

# Enhancement of the efficacy of mitomycin C-mediated apoptosis in human colon cancer cells with RNAi-based thioredoxin reductase 1 deficiency

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**Abstract.** Thioredoxin reductase 1 (Trr1) is an antioxidant and redox regulator that functions in governing the cellular redox state and survival against oxidative insults in mammals. However, this selenoprotein is also overexpressed in various forms of malignant cancers, leading to the hypothesis that Trr1 may be a potential target for cancer therapy. A quinone anticancer drug, mitomycin C (MMC), has been clinically used in the treatment of several types of tumors, including those of the colon. MMC exerts its activity via ROS induction and further results in DNA cross-linkage. To evaluate the significant role of Trr1 in MMC resistance in human colon cancer (RKO) cells, specific reduction in the expression of Trr1 was achieved using short-hairpin RNA (shRNA)-based interference. Our results showed that stable Trr1 shRNA knockdown manifested higher cellular susceptibility to MMC in comparison to that in wild-type cells. In addition, increased intracellular ROS accumulation appeared in the Trr1 shRNA knockdown cells compared to the RKO wild-type cells, in proportion to a relatively higher fraction of the DNA damage reporter protein phosphorylated histone 'γ-H2AX'. Notably, a neutral comet assay demonstrated that DNA double-strand breaks were highly induced in the Trr1-deficient cancer cells in the presence of MMC, presumably stimulating cancer cell death. Our results also revealed that MMC-induced apoptosis was associated with enhancement of oxidative damage to DNA. These results suggest that the specific knockdown of Trr1 expression via shRNA vector interference technology may be a potent molecular strategy by which to enhance the effectiveness of MMC-mediated killing in human colon cancer cells, through acceleration of double-strand DNA damage-oxidative stress as a trigger for apoptosis. This implies that Trr1 may be a prime

target for enhancing the effectiveness of MMC chemotherapy in combination with specific RNA interference.

## Introduction

Mitomycin C (MMC) is a quinone-containing antibiotic isolated from *Streptomyces caespitosus*, with a wide spectrum of antitumor activities against several tumor types, including colon, breast and head and neck (1). Notably, MMC is used as a prototype drug to study the mechanisms associated with bioreductive drug activation. This drug is reductively activated to 2,7-diaminomitosene, which cross-links DNA and subsequently leads to cell death (1,2). Apart from the mechanism of MMC-induced DNA cross-linkage, its mode of action has been also associated with the formation of DNA monoadducts and free radical-induced DNA strand breaks (3,4). However, MMC has also been associated with significant adverse side effects in cancer patients, including cardiotoxicity, hematologic toxicity and renal toxicity (5). Thus, combined cancer therapy may alternatively allow a lower dosage of MMC treatment, while ensuring its continued effectiveness in killing cancer cells.

Among the 25 selenoproteins identified in the human and 24 in rodents (6), thioredoxin reductase 1 (Trr1) is the most well-characterized selenoprotein in mammalian cells and plays an essential role in the thioredoxin system as an enzyme that governs the redox state and maintains the function of thioredoxin by keeping this protein in a reduced state (7). In mammals, Trr1 acts as a redox regulator with a major role in several biological processes, e.g., cell proliferation, transcription, angiogenesis, embryogenesis and DNA repair, as well as antioxidant defense with an antioxidant activity (8).

However, Trr1 has a deleterious effect with a role in promoting cancer as its overexpression has been manifested in many types of cancers with characteristics of malignant phenotypes, including resistance to anticancer drugs (9,10). Resistance to MMC is induced and developed in cancer cells, such as colon cancer, leading to the limitation of such chemotherapeutic agents in clinical use (11-13). A number of possible mechanisms associated with MMC resistance include an elevated level of protective agents (e.g., glutathione) and increased drug efflux.

Extensive studies have revealed that targeting Trr1 inhibition with a number of anticancer drugs and potent

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inhibitors alters cancer-related properties of malignancy (14,15). Notably, a previous study found that reduction of Trr1 in animal lung carcinoma cells caused a reversal of the characteristic malignant phenotype (16). Furthermore, animals injected with stably transfected Trr1 siRNA cells were found to exhibit a strong reduction in tumor progression and metastasis compared to control animals (16). Accordingly, a previous study found that an animal cancer cell line exhibited morphological changes upon Trr1 knockdown. These characteristic changes included loss of self-sufficient growth, defective progression to the S phase and decreased expression of DNA polymerase  $\alpha$  (17). Thus, it is worth noting that Trr1 is essential for tumor growth in animal models. It has also been discovered that Trr1 is uniquely overexpressed in cancer cells. These points may lead to the proposal that Trr1, strongly acting as a pro-cancer protein, is a potential target for anticancer therapy.

In this regard, researchers have demonstrated that the overexpression of Trr1 has a strong impact on controlling the process of malignancy (17,18). Therefore, investigation of the significant role of an endogenously high expression of selenoprotein Trr1 on MMC resistance in a human colon cancer cell line (RKO) using knockdown of Trr1 is warranted. The present study hypothesized that the knockdown of the expression level of Trr1 using a specific shRNA viral vector improves MMC efficacy in killing human colon cancer RKO cells. Importantly, we revealed that decreasing the elevated level of Trr1 expression in human colon cancer cells enhances the effectiveness of MMC-induced apoptosis, possibly via an increase in free radical-mediated double-strand damage to DNA.

## Materials and methods

**Cell culture.** RKO human colon cancer cells (ATCC no. CRL-257) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), while Trr1 shRNA knockdown cells were grown in RPMI-1640 medium containing 10% FBS and 0.5  $\mu\text{g}/\text{ml}$  puromycin. The cultures were incubated at 37°C in a humidified atmosphere of 5%  $\text{CO}_2/95\%$  air.

**Stable knockdown of Trr1 using a short-hairpin RNA vector.** The *Escherichia coli* clone, which harbors retroviral vector pSM2c containing the Trr1-specific short-hairpin RNA (shRNA) target, was provided from Open Biosystems (USA) and subjected to plasmid extraction using the HiSpeed Plasmid Midi kit (Qiagen, Germany). The purified plasmids were then submitted to sequencing to verify the Trr1-specific shRNA target (sense, CATCCCGGTGACAAAGAA and antisense, CATCCCTGGTGACAAAGAA). Stably transfected Trr1 shRNA (knockdown) cells were prepared using transfection reagent Fugen6 (Roche, Germany) according to the manufacturer's instructions. Briefly,  $5 \times 10^4$  RKO cells in a 2-ml suspension were seeded in each well of a 6-well plate. Subsequently, the 12-h cultured cells were transfected with the Trr1-specific shRNA construct using Fugen6, and selection of puromycin-resistant clones was performed in the presence of 0.5  $\mu\text{g}/\text{ml}$  puromycin after 48 h of incubation. The reduced Trr1 expression level in each puromycin-resistant clone was verified by Western blotting.

**Western blot analysis.** The Trr1 shRNA transfectant and wild-type cells were harvested by centrifugation at 1,500 rpm for 5 min and then lysed in RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1% (v/v) Triton-100, 0.1% (v/v) sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail]. Cell lysates (40  $\mu\text{g}$ ) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 60 V for 180 min and transferred onto polyvinylidene fluoride (PVDF) membranes. Blotted membranes were blocked with a blocking buffer [Tris-buffered saline (TBS), 5% skim milk, 0.02%  $\text{NaN}_3$  and 0.001% Tween-20] at 25°C for 2 h. After washing, the Trr1 protein was immunologically detected using a goat polyclonal antibody against Trr1 at a 1:2,000 dilution ratio (Santa Cruz Biotechnology, USA). A peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) was used as a secondary antibody. Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) Plus Western blotting detection system (GE Healthcare, UK). GAPDH was used as a control for protein loading. The entire procedure was performed three times independently.

**Cell viability assay.** Cell viability was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a cell proliferation kit (Roche). Briefly,  $1 \times 10^4$  cells in a 100- $\mu\text{l}$  suspension were seeded in each well of 96-well plates. After 18 h of incubation, both RKO and Trr1 shRNA knockdown cells were treated with various concentrations of MMC (0, 0.5, 1, 2.5, 5, 10 and 20  $\mu\text{M}$ ) for 1 h, following an additional 24-h incubation in fresh selective media. After incubation, MTT-labeling reagent was added at a final concentration of 0.5 mg/ml to each culture well and further incubated for 4 h. Subsequently, 200  $\mu\text{l}$  of solubilization solution was added to dissolve formazan crystal-forming products. The percentage of cell survival was quantified by measuring the absorbance at 595 nm (A595) with a microplate reader 680 (Bio-Rad, USA).

**Acridine orange staining.** The apoptotic fraction was evaluated as morphological change indicator of apoptosis using acridine orange (AO) staining. The RKO and Trr1 shRNA knockdown cells ( $6 \times 10^5$  cells in a 3-ml suspension) were seeded in 60-mm dishes. The cells were harvested after a 24-h (5  $\mu\text{M}$ ) MMC treatment, following incubation in a selective fresh media for another 24 h. The cell pellets were resuspended in AO staining solution (Sigma, USA). The stained cells were dropped onto a glass slide and observed under a fluorescence microscope (Nikon). The apoptotic fraction was determined by cell counting.

**Measurement of reactive oxygen species (ROS).** The detection of ROS was determined as a measure of intracellular accumulation of free radicals in the MMC-treated Trr1 shRNA knockdown cells compared to the RKO cells. The ROS level was evaluated using 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Molecular Probes, Eugene, OR, USA) as an ROS indicator. In brief,  $6 \times 10^5$  RKO and Trr1 shRNA knockdown cells in a 3-ml suspension were seeded in 60-mm dishes and treated without

and with 5  $\mu$ M MMC (Sigma) for 24 h, and then exposed to 0.5  $\mu$ M CM-H2DCFDA in PBS for 20 min at 37°C in the dark. Subsequently, the cells were washed with PBS and examined under a fluorescence microscope (Nikon). Image analysis was carried out with NIS-Elements BR 3.0 software (Nikon).

**$\gamma$ -H2AX detection.** The formation of phosphorylated histone H2AX ( $\gamma$ -H2AX) foci was determined using the immunofluorescence staining approach (19). Briefly, either RKO or Trr1 shRNA knockdown cells grown on glass coverslips were treated without and with 5  $\mu$ M MMC (Sigma) for 24 h, following incubation in selective fresh media for an additional 12 h. The cells were fixed with chilled methanol at -20°C for 30 min and rinsed with chilled acetone for a few seconds. After washing with PBS, the cells were incubated with an anti- $\gamma$ -H2AX primary antibody (Active Motif, USA) at 4°C overnight and an anti-rabbit-cy3 secondary antibody (Jackson ImmunoResearch, USA) at room temperature for 1 h. After washing with PBS, the cells were mounted with mounting solution containing DAPI nuclear stain. The coverslips were placed face-down onto glass slides, and the foci were visualized under fluorescence microscope (Nikon).

**Neutral single-cell gel electrophoresis (SCGE or comet assay).** The comet assay under neutral conditions mainly allows the detection of DNA double-strand breaks which basically trigger apoptotic processes, but not necrotic events (20,21). In brief, the RKO and Trr1 shRNA knockdown cells ( $6 \times 10^5$  cells in a 3-ml suspension) were seeded in 60-mm dishes. After 24 h of 5  $\mu$ M MMC treatment following an additional 24-h of fresh media incubation, the cells were harvested by trypsinization, resuspended in 1 ml resuspending buffer [Hanks' Balanced Salt Solution (HBSS), 20 mM EDTA and 10% DMSO freshly added 1 h prior to use] and chilled on ice. Cells ( $1 \times 10^4$  in 10  $\mu$ l) were embedded in 90  $\mu$ l low-melting point agarose (0.5% in PBS at 37°C) onto agarose-coated (1.0% in PBS) and dried glass slides that were submerged for 1 h in pre-chilled lysis buffer pH 9.5 [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base; 1 h prior to use 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulphoxide (DMSO) were added]. Slides were electrophoresed at 25 V for 1 h at 4°C in electrophoresis buffer pH 9.0 [100 mM Tris-HCl (pH 9.0) and 300 mM sodium acetate]. Afterwards, DNA precipitation was carried out in 90% ethanol containing 1 M ammonium acetate at 4°C for 30 min. After ethanol dehydration, the slides were subjected to air drying and then SYBR Gold staining (1:10,000 dilution ratio in TE buffer, pH 7.6) (Invitrogen, USA). At this step, the slides could be stored in a refrigerator in light-tight boxes without any loss of assay sensitivity. Finally, the stained DNA was visualized under a fluorescence microscope (Nikon). One hundred cells per slide were scored.

## Results

**Induced cellular sensitivity via Trr1 inhibition under MMC treatment.** Trr1 was targeted for knockdown in the RKO cells by transfection using a Trr1-specific shRNA retroviral vector. The decreased protein level of Trr1 was confirmed by Western blotting. As shown in Fig. 1A, the protein level of Trr1 was markedly reduced in the Trr1 shRNA transfectant

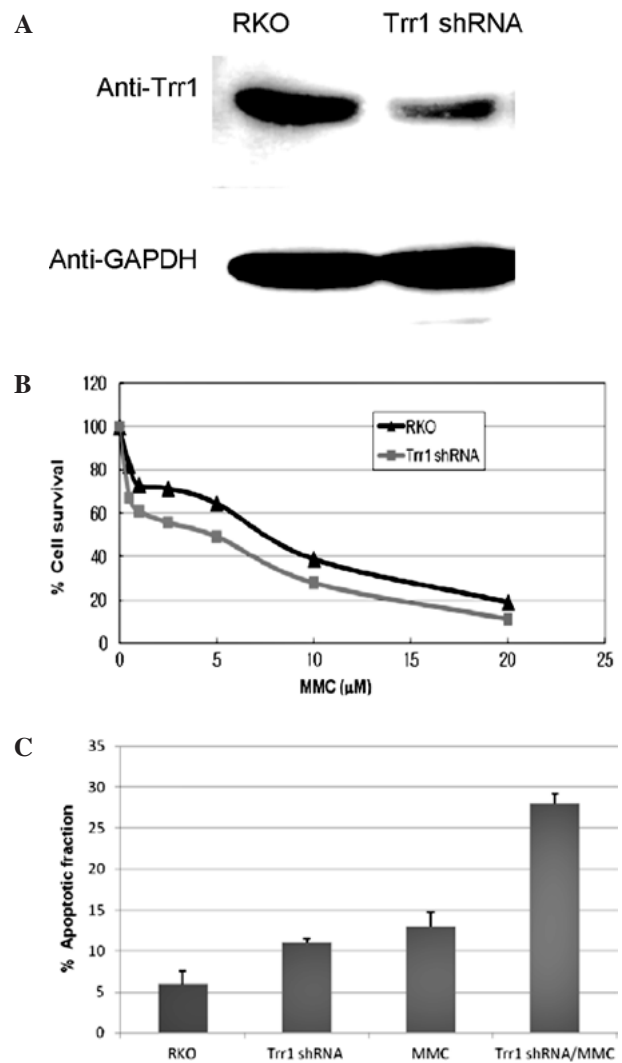


Figure 1. Cell viability and apoptosis following MMC treatment. (A) Stable Trr1 knockdown in RKO cells was successfully achieved by specific shRNA viral vector transfection. The specific Trr1 knockdown was verified by Western blotting in stable Trr1 shRNA-expressing RKO cells. (B) Cell viability was evaluated by an MTT assay. After a 1-h exposure to increasing concentrations of MMC (0-20  $\mu$ M) following a 24-h incubation in fresh media, the Trr1 shRNA knockdown cells exhibited higher sensitivity to MMC in comparison to wild-type RKO cells. (C) Apoptotic body formation was detected by AO staining. After a 24-h exposure to 5  $\mu$ M MMC following 24 h of incubation in fresh media, MMC-induced apoptosis was significantly enhanced in the Trr1 shRNA knockdown cells relative to wild-type RKO cells. The apoptotic fraction was quantified by cell counting.

cells relative to the wild-type RKO cells, while there was apparently no difference in the expression level of the internal control GAPDH between the Trr1 shRNA transfectant and the wild-type RKO cells. This indicated that suppression of Trr1 expression via shRNA transfection in the colon cancer RKO cells was successfully conducted.

Highly expressed Trr1 is noted in many cancer types with malignant phenotypes, including resistance to anticancer drugs (9,10). This raises the question whether Trr1 suppression via shRNA leads to enhanced MMC-mediated cytotoxicity in colon cancer RKO cells. An MTT assay was performed to evaluate cell viability towards MMC. The result showed that the Trr1 shRNA knockdown cells displayed higher sensitivity to MMC treatment compared to the wild-type RKO cells (Fig. 1B). This suggests that Trr1 may be a potent mediator for

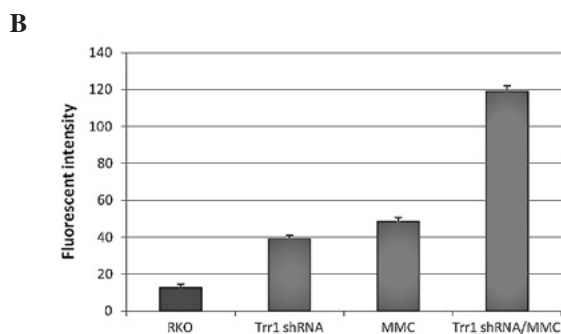
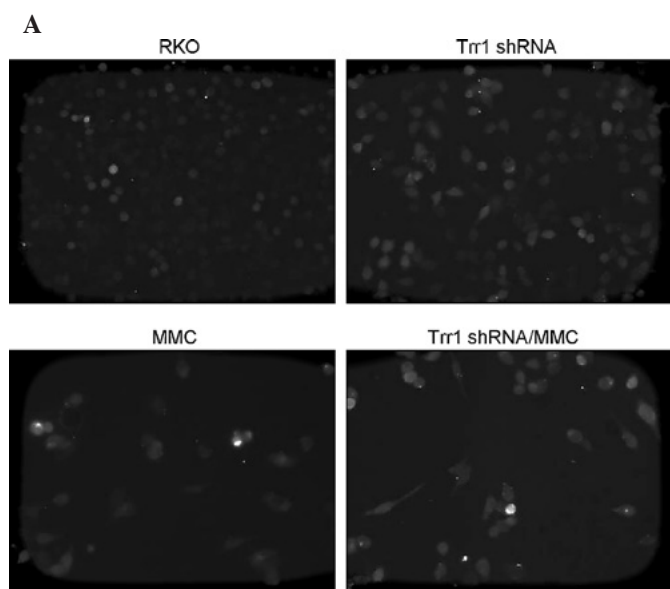


Figure 2. An increase in intracellular ROS in response to MMC. (A) The intracellular level of ROS was measured by H2DCFDA staining. After a 24-h exposure to 5  $\mu$ M MMC, the Trr1 shRNA knockdown cells significantly exhibited increased fluorescence compared to that of the wild-type RKO cells. This indicated the intracellular accumulation of ROS, which is a potent mediator of apoptosis. (B) Average fluorescence intensity of the H2DCFDA-stained cells was quantified.

cell survival caused by MMC toxicity in colon cancer RKO cells. Additionally, the Trr1 shRNA knockdown cells also showed a higher rate of MMC-induced apoptosis, relative to the wild-type cells (Fig. 1C). This implies that the inhibition of Trr1 expression is potentially effective for inducing apoptosis in colon cancer RKO cells, as a tentative approach to enhance the antitumor effects of MMC treatment.

#### *Increased ROS level with Trr1 deficiency in response to MMC.*

As MMC is one of the quinone anticancer agents to generate free radicals (22,23), enhancement of the ROS level in the Trr1-deficient cells in comparison to wild-type RKO cells was assessed. The intracellular ROS was detected using fluorescent probe CM-H2DCFDA. Exposure to 5  $\mu$ M MMC for 24 h induced the generation of intracellular ROS to a greater extent in the Trr1 shRNA knockdown cells as compared to that in the wild-type RKO cells (Fig. 2). This implicates the intracellular accumulation of ROS in the Trr1 shRNA knockdown. Therefore, Trr1 deficiency may be a potent mediator by which to sensitize colon cancer RKO cells to MMC treatment via enhancement of ROS-mediated oxidative damage.

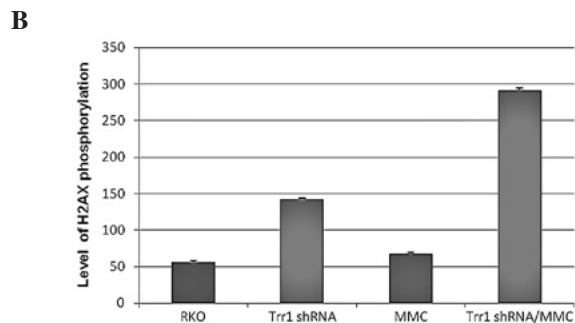
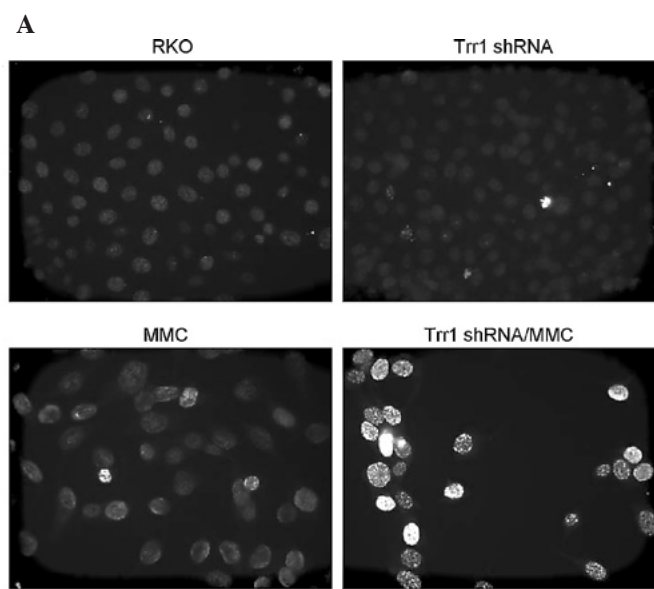


Figure 3. Augmentation of MMC-induced DNA damage. (A) The formation of nuclear foci was determined by  $\gamma$ -H2AX immunofluorescence staining. After 12 h of incubation in fresh media after a 24-h (5.0  $\mu$ M) MMC exposure, Trr1 shRNA knockdown cells exhibited a higher focus formation compared to that of the wild-type RKO cells. The formation of fluorescent  $\gamma$ -H2AX foci was observed under a fluorescence microscope. (B) Analysis of fluorescent foci was carried out using a Nikon Eclipse 50i microscope. The relative fluorescence signal of the immunostained cells was representative of the degree of DNA double-strand damage.

*Elevated  $\gamma$ -H2AX foci as a biomarker of DNA damage in Trr1 knockdown cells upon MMC exposure.* DNA is a target molecule for underlying molecular mechanisms of MMC toxicity in antitumor therapy (24,25). Accordingly, the accumulation of intracellular ROS generating oxidative damage to cellular components, particularly DNA, was observed in the Trr1 shRNA knockdown cells (Fig. 2). Hence, MMC-induced DNA damage in Trr1 shRNA knockdown cells was examined in comparison to wild-type RKO cells using  $\gamma$ -H2AX immunostaining. Phosphorylated nuclear histone protein variant H2AX ' $\gamma$ -H2AX' causes recruitment of DNA repair protein complexes at sites flanking DNA strand breaks (19). As shown in Fig. 3, the Trr1 shRNA knockdown cells exhibited a marked increase in DNA strand breaks compared to that in the wild-type RKO cells after MMC treatment.

*Increased DNA double-strand breaks via Trr1 inhibition under MMC treatment.* In addition to the  $\gamma$ -H2AX staining, a neutral comet assay was also utilized to evaluate the relative occurrence of DNA double-strand breaks at the individual cell

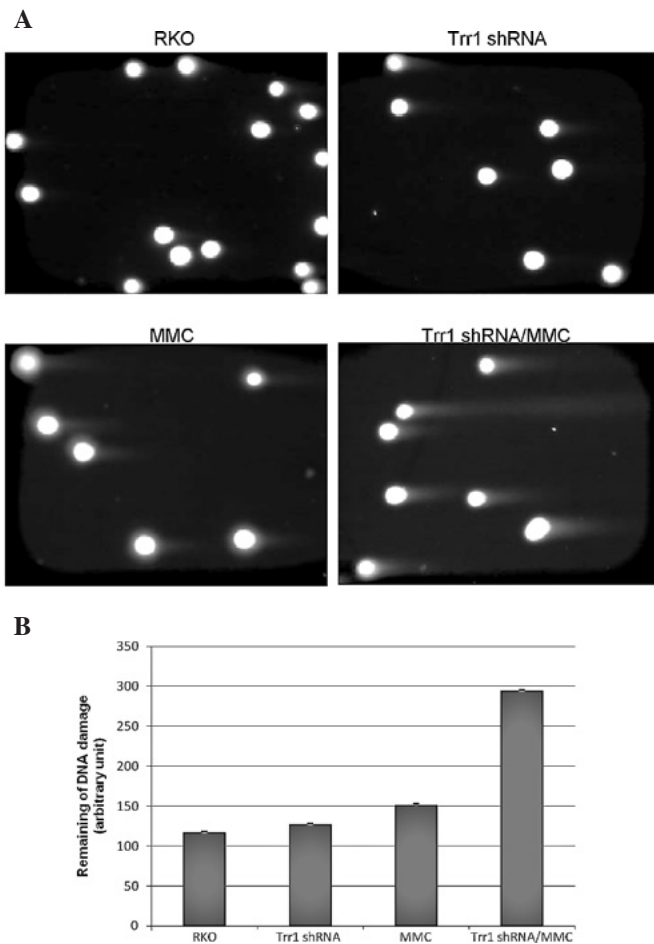


Figure 4. Induction of DNA double-strand breaks after MMC exposure. (A) DNA double-strand breaks were specifically evaluated as DNA fiber extension with separated fragments by a neutral comet assay. After 12 h of incubation in fresh media after a 24-h ( $5.0 \mu\text{M}$ ) MMC exposure, Trr1 shRNA knockdown cells exhibited a higher degree of DNA damage as shown by comet tail intensity compared to that of the wild-type RKO cells. (B) The DNA damage of the SYBR Gold-stained cells was quantified and remained average.

level. After MMC exposure, the Trr1 shRNA knockdown cells showed significantly increased DNA double-strand breaks, represented by a much greater extent of stretched DNA, compared to the wild-type RKO cells (Fig. 4).

The results overall demonstrated that Trr1 suppression sensitizes colon cancer RKO cells to MMC exposure through augmentation of DNA double-strand damage, probably leading to enhanced cell death.

## Discussion

The potent antitumor agent, MMC, is extensively used against several tumor types, such as colon and breast (2,26). MMC is capable of inducing ROS production, leading to enhanced cancer apoptosis via DNA strand damage (27). However, the development of MMC resistance in colon cancer cell lines is an obstacle for cancer therapy (12,13). In addition to cancer prevention, Trr1 has an opposing role in promoting cancer, as Trr1 expression has been shown to be elevated in primary human malignancies arising in the breast, thyroid, prostate and liver, as well as a number of human cancer cell lines

(9,28). Therefore, the potential association of increased Trr1 and MMC resistance in cancer can be postulated.

One significant finding of this study is the initial demonstration that a reduction in the expression of selenoprotein Trr1 potentiates MMC action on cancer apoptosis through ROS-induced DNA double-strand damage in human colon cancer RKO cells. In the present study, stable down-regulated expression of Trr1 was performed in RKO cells using shRNA vector-based interference technology (Fig. 1A). Our observation revealed that stable Trr1-deficient cells exhibited a higher intracellular ROS level compared to wild-type RKO cells after 24 h ( $5 \mu\text{M}$ ) of MMC exposure, implying that Trr1 deficiency enhances MMC-induced ROS (Fig. 2).

To subsequently investigate whether there is an association between MMC-induced oxidative stress and DNA damage in the Trr1 shRNA knockdown cells following MMC treatment, the DNA strand breaks were determined using  $\gamma\text{-H2AX}$  staining and a neutral comet assay. Upon MMC exposure, the DNA strand breaks became significantly increased in the Trr1 knockdown cells relative to that in the wild-type cells (Figs. 3 and 4). Accordingly, the extent of DNA damage was also correlated with the level of generated ROS as an oxidative stress indicator (Figs. 2, 3 and 4). This demonstrated that the elevated expression of Trr1 in the human colon cancer RKO cells facilitated cellular protection against oxidative damage to DNA. Taken together, the antitumor property via Trr1 suppression is apparently attributed to the stimulation of the ROS-mediated DNA damage process, particularly DNA double-strand breakage (Fig. 4).

Since Trr1 is recognized as an anticancer drug target, recent study has focused on the importance of Trr1 in drug-specific cytotoxic efficacy in response to various anticancer agents in the human lung carcinoma A549 cell line expressing a very high basal level of Trr1 (29). In accordance with these observations, our results showed for the first time that cellular sensitivity to MMC was dramatically increased in the Trr1 shRNA knockdown cells relative to the wild-type RKO cells (Fig. 1B) and consequently led to induction of cancer apoptosis (Fig. 1C). One strong possible explanation why shRNA inhibition of Trr1 gave rise to the induction of MMC-mediated cytotoxicity and concomitant apoptosis is the enhancement of DNA damage caused by an increase in ROS generation (Figs. 2, 3 and 4). Our data first demonstrated that suppression of Trr1 accelerated intracellular accumulation of DNA double-strand breaks in the presence of MMC, strongly evidenced by the neutral comet assay (Fig. 4). This suggested that specific knockdown of Trr1 enhanced apoptosis of cancer cells treated with MMC by stimulation of DNA double-strand damage. In accordance with a supportive study, DNA double-strand breaks are the most severe lesions and lead to cell lethality (30).

In conclusion, our observations provide compelling evidence that a specific reduction in Trr1 effectively sensitizes human colon cancer RKO cells to MMC treatment. MMC treatment combined with Trr1 deficiency may limit the side effects noted in normal cells, while enhancing the effectiveness against cancer cells when compared with the efficacy of classical MMC treatment. We found, for the first time, that the enhancement of MMC-mediated apoptosis in the Trr1 shRNA knockdown cells may result from an

increase in ROS-generated DNA double-strand breaks. Therefore, inhibition of Trr1 via shRNA interference may be a promising strategy for improving the MMC killing efficacy in cancer therapy.

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