TNFR2 promotes Adriamycin resistance in breast cancer cells by repairing DNA damage

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Abstract. In recent years, tumor necrosis factor receptor 2 (TNFR2) has attracted increasing attention for its important roles in promoting proliferation, migration and angiogenesis in several types of cancer. However, its role in drug resistance remain unclear. In the present study, TNFR2 expression levels in MDA-MB-231 and MCF-7 breast cancer cells were demonstrated to be associated with Adriamycin (ADM) resistance. Silencing of TNFR2 in MCF-7 cells significantly inhibited ADM resistance, while overexpression of TNFR2 in MDA-MB-231 cells significantly enhanced ADM resistance. ADM treatment induced phosphorylation of the histone family 2A variant X (pH2AX), an established marker of DNA damage. Silencing of TNFR2 in MCF-7 cells further induced pH2AX levels but inhibited the expression of the DNA damage repair protein, poly(ADP-ribose) polymerase (PARP). By contract, overexpression of TNFR2 in MDA-MB-231 cells decreased pH2AX levels and enhanced PARP expression. Of note, treatment with the PARP inhibitor ABT888 significantly abrogated the effects of TNFR2 on pH2AX expression. On a molecular mechanism level, TNFR2 significantly affected the phosphorylation of AKT serine/threonine kinase 1 (AKT) in both cell lines, and treatment with the AKT inhibitor LY294002 effectively abrogated TNFR2-induced PARP expression. A drug resistance assay demonstrated that treatment with either LY294002 or ABT888 inhibited ADM resistance in breast cancer cells, and combination treatment with both LY294002 and ABT888 exhibited a significantly stronger inhibition effect on ADM resistance. The present results indicated that TNFR2 promoted ADM resistance in breast cancer cells by regulating the DNA damage repair protein PARP via the AKT signaling pathway.

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Introduction

Breast cancer is one of the most common malignant cancers in women worldwide (1). Its occurrence and development is a complicated process and can be influenced by many factors, including abnormal expression of cell surface receptors, abnormal activation of intracellular signal transduction pathways and gene mutations (2-5). In recent years, with significant progress in novel chemotherapy regimens and proper combination of various therapeutic methods including surgery, endocrine therapy, molecular targeted therapy, chemotherapy and radiotherapy, the overall patient survival has improved to some extent (6,7). As a member of anthracyclines, Adriamycin (ADM) has been widely used in different types of tumors due to its strong antitumor effects (8-11). Especially in breast cancer, ADM has become the cornerstone of many therapy regimens with very good therapy outcomes (12,13). Unfortunately, drug resistance for ADM, which usually occurs in most cases following a period of treatment, restricts its further application and results in poor long-term therapy outcomes (14). Therefore, there is an urgent need for novel strategies to overcome drug resistance that will lead to better prognosis for patients.

Tumor necrosis factor receptor (TNFR) 2 is a member of the TNFR family, and it is important in tumor progression and prognosis, by regulating the malignant behavior of tumor cells via stimulating AKT serine/threonine kinase 1 (AKT) or nuclear factor (NF)-κB signaling pathways (15). However, its role in ADM resistance of breast cancer has not been reported. Aberrant stimulation of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, DNA damage repair and cancer stemness are considered established events responsible for drug resistance in many types of tumors (16-18). But, whether TNFR2 could induce drug resistance through regulating DNA repair or cancer stemness remains unknown.

In the present study, the role of TNFR2 in drug resistance was explored from the perspective of its effect on the DNA repair mechanism. The results demonstrated that TNFR2 induced ADM resistance in breast cancer cells, by enhancing DNA damage repair via regulating the DNA repair protein, poly(ADP-ribose) polymerase (PARP). Furthermore, the AKT signaling pathway was demonstrated to be required for TNFR2-induced PARP expression.

Materials and methods

Cell culture. Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured in minimum essential medium (MEM) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell transfection. Cells were plated in a 6-well plate at $4x10^5$ cells per well. After 24 h, plasmid pReceiver-M77-TNFR2 (410 ng/μl) (EX-A0254-M77; GeneCopoeia, Inc., Rockville, MD, USA) and control plasmid (320 ng/μl) were transfected into MDA-MB-231 cells to upregulate TNFR2 expression; plasmid psi-U6-GFP-TNFR2-sh (380 ng/μl) (RSH052309-CU6; GeneCopoeia, Inc.) and control plasmid (440 ng/μl) were transfected into MCF-7 cells to down-regulate TNFR2 expression. All procedures were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and empty vectors were used as a control. After 48 h, cells were harvested and transfection efficiency was determined using western blot analysis. Sequence silencing TNFR2, 5'-TTGACACCCTACAAGCCAGAA-3'; sequence as control plasmid, 5'-GTTCTGCGAACGTGTCACGT-3'.

Western blotting. Cells were washed twice in PBS and lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1% protease inhibitor. Protein concentration was measured by spectrophotometry (ND-1000; Nano Drop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein (200 μg) was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Following blocking in TBS/0.1% Tween-20 containing 5% non-fat dry milk for 1 h at room temperature, the membrane was incubated with primary antibodies (listed in Table I) at 4˚C overnight and then with horseradish peroxidase-conjugated secondary antibody (ab97023/ab6802; 1:5,000; Epitomics; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Finally, signals on the membrane were visualized by enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.) and measured by Image-Pro software (version 5.1; Media Cybernetics, Inc., Rockville, MA, USA).

Drug resistance assay. Cells were plated in 96-well plates in triplicate in MEM supplemented with 10% FBS at 8,000 cells per well. After 24 h, the medium was replaced with MEM containing 0.02, 0.08, 0.32, 1.28, 5.12, or 20.48 μmol/l ADM for 48 h and then MTT was dissolved in dimethyl sulfoxide (M1020-500T; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) and MTT assay was performed at 490 nm wavelength. The survival curves were constructed and the half maximal inhibitory concentration (IC50) was calculated. The experiment was repeated at least three times.

Statistical analysis. Data were expressed as mean ± standard deviation. SPSS 13.0 software (SPSS, Inc. Chicago, IL, USA) was used for statistical analysis. IC50 was calculated by regression analysis. Significance of differences between two groups was analyzed by Student two-tailed t-test. Significance of differences between multiple groups was analyzed by one-way analysis of variance followed by Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TNFR2 expression levels are associated with ADM resistance in breast cancer cells. Firstly, the protein expression levels of TNFR2 were detected in the breast cancer cell lines MCF-7 and MDA-MB-231. As illustrated in Fig. 1A and B, TNFR2 protein expression levels were significantly higher in MCF-7 cells compared with MDA-MB-231 cells, by ~3-fold. Of note, ADM resistance of MCF-7 cells was also significantly higher compared with MDA-MB-231 cells (Fig. 1C). The IC50 was 0.505±0.028 and 0.331±0.039 μmol/l for MCF7 and MDA-MB-231 cells respectively, which was a significant difference ($P<0.05$; Fig. 1D). These results suggested a potential correlation between TNFR2 expression and AMD resistance in breast cancer cells. In order to further explore this hypothesis, TNFR2 expression was silenced in MCF-7 cells by shRNA (Fig. 1E). The cell survival rate of TNFR2-deficient MCF-7 cells declined significantly following ADM treatment compared with control MCF-7 cells (Fig. 1F), with the IC50 decreasing from 0.649±0.06 μmol/l in the control cells to 0.353±0.054 μmol/l in the TNFR2-deficient MCF-7 cells ($P<0.05$; Fig. 1G). By contrast, overexpressing TNFR2 in MDA-MB-231 cells (Fig. 1H) significantly increased the cell survival rate following ADM treatment (Fig. 1I), with the IC50 increasing from 0.339±0.087 μmol/l in the control cells to 0.769±0.075 μmol/l in the TNFR2-overexpressing cells ($P<0.05$; Fig. 1J). These results demonstrated that TNFR2 promoted ADM resistance in breast cancer cells.

TNFR2 inhibits ADM-induced pH2AX expression. As illustrated in Fig. 2A, phosphorylation of histone family 2A variant X (pH2AX), which is indicative of DNA damage by double strand breakage, increased by ~5-fold in ADM-treated MCF-7 cells compared with untreated MCF-7 cells. No changes were observed in the levels of total histone family 2A variant X (H2AX). When TNFR2 expression was silenced in MCF-7 cells, pH2AX expression was further increased (Fig. 2A). By contrast, TNFR2 overexpression in MDA-MB-231 cells resulted in a ~5-fold decrease in pH2AX expression compared with control MDA-MB-231 cells treated with ADM alone (Fig. 2B). These results suggested that TNFR2 reduces the levels of DNA damage.

TNFR2 inhibits pH2AX expression through regulation of PARP. As illustrated in Fig. 3A, TNFR2 overexpression significantly inhibited pH2AX expression in MDA-MB-231 cells following ADM treatment, but significantly increased PARP expression. By contrast, the pH2AX increase induced by TNFR2 silencing in MCF-7 cells was accompanied by PARP expression inhibition (Fig. 3B). No changes were observed to O6-methylguanine-DNA methyltransferase (MGMT; data not shown). When the PARP inhibitor ABT888 was used, TNFR2 silencing in MCF-7 cells did not result in

Table I. Primary antibodies used in western blot analyses.

Protein	Cat. no.	Final dilution	Supplier
TNFR2	ab8161	1:1,000	Abcam, Cambridge, MA, USA
pH2AX	ab22551	1:1,000	Abcam, Cambridge, MA, USA
PARP	13371-1-AP	1:1,000	Wuhan Sanying Biotechnology, Wuhan, China
MGMT	17195-1-AP	1:2,000	Wuhan Sanying Biotechnology, Wuhan, China
p-ERK1/2	ab214362	1:1,000	Abcam, Cambridge, MA, USA
ERK1/2	9102	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
p-AKT	13038	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
AKT	4685	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
GAPDH	Ab181602	1:2,000	Abcam, Cambridge, MA, USA

TNFR2, tumor necrosis factor receptor 2; pH2AX, phosphorylated histone family 2A variant X; PARP, poly(ADP-ribose) polymerase; MGMT, O6-methylguanine-DNA methyltransferase; p-, phosphorylated; ERK, extracellular signal-regulated kinase; AKT, AKT serine/threonine kinase 1.

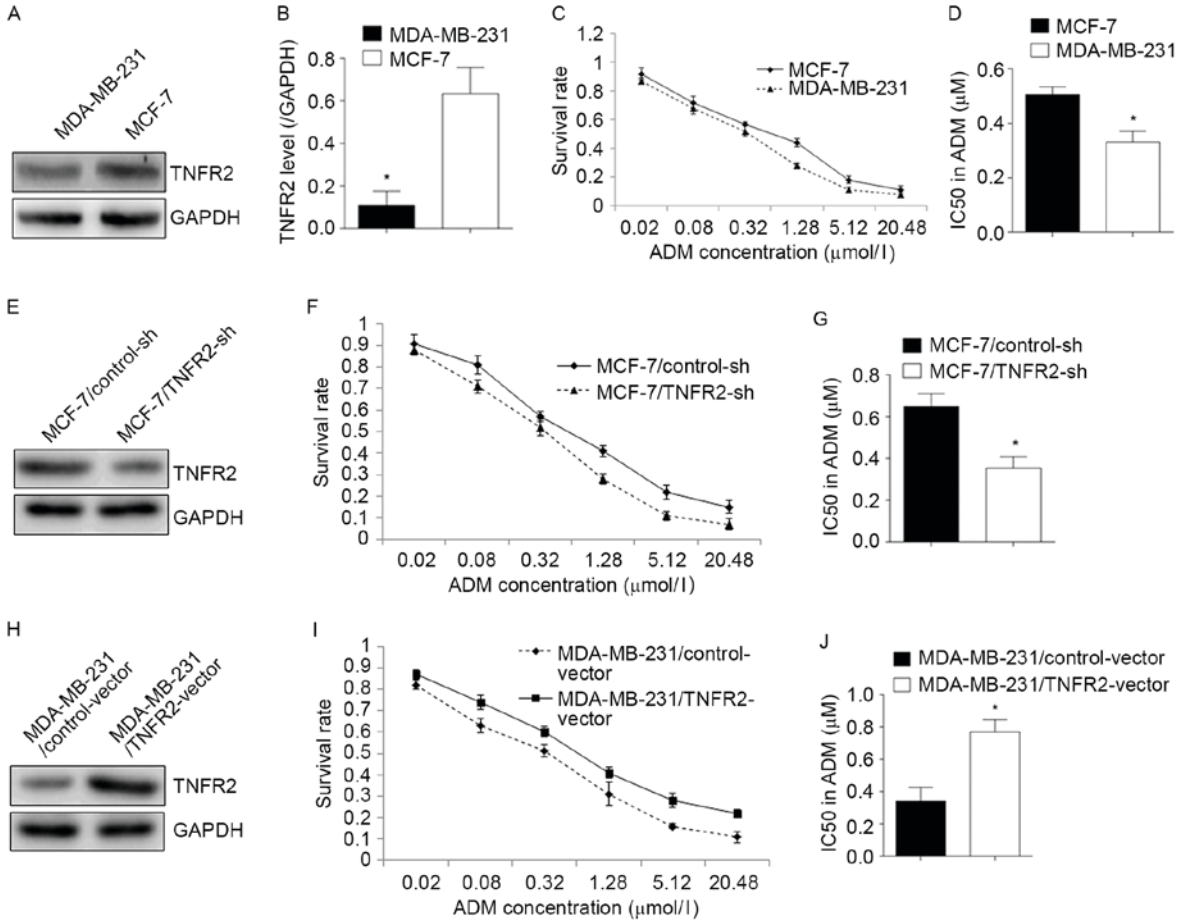

Figure 1. ADM resistance assay in breast cancer cells. (A) Representative blots and (B) quantification of TNFR2 protein expression levels in MDA-MB-231 and MCF-7 cells, as assessed by western blotting. GAPDH was used as a loading control. (C) ADM resistance abilities of MDA-MB-231 and MCF-7 cells. (D) Quantification of IC50 values for MDA-MB-231 and MCF-7 cells following ADM treatment. (E) TNFR2 protein expression levels in MCF-7 cells treated with either control or TNFR2-specific shRNA (TNFR2-sh). (F) ADM resistance abilities and (G) IC50 values of control and TNFR2-sh MCF-7 cells. (H) TNFR2 protein expression levels in MDA-MB-231 cells treated with either control empty vector or a TNFR2-overexpressing vector. (I) ADM resistance abilities and (J) IC50 values of control and TNFR2-overexpressing MDA-MB-231 cells. *P<0.05. ADM, Adriamycin; TNFR2, tumor necrosis factor receptor 2; shRNA, short hairpin RNA; IC50, half maximal inhibitory concentration.

any significant changes of pH2AX expression following ADM treatment (Fig. 3C). Similarly, pH2AX levels in MDA-MB-231 cells were not affected by TNFR2 overexpression following ADM treatment in the presence of the PARP inhibitor ABT888 (Fig. 3D). A drug resistance assay demonstrated that the increase in survival rate for MDA-MB-231 cells induced

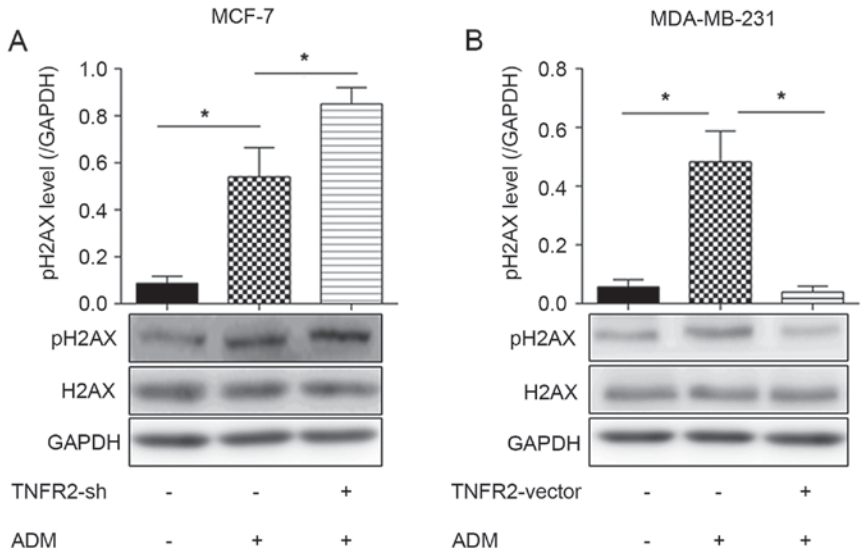

Figure 2. TNFR2 inhibits ADM-induced pH2AX expression. Protein expression levels of pH2AX and H2AX were assessed by western blotting in (A) control and TNFR2-knockdown (TNFR2-sh) MCF-7 cells, and in (B) control and TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells, with or without ADM treatment. GAPDH was used as a loading control. *P<0.05. TNFR2, tumor necrosis factor receptor 2; ADM, Adriamycin; H2AX, histone family 2A variant X; p-, phosphorylated; sh, short hairpin RNA.

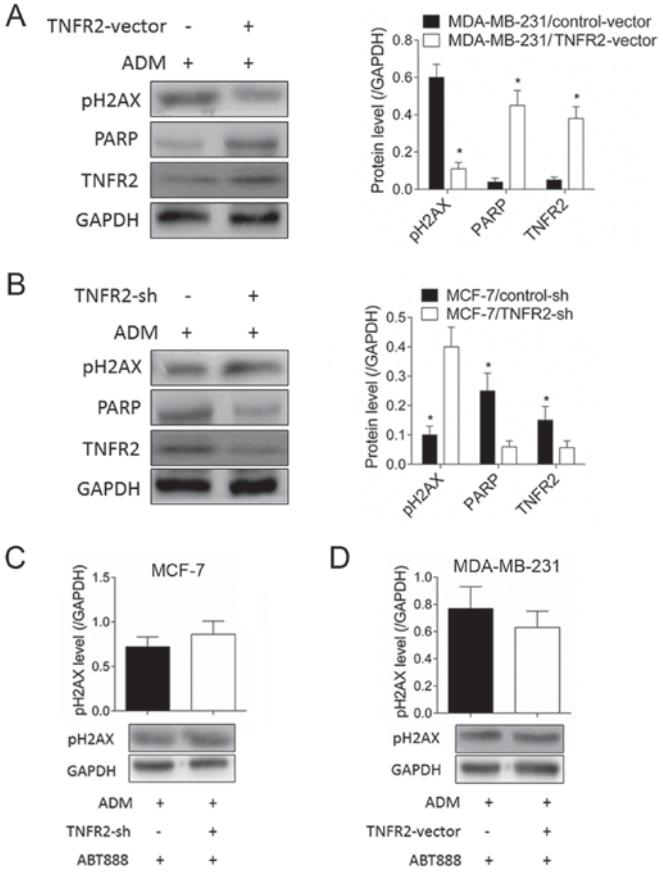

Figure 3. TNFR2 inhibits pH2AX expression through PARP. Protein expression levels of pH2AX and PARP were assessed in (A) control and TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells, and in (B) control and TNFR2-knockdown (TNFR2-sh) MCF-7 cells, with or without ADM treatment. The effect of the PARP inhibitor ABT888 on the ADM-induced expression levels of pH2AX was assessed in (C) control and TNFR2-knockdown (TNFR2-sh) MCF-7 cells, and in (D) control and TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells. GAPDH was used as a loading control. *P<0.05. TNFR2, tumor necrosis factor receptor 2; pH2AX, phosphorylated histone family 2A variant X; PARP, poly(ADP-ribose) polymerase; sh, short hairpin RNA; ADM, Adriamycin.

by TNFR2 overexpression declined significantly following addition of ABT888 (Fig. 4A), with the IC50 declining from 0.756±0.117 to 0.384±0.071 μmol/l (P<0.05; Fig. 4B). These results suggested that TNFR2 affected pH2AX expression partly by regulating PARP.

TNFR2 promotes PARP expression via AKT signaling. To further study the potential molecular mechanism responsible for PARP expression, AKT and extracellular signal-regulated kinase (ERK) were examined as candidate signal targets. As illustrated in Fig. 5A, TNFR2 overexpression in MDA-MB-231 cells significantly stimulated phosphorylation of AKT, but no change ERK phosphorylation was observed. By contrast, TNFR2 silencing in MCF-7 cells significantly inhibited phosphorylation of AKT, while again no change was observed in ERK phosphorylation (Fig. 5B). To further confirm that AKT activation mediated PARP expression, the AKT inhibitor LY294002 was used. The results demonstrated that PARP upregulation induced by TNFR2 overexpression in MDA-MB-231 cells was significantly inhibited by addition of the AKT inhibitor LY294002 (Fig. 5C). A drug resistance assay demonstrated that increase in survival rate of MDA-MB-231 cells induced by TNFR2 overexpression declined significantly following addition of LY294002 (Fig. 4A), with the IC50 declining from 0.756±0.117 to 0.304±0.08 μmol/l (P<0.05; Fig. 4B). Furthermore, combination treatment of LY294002 and ABT888 inhibited the IC50 of TNFR2-overexressing MDA-MB-231 cells from 0.756±0.117 to 0.176±0.07 μmol/l, with was significantly lower than either LY294002 or ABT888 treatment alone (Fig. 4B). These results indicated that TNFR2 promoted PARP expression via AKT signaling.

Discussion

Previous studies have reported two possible mechanisms responsible for the antitumor effects of ADM: Inhibition of

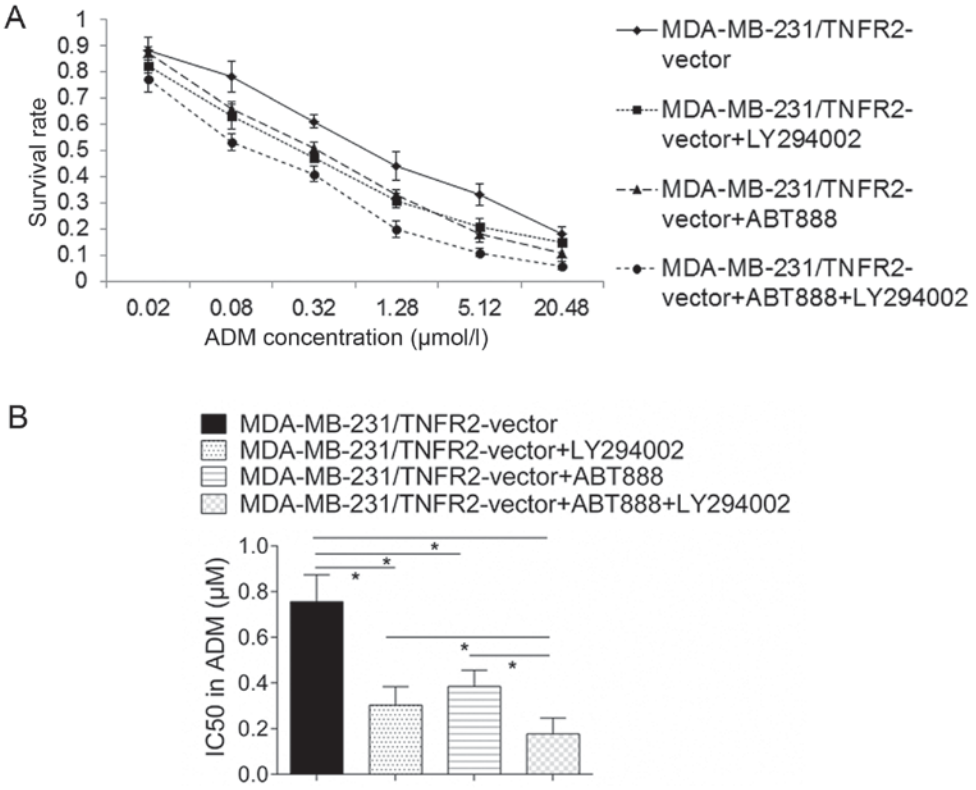

Figure 4. LY294002 and ABT888 inhibit ADM resistance induced by TNFR2. TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells were challenged with increasing concentrations of ADM, in the absence or presence of LY294002 and/or ABT888 inhibitors. (A) Survival rate. (B) IC50 values. *P<0.05. ADM, Adriamycin; TNFR2, tumor necrosis factor receptor 2; IC50, half maximal inhibitory concentration.

DNA transcription and replication by intercalating between DNA base pairs, and induction of DNA double strand breakage by generating oxygen free radicals (19). Therefore, ADM resistance studies may focus on the DNA damage repair mechanism.

TNFR2, which differs from TNFR1 mainly due to the absence of death domain in its structure, promotes survival, proliferation, migration and invasion in multiple types of cancer. Tanimura *et al* (20) reported that TNF-α promotes invasiveness of cholangiocarcinoma cells via TNFR2. In addition, Yang *et al* (21) reported that progranulin promotes proliferation and angiogenesis of colorectal cancer cells through TNFR2. However, studies about the role of TNFR2 in drug resistance are limited and remain controversial. Zhang *et al* (22) reported that apoptotic response of colorectal cancer cells to 5-fluorouracil is mediated by induced TNFR2, implying negative regulation of TNFR2 in drug resistance. Sprowl *et al* (23) reported that TNFR2 expression was upregulated in ADM resistant MCF-7 cells, this suggested a possible correlation between TNFR2 and drug resistance, but did not confirm the role of TNFR2 in drug resistance, and the underlying mechanism remains to be elucidated. In the present study, it was demonstrated that MCF-7 cells with higher TNFR2 expression exhibited stronger ADM resistance than MDA-MB-231 cells in which TNFR2 expression was significantly lower. Furthermore, overexpression of TNFR2 in MDA-MB-231 cells enhanced ADM resistance, while silencing of TNFR2 in MCF-7 cells weakened ADM resistance. These results indicate that TNFR2 is important in ADM resistance of breast cancer cells. The present findings are in contrast to the findings of Zhang *et al* (22) for colorectal cancer cells. It is possible that different types of tumors and different drugs may involve different pharmacological mechanisms and pathways regulating resistance.

The mechanism of ADM resistance is complicated and TNFR2 effect on cell survival and proliferation may be partly responsible for this. In addition, integrity of DNA is crucial for cell survival (24). Chemotherapy drugs kill tumor cells by destroying their DNA, but tumor cells can repair DNA damage by activating the DNA damage repair mechanism, resulting in drug resistance (25). To date, there are no reports on the effect of TNFR2 on DNA damage repair. H2AX is a subtype of the core histone 2A and it is localized to human chromosome 11q23 (26). Post-translational modification of H2AX, such as phosphorylation, methylation and acetylation, usually happens following DNA double strand breakage. Because H2AX is the first substrate for phosphorylation following DNA double strand breakage, phosphorylation of H2AX is routinely used as a marker of DNA damage for recruitment of repair factors and chromosome remodeling factors, thus maintaining genome stability (27). In the present study, pH2AX was detected following ADM treatment, confirming the presence of DNA damage induced by ADM. TNFR2 overexpression in MDA-MB-231 cells significantly decreased pH2AX levels, while TNFR2 silencing in MCF-7 cells significantly induced the levels of pH2AX, following ADM challenge. These results suggested that TNFR2 affects the levels of DNA damage induced by ADM in breast cancer cells.

MGMT and PARP are both important DNA repair proteases (28,29). To test whether TNFR2 could repair DNA damage

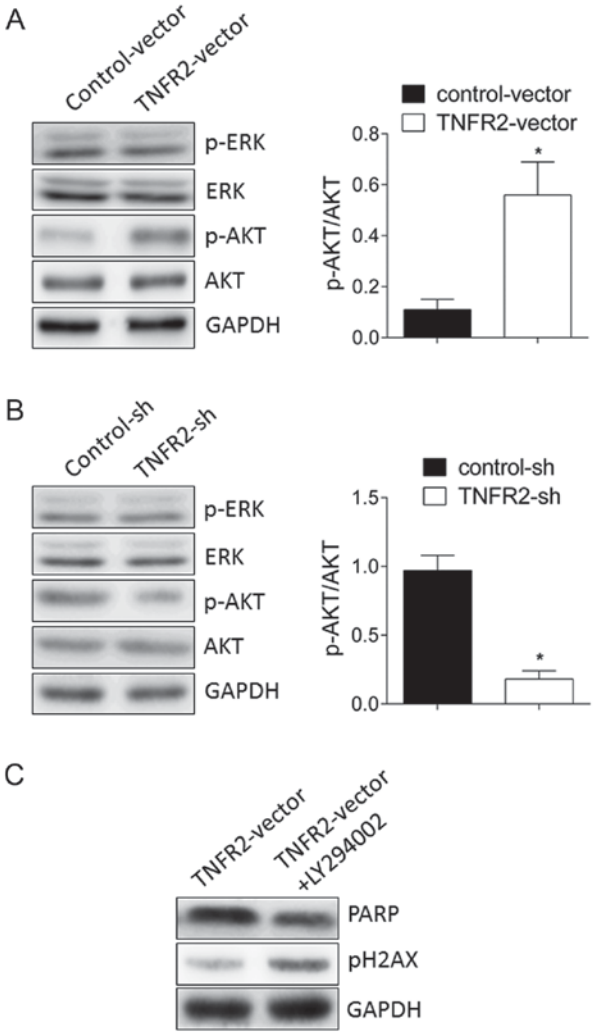

Figure 5. TNFR2 promotes PARP expression via the AKT signaling pathway. Expression levels of total and phosphorylated ERK and AKT were assessed by western blotting in (A) control and TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells, and in (B) control and TNFR2-knockdown (TNFR2-sh) MCF-7 cells. (C) Protein expression levels of PARP and pH2AX were assessed in TNFR2-overexpressing (TNFR2-vector) MDA-MB-231 cells in the absence or presence of the AKT inhibitor LY294002. GAPDH was used as a loading control. *P<0.05. TNFR2, tumor necrosis factor receptor 2; PARP, poly(ADP-ribose) polymerase; AKT, AKT serine/threonine kinase 1; ERK, extracellular signal-regulated kinase; pH2AX, phosphorylated histone family 2A variant X; p-, phosphorylated; sh, short hairpin RNA.

by regulating DNA repair proteins, we examined the expression levels of MGMT and PARP. The results demonstrated that TNFR2 overexpression in MDA-MB-231 cells significantly upregulated PARP expression, and TNFR2 silencing in MCF-7 cells significantly inhibited PARP expression, following ADM challenge. No changes were observed in MGMT expression (data not shown). pH2AX expression levels exhibited opposite trends to PARP expression levels, when TNFR2 was altered. In addition, when the PARP inhibitor ABT888 was used, no significant change was observed in pH2AX levels in TNFR2-overexpressing and control MDA-MB-231 cells following ADM treatment. Similarly, no significant change was observed in pH2AX levels in TNFR2-silenced and control MCF-7 cells following ADM treatment. These results indicated that TNFR2 inhibited DNA damage partly through PARP. However, other DNA damage repair proteins may also be required and further studies will be needed to fully explore the role of TNFR2 in DNA damage repair mechanisms.

AKT and ERK are important signaling pathways for various cellular functions, including survival, proliferation, and migration in multiple types of tumors (30-32). Yang *et al* (21) have reported that blocking TNFR2 significantly inhibited activation of AKT signaling induced by progranulin, but no change was observed in ERK phosphorylation. In the present study, TNFR2 overexpression was also demonstrated to activate AKT signaling. In addition, the AKT inhibitor LY294002 inhibited PARP expression. These results suggested that TNFR2 promoted PARP expression via AKT signaling, which is consistent to the study by Yang *et al* (21). A drug resistance assay demonstrated that both ABT888 and LY294002 treatments alone enhanced the sensitivity of MDA-MB-231 cells to ADM, and the combination treatment had a synergistic effect, suggesting that a similar combination may be beneficial for treatment of breast cancer. Of course, further studies on the potential side effects and long-term benefits for such a drug combination will be necessary for further consideration of these results in the clinic.

In conclusion, the present study demonstrated a role of TNFR2 in ADM resistance of breast cancer cells. This effect of TNFR2 was partly mediated by the induction of the DNA damage repair protease PARP via the AKT signaling pathway. The present results may enrich our understanding regarding the role of TNFR2 in breast cancer and in drug resistance and may provide novel therapy targets for breast cancer treatment.

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