with any changes in apoptosis, which may be due to the resistance of MDA-MB-468 cells to TRAIL (Fig. 1, lane 2). By contrast, BBR decreased ERK phosphorylation and markedly increased p38 phosphorylation (Fig. 1). In addition, the BBR/TRAIL combination further enhanced p38 phosphorylation and decreased ERK phosphorylation. TAK1 inhibition using the specific inhibitor 5Z-7-oxozeaenol demonstrated an inhibition of the combination therapy-mediated upregulation of p38; comparably, decreased ERK phosphorylation was

observed following 5Z-7-oxozeaenol treatment. This indicated that TAK1 was involved in the regulation of the phosphorylation of p38 and ERK in MDA-MB-468 cells. However, TAK1 inhibition did not induce any changes in combination therapy-mediated apoptosis (Fig. 1, lane 5 vs. 4). In order to further demonstrate the effect of p38 and ERK inhibition on cell apoptosis, cells were pretreated with specific inhibitors, SB203580 and U0126, respectively. The results revealed that p38 inhibition enhanced apoptosis in cells treated with BBR/TRAIL combination therapy, whereas ERK inhibition did not exhibit notable effects on apoptosis (Fig. 1).

p38 MAPK rescues cancer cells from BBR/TRAIL combination therapy. In order to confirm the efficient inhibition of p38 in EGFR-expressing cells, the previous experiment was repeated with EGFR tyrosine kinase inhibition instead of TAK1 inhibition. EGFR tyrosine kinase is well-known to be crucial for cell survival and proliferation (18). Although blocking the EGFR tyrosine kinase may indirectly block ERK and EGFR threonine phosphorylation, the present results demonstrated that p38 inhibition downregulated EGFR serine phosphorylation as well as enhanced apoptosis compared with that of EGFR tyrosine kinase inhibition. In addition, ERK inhibition did not promote apoptosis in MDA-MB-468 cells (Fig. 2A). This confirmed that p38, but not ERK, may contribute to cell apoptosis in EGFR-expressing MDA-MB-468 cells. In order to confirm these results, the effects of the EGFR tyrosine kinase inhibitor and p38 inhibitor were examined in EGFR-mutant
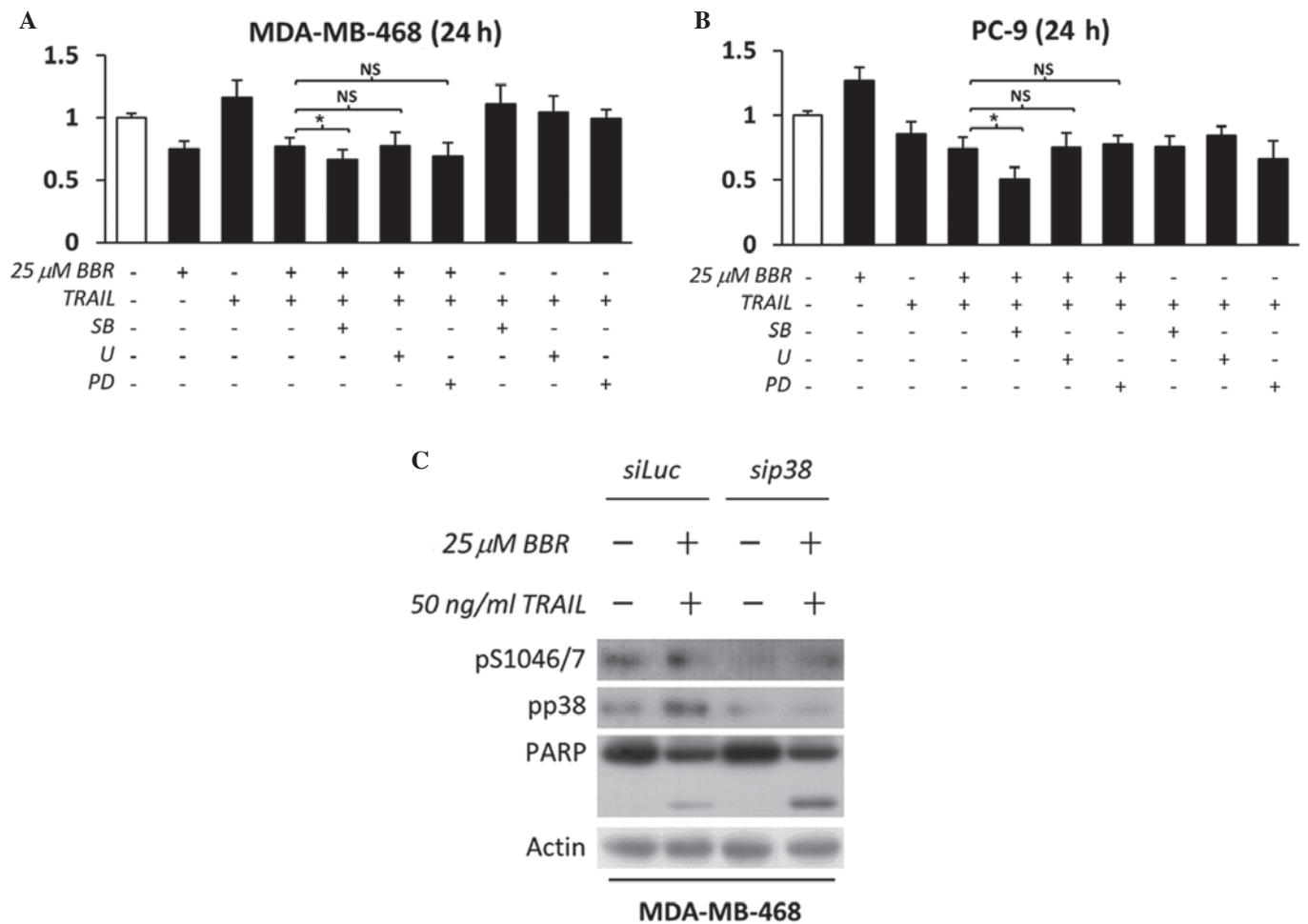
Figure 3. p38 inhibition enhances the apoptotic effect mediated by BBR/TRAIL combination therapy in EGFR-expressing cancer cells. (A) EGFR-overexpressing MDA-MB-468 and (B) mutant EGFR-overexpressing PC-9 cells were pretreated with dimethyl sulfoxide, 1 μM PD (EGFR tyrosine kinase inhibitor), 10 μM SB (p38 inhibitor) or 5 μM U (extracellular signal-regulated kinase inhibitor) for 30 min, followed by incubation with 25 μM BBR for 90 min. Cells were then further incubated in the presence or absence of 50 ng/ml recombinant human TRAIL for 24 h in the presence or absence of specific inhibitors.. Absorbance was measured following incubation with WST-1 reagent and cell viability was calculated as a percentage relative to the untreated control cells. Data are expressed as the mean ± standard deviation of triplicate experiments. *P<0.05 between groups; NS, not significant. (C) MDA-MB-468 cells were transfected with Luc (control) or p38α siRNA in the presence or absence of BBR/TRAIL. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; BBR, berberine; EGFR, epidermal growth factor receptor; siRNA, small interfering RNAs; PD, PD153035; SB, SB203580; U, U0126; PARP, poly-(adenosine diphosphate-ribose) polymerase 1; Luc, luciferase.

non-small-cell lung carcinoma (NSCLC) PC-9 cells, the results of which demonstrated a comparable pattern to that of the EGFR-expressing breast cancer cells (Fig. 2B). The consequences of enhancing cellular apoptosis following combination therapy were demonstrated using cell viability assays in these EGFR-overexpressing cell lines; MDA-MB-468 and PC-9. In this experiment, cells were exposed to a lower concentration of BBR, in addition to TRAIL, and the specific inhibitors for extended time periods in order to monitor their significance on cell viability. As shown in Fig. 3A and B, only p38 inhibition in addition to BBR/TRAIL therapy induced a significant decrease in cell viability, compared to ERK inhibition or EGFR inhibition. In addition, the specificity of p38 action was confirmed using specific knockdown of p38 with siRNA. The results demonstrated that p38 knockdown induced EGFR serine down-regulation, which resulted in the cleavage of caspase-substrate, PARP-1 (Fig. 3C); this therefore confirmed the value of using p38 inhibition in conjunction with BBR/TRAIL combination therapy in EGFR-expressing cancer cells.

Discussion

A previous study demonstrated the role of BBR in synergizing TRAIL action in TRAIL-sensitive MDA-MB-231 TNBC cells, as well as sensitizing the TRAIL-resistant MDA-MB-468 TNBC cells to TRAIL (1). In addition to the difference in TRAIL sensitivity pattern, MDA-MB-468, and not MDA-MB-231 cells, were reported to preferentially over-express EGFR (1). Furthermore, through comparing data from the publicly available microarray (GSE41313; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41313), it was revealed that MDA-MB-468 exhibited the highest expression of EGFR among other breast cancer cells (data not shown). In the present study, the role of MAPK in cellular apoptosis was investigated in EGFR-expressing cells. MDA-MB-468 cells were primarily used in the present study, with a secondary focus on PC-9 EGFR-mutant NSCLC cells. The selected cell lines facilitated the exploration of the present findings regardless of the EGFR status.

In concurrence with previous studies (12,14,19), the present study suggested that the role of p38 in EGFR serine phosphorylation is of increasing importance for cell survival. In order to explore the role of p38 in BBR/TRAIL-mediated apoptosis, independent of the EGFR-expression status, the present study used MDA-MB-468 and PC-9 cells; the results of which confirmed the role of p38 in counteracting BBR/TRAIL-mediated apoptosis. The role of p38 in EGFR serine was not thoroughly investigated in the present study; however, these results provided certain evidence for this reproducible association. It was therefore suggested that p38 may counteract the BBR/TRAIL-mediated apoptosis as well as suppress the apoptotic effect of other therapies.

The role of p38 in cell death/survival has been a controversial issue for decades. We believe that the type, strength and duration of specific stimuli towards p38 may significantly affect the eventual outcome experienced by the cell. However, in cancer cells expressing EGFR, growing evidence indicates the prominence of the p38-EGFR serine axis on cell survival. Our recent studies demonstrated the role of various stimuli on the activation of this survival axis (12,18). Notably, in a recent study, we demonstrated a similar apoptotic response subsequent to inhibiting the p38-EGFR serine axis (14). Therefore, studies involving the use of specific p38 inhibitors may be of potential future use in anticancer combination therapies.

Acknowledgements

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