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Down-regulation of MSH2 expression by Hsp90 inhibition enhances cytotoxicity affected by tamoxifen in human lung cancer cells

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ABSTRACT

The anti-estrogen tamoxifen has been used worldwide as an adjuvant hormone therapeutic agent in the treatment of breast cancer. However, the molecular mechanism of tamoxifen-induced cytotoxicity in non-small cell lung cancer (NSCLC) cells has not been identified. Human MutS homolog 2 (MSH2), a crucial element of the highly conserved DNA mismatch repair system, and expression of MSH2 have been down-regulated by Hsp90 function inhibition in human lung cancer. Therefore, in this study, we examined whether MSH2 plays a role in the tamoxifen and Hsp90 inhibitor-induced cytotoxic effect on NSCLC cells. The results showed that treatment with tamoxifen increased MSH2 mRNA and protein levels. The combination treatment with PI3K inhibitors (LY294002 or wortmannin) or knockdown AKT expression by specific small interfering RNA could decrease tamoxifen-induced MSH2 expression. Both knocking down MSH2 expression and co-treatment of PI3K inhibitors enhanced the cytotoxicity and cell growth inhibition of tamoxifen. Compared to a single agent alone, tamoxifen combined with an Hsp90 inhibitor resulted in cytotoxicity and cell growth inhibition synergistically in NSCLC cells, accompanied with reduced MSH2 expression. These findings may have implications for the rational design of future drug regimens incorporating tamoxifen and Hsp90 inhibitors for the treatment of NSCLC.

1. Introduction

Tamoxifen, an estrogen receptor (ER) antagonist, has been used as therapy against breast cancer [1]. However, it also has been confirmed that tamoxifen could promote cell death in non-small cell lung cancer (NSCLC) [2]. A close relationship between estrogen and the risk of NSCLC cancers has been verified recently in a series of prospective studies [2–4]. However, the presence of ERs in human lung tumor cells has been controversial. Rather than ERα, ERβ is expressed in the major part of NSCLC cells [5]. Some studies indicated that estrogen contributes greatly to both the genesis and development of NSCLC by either triggering cell proliferation or inhibiting apoptosis; this is generally similar to the finding in breast cancers [6,7]. However, the detailed mechanism of the induction of a cytotoxic effect by tamoxifen in NSCLC is still unclear.

Human MutS homolog 2 (MSH2), a crucial element of the highly conserved DNA mismatch repair (MMR) system, maintains genetic integrity by correcting DNA replication errors [8]. The function of MSH2 is to recognize DNA mismatches and then help recruit DNA repair proteins to the mismatched site [8]. A previous study indicated that MSH2 is one of the promising markers of the benefit of adjuvant cisplatin-based chemotherapy for NSCLC [9]. However, whether MSH2 also plays a role in the tamoxifen-induced cytotoxic effect in NSCLC cells needs further examination.

Heat shock protein 90 (Hsp90), a molecular chaperone, is an exciting new target in cancer therapy, and is required for conformational folding and maintaining the stability of numerous client proteins, including the proper folding and intracellular disposition of multiple proteins involved in cell signaling and survival [10,11]. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) can interfere with the function of Hsp90 [12] through binding to a conserved ATP interaction pocket of the NH_2-terminal domain [13]. Previous studies have indicated that Hsp90 inhibition can trigger the destabilization and degradation of client proteins such as AKT, Raf-1, cyclin D and ErbB [14]. The phosphatidylinositol 3′-kinase (PI3K)-AKT signal has been proved to be a therapeutic target for tamoxifen-resistant breast cancer cells [15].

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Based on previous studies, we wanted to examine the interactions between tamoxifen and the Hsp90 antagonist 17-AAG in human NSCLC cells in relation to effects on MSH2 expression and cytotoxic effects. We proposed a novel mechanism of a combination of tamoxifen and Hsp90 inhibitor that can synergistically inhibit cell proliferation and reduce cell viability, both of which are correlated with down-regulation of AKT-mediated MSH2 expression in A549 and H1703 cells.

2. Materials and methods

2.1. Chemicals, cells, and cell culture

Tamoxifen, 17-AAG, actinomycin D, and cycloheximide were purchased from Sigma Chemical (St. Louis, MO). LY294002 and wortmannin were purchased from Calbiochem–Novabiochem (San Diego, CA, USA). Human lung carcinoma cells A549 and H1703 were obtained from the American Type Culture Collection (Manassas, VA) and the cells were cultured at 37° C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal calf serum (10%).

2.2. Western blot analysis

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described [16]. The specific phospho-AKT (Ser^{473}) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against MSH2 (N-20) (sc-494), AKT (H-136) (sc-8312), ERβ (H-150) (sc-8974), and actin (I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Transfection with AKT-CA vector or small interfering RNA

Exponentially growing human lung cancer cells (10^6) were plated for 18 h, and then constitutively active AKT expression plasmid (AKT-CA), which harbored a consensus myristylation domain that replaced the 4–129 amino acids of wild-type AKT, were transfected into cells using Lipofectamine (Invitrogen). Cells were transfected with siRNA duplexes (200 nM) by using Lipofectamine 2000 (Invitrogen) for 24 h.

2.4. Quantitative real-time polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed using an ABI Prism 7900HT according to the manufacturer's instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). For each sample, data was normalized to the housekeeping gene GAPDH.

2.5. MTS assay

Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 μL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 2 h, and the absorbance was measured at 490 nM using an ELISA plate reader (Biorad Technologies, Hercules, CA).

2.6. Combination index analysis

The cytotoxicity induced by the combined treatment with tamoxifen and/or 17-AAG was compared with the cytotoxicity induced by each drug using the combination index (*CI*), where $CI < 0.9$, $CI = 0.9–1.1$, and $CI > 1.1$ indicated synergistic, additive, and antagonistic effects, respectively. The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK).

2.7. Trypan blue dye exclusion assay

Cells were treated with tamoxifen and/or 17-AAG for 24 h. Trypan blue dye can be excluded from living cells, but is able to penetrate dead cells. The proportion of dead cells was determined by hemocytometer, counting the number of cells stained with trypan blue.

2.8. Statistical analyses

For each protocol, 3 or 4 independent experiments were performed. Results were expressed as the mean ± SEM. Statistical calculations were performed using SigmaPlot 2000 software (Systat Software, San Jose, CA). Differences in measured variables between the experimental and control groups were assessed by unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Tamoxifen increased MSH2 protein and mRNA expression in an AKT activation-dependent manner

To investigate if tamoxifen had any effect on MSH2 expression, we first assessed A549 or H1703 cells treated with tamoxifen (0.5–5 μM) for 24 h, and real-time PCR was used for determination of the MSH2 mRNA level. The protein levels of MSH2 were determined by Western blot analysis. Tamoxifen increased MSH2 mRNA and protein expression and was accompanied with an increase in phospho-AKT protein levels (Supplementary Fig. 1A and B).

Next, we proposed that the activation of AKT was involved in the up-regulation of MSH2 expression in tamoxifen-exposed A549 and H1703 cells. These cell lines were pretreated with PI3K inhibitors (LY294002 or wortmannin), and the MSH2 protein and mRNA levels in tamoxifen-exposed A549 or H1703 cells could be decreased (Fig. 1A–D). Also, knockdown of AKT expression by specific si-AKT RNA decreased MSH2 protein and mRNA levels in tamoxifen-exposed A549 and H1703 cells (Fig. 1E and F). However, enforced expression of constitutive AKT (AKT-CA) vectors in A549 and H1703 cells could enhance cellular MSH2 protein and mRNA expression (Fig. 1G and H). Of interest, knockdown of ERβ expression decreased the cellular and tamoxifen-elicited MSH2 protein and mRNA levels in A549 and H1703 cells (Fig. 1I and J). Therefore, we suggested that tamoxifen increased MSH2 expression was correlated with AKT activation and ERβ expression.

3.2. Knockdown of MSH2 enhanced the cytotoxicity and growth inhibition in tamoxifen-exposed NSCLC cells

We next examined the effect of siRNA-mediated MSH2 knockdown on tamoxifen-induced cytotoxicity and cell growth inhibition in NSCLC cells. At 24 h post-transfection, real-time RT-PCR and Western blot analysis showed a decrease in MSH2 mRNA and protein in tamoxifen-treated A549 and H1703 cells; however, tamoxifen-induced AKT activation was not affected (Fig. 2A and B). Furthermore, suppression of MSH2 expression by si-MSH2 RNA resulted in increased sensitivity to tamoxifen,

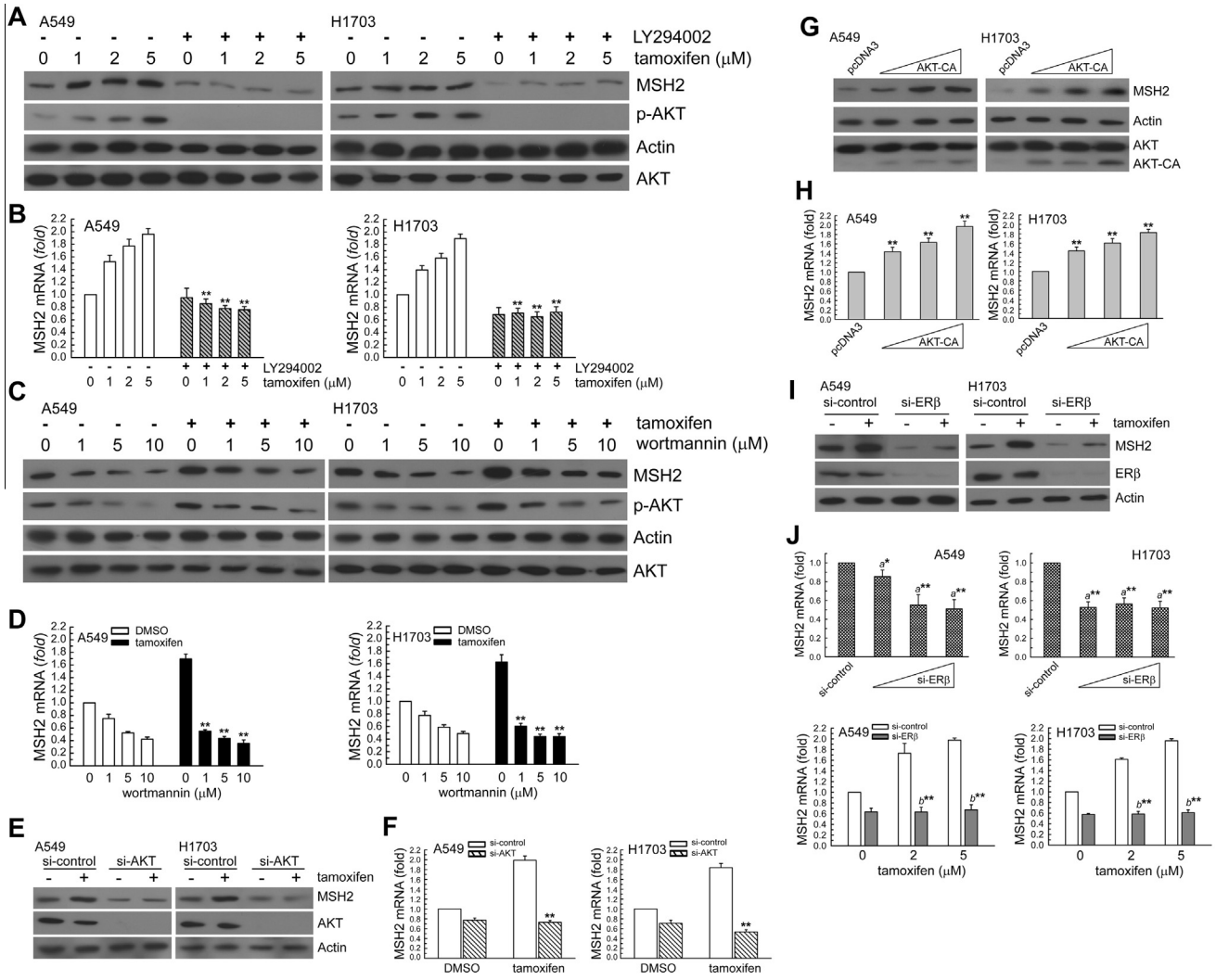

Fig. 1. Tamoxifen increased MSH2 expression via AKT activation in NSCLC cells. (A and B) LY294002 (5 μM) was added to A549 or H1703 cells for 1 h before tamoxifen treatment for 24 h. The results (mean ± SEM) were from 3 independent experiments. (C and D) Wortmannin was added to cells for 1 h before tamoxifen (5 μM) treatment for 24 h. The results (mean ± SEM) were from 3 independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with tamoxifen–DMSO or a tamoxifen–LY294002/wortmannin combination. After treatment, the cell extracts were examined by Western blot (A, C) and real-time PCR (B, D) for determination of MSH2 protein and mRNA levels, respectively. (E and F) A549 or H1703 cells were transfected with si-AKT RNA. After incubation for 24 h, the cells were treated with 5 μM tamoxifen for 24 h. After treatment, the cell extracts were examined by Western blot and real-time PCR for determination of MSH2 protein and mRNA levels, respectively. The results (mean ± SEM) were from 3 independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with tamoxifen in si-AKT RNA or si-scrambled RNA-transfected cells. (G and H) A549 or H1703 cells (5×10^5) were transfected with 1, 2, 5 μg of AKT-CA vectors. After incubation for 24 h, the cell extracts were examined by Western blot and real-time PCR for determination of MSH2 protein and mRNA levels, respectively. $^{**}p < 0.01$ using Student's *t*-test for comparison between cells transfected with pcDNA3 or AKT-CA vector. (I and J) The cells were transfected with siRNA duplexes (25, 50, 100 nM) specific to ERβ or scrambled (control) in complete medium for 24 h prior to treatment with tamoxifen (5 μM) in complete medium for 24 h; the cell extracts were examined by Western blot and real-time PCR for determination of MSH2 protein and mRNA levels, respectively. $^{a**}p < 0.01$, $^{*}p < 0.05$ using Student's *t*-test for comparison between cells transfected with si-ERβ RNA or si-scrambled RNA. $^{b**}p < 0.01$ using Student's *t*-test for comparison between cells treated with tamoxifen in si-ERβ RNA or si-scrambled RNA-transfected cells.

compared to si-control transfected cells (Fig. 2C). More inhibition of cell growth was induced by the combination of MSH2 siRNA and tamoxifen than by tamoxifen alone in A549 or H1703 cells (Fig. 2D). Therefore, down-regulation of MSH2 expression increased the cytotoxicity and growth inhibition in tamoxifen-exposed NSCLC cells.

3.3. Blocking AKT activation enhanced tamoxifen-induced cytotoxicity and growth inhibition

Next, the role of AKT and ERβ in the cytotoxic effect of tamoxifen was examined. In Fig. 2E and F, co-treatment with LY294002 or wortmannin significantly decreased cell viability in tamoxifen-exposed A549 or H1703 cells, compared with tamoxifen treatment alone. Both LY294002 and wortmannin could enhance growth inhibition individually after treatment with tamoxifen (Fig. 2G). Moreover, knockdown ERβ expression enhanced the sensitivity of tamoxifen in both A549 and H1703 cells (Fig. 2H). Taken together, inactivation of the PI3K-AKT-MSH2 signals could enhance tamoxifen-induced cytotoxicity and growth inhibition in NSCLC cells.

3.4. Combination treatment with 17-AAG enhanced the cytotoxic effect and growth inhibition of tamoxifen

Down-regulation of AKT has been associated with administration of Hsp90 antagonists, and recent evidence suggests that 17-AAG might cooperate to antagonize tumor cell survival by inhibiting the AKT-MSH2 pathway. To test this possibility, we

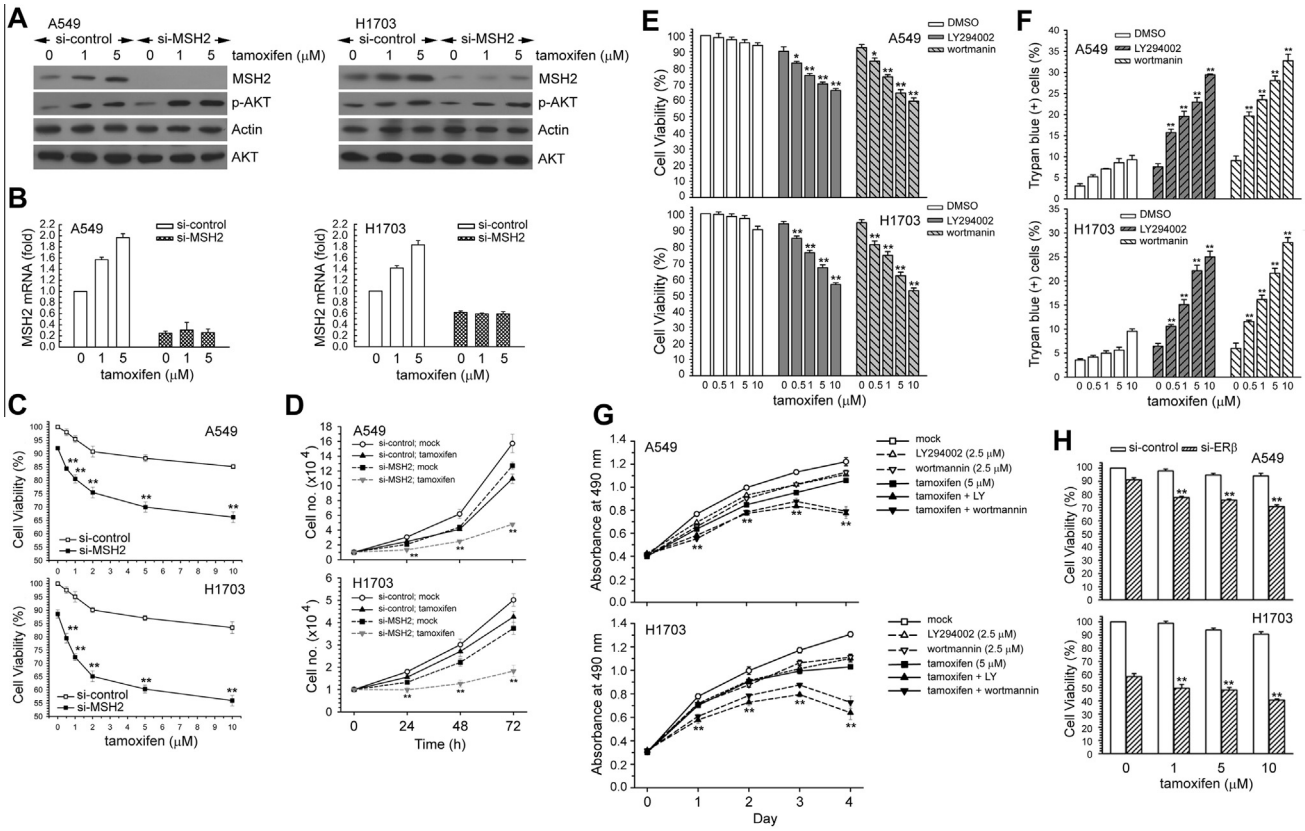

Fig. 2. Knockdown of MSH2 expression by si-RNA transfection enhanced the cytotoxicity induced by tamoxifen. (A and B) Cells were transfected with siRNA duplexes (200 nM) specific to MSH2 or scrambled (control) in complete medium for 24 h prior to treatment with tamoxifen (1 or 5 μM) in complete medium for 24 h; total RNA was isolated and subjected to real-time PCR for MSH2 mRNA expression (A). Whole-cell extracts were collected for Western blot analysis using specific antibodies against MSH2, phospho-AKT, AKT, and actin (B). (C) After the above-mentioned treatment, cytotoxicity was determined by MTS assay. (D) After the cells were transfected with si-MSH2 or si-scrambled RNA, the cells were treated with tamoxifen (5 μM) for 24, 48, and 72 h, after which living cells were determined by trypan blue exclusion assay. The results (mean ± SEM) were from 3 independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between cells treated with tamoxifen in si-MSH2 RNA or si-scrambled RNA-transfected cells. (E and F) A549 or H1703 cells were pretreated with LY294002 (5 μM) or wortmaninn (5 μM) for 1 h and then co-treated with tamoxifen for 24 h. Cytotoxicity was determined by the MTS assay (E) and trypan blue exclusion assay (F). $^{**}p < 0.01$ using Student's *t*-test for comparison between cells pretreated with or without LY294002/wortmaninn in tamoxifen-exposed cells. (G) Cells were treated with tamoxifen (5 μM) and/or LY294002/wortmaninn (2.5 μM) for 1–4 days, after which living cells were determined by MTS assay. $^{**}p < 0.01$ using the Student's *t*-test for comparison between cells treated with tamoxifen alone or with a tamoxifen and LY294002/wortmaninn combination. (H) A549 or H1703 cells were transfected with siRNA duplexes (200 nM) specific to ERβ or scrambled (control) in complete medium for 24 h prior to treatment with tamoxifen in complete medium for 24 h; cytotoxicity was determined by the MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with tamoxifen in si-ERβ RNA or si-scrambled RNA-transfected cells.

examined interactions between 17-AAG and tamoxifen in human NSCLC cells, emphasizing their effects on cytotoxicity of NSCLC cells. 17-AAG and tamoxifen were combined at a ratio of 1:10 and MTS assay was used to analyze cell viability. Combined treatment with 17-AAG and tamoxifen for 24 h resulted in a greater loss of cell viability in A549 and H1703 cells than treatment with either 17-AAG or tamoxifen alone (Fig. 3A). The *CI* values for 17-AAG and tamoxifen were <1, indicating the combination treatment had a synergistic effect (Fig. 3B). In addition, A549 and H1703 cells were exposed to 17-AAG and/or tamoxifen, and cell proliferation was determined 1–4 days after exposure to the drugs. 17-AAG and tamoxifen co-treatment had a greater cell growth inhibition effect than either treatment alone (Fig. 3C). The results showed that combined 17-AAG and tamoxifen has a synergistic cytotoxic effect on human NSCLC cells.

3.5. 17-AAG enhanced down-regulation of MSH2 protein and the mRNA level in tamoxifen-treated human lung cancer cells

In order to assess the mechanism of the synergistic effects, we hypothesized that 17-AAG would affect MSH2 expression. To test this hypothesis, A549 and H1703 cells were exposed to various concentrations of tamoxifen and 17-AAG for 24 h. 17-AAG suppressed the phospho-AKT and MSH2 protein levels in tamoxifen-exposed NSCLC cells (Supplementary Fig. 2A). Moreover, the results from real-time PCR analysis showed that 17-AAG decreased tamoxifen-induced MSH2 mRNA levels in A549 and H1703 cells (Supplementary Fig. 2B). Next, we examined the possible mechanisms for post-transcriptional regulation of MSH2 transcripts under tamoxifen and/or 17-AAG treatment. To evaluate the stability of MSH2 mRNA in tamoxifen and/or 17-AAG-exposed A549 or H1703 cells, we treated these cells with actinomycin D to block *de novo* RNA synthesis and then measured the levels of existing MSH2 mRNA using quantitative real-time PCR. After actinomycin D co-exposure, lower levels of MSH2 mRNA were observed after 17-AAG and tamoxifen treatment than in tamoxifen-alone cells (Supplementary Fig. 2C). Then, cycloheximide (an inhibitor of *de novo* protein synthesis) was added to tamoxifen alone or tamoxifen-17-AAG combination for 4, 8, and 12 h, and the remaining MSH2 protein was analyzed by Western blot. 17-AAG enhanced the MSH2 protein instability in tamoxifen-exposed A549 and H1703 cells (Supplementary Fig. 2D). Therefore, 17-AAG triggered

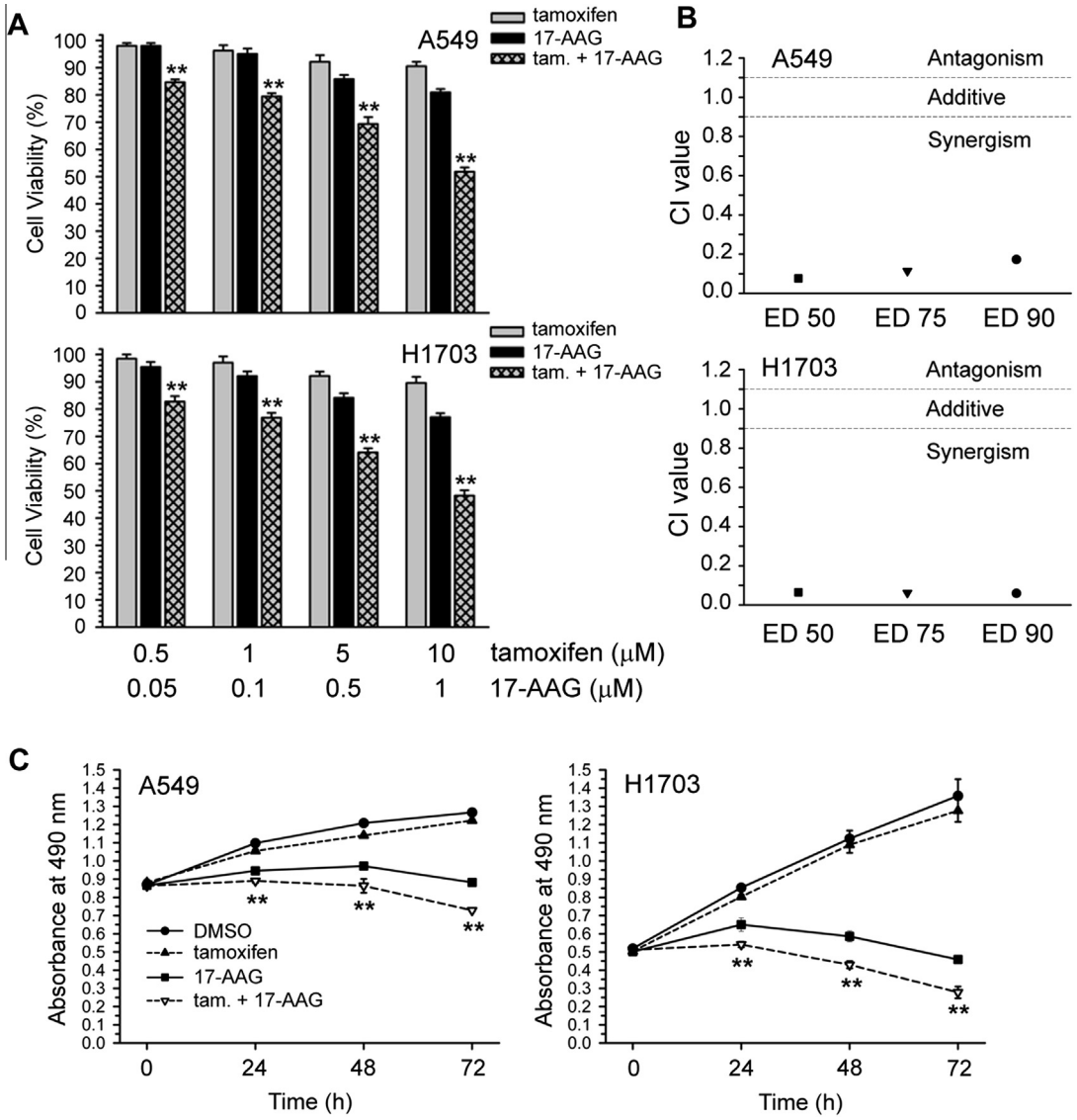

Fig. 3. 17-AAG co-treatment with tamoxifen synergistically enhanced cytotoxicity. (A) 17-AAG and tamoxifen were combined at a ratio of 1:10. After treatment for 24 h, the MTS assay was used to analyze cell viability. (B) The mean CI values at a fraction affected (FA) of 0.50, 0.75, 0.90 for tamoxifen and 17-AAG combined treatment were averaged for each experiment and used to calculate the mean between experiments. Points and columns, mean values obtained from 3 independent experiments; bars, standard error (SE). (C) Cells were treated with tamoxifen (1 μM) and/or 17-AAG (0.2 μM) for 24, 48, and 72 h, after which living cells were determined by MTS assay. $^{**}p < 0.01$ using the Student's *t*-test for comparison between cells treated with a drug alone or with a tamoxifen/17-AAG combination.

down-regulation of MSH2 expression in tamoxifen-exposed cells through increased mRNA and protein instability.

3.6. Transfection with AKT-CA or MSH2 vectors enhanced the MSH2 protein level as well as the cell survival suppressed by 17-AAG and tamoxifen

We used enforced expression of the AKT-CA vector in A549 and H1703 cells to gain insights into the functional role of the PI3K-AKT pathways in tamoxifen and 17-AAG-mediated lethality. Overexpression of AKT-CA could rescue the cellular MSH2 protein and mRNA levels that were suppressed by 17-AAG and tamoxifen (Fig. 4A and B). In addition, AKT-CA or MSH2 expression vectors transfection could rescue A549 and H1703 cell viability after being decreased by 17-AAG and tamoxifen (Fig. 4C–F). These results indicated that 17-AAG decreased MSH2 mRNA and protein levels through AKT inactivation, and thus enhanced the cytotoxicity of tamoxifen in A549 or H1703 cells.

4. Discussion

This study provides new insight into the mechanism of Hsp90 inhibition in down-regulating the expression of MSH2 to enhance the cytotoxic effect of tamoxifen in NSCLC cells. The results showed that tamoxifen treatment increased the protein levels of phospho-AKT and MSH2 in A549 and H1703 cells. The previous study indicated that the inhibition of PI3K-AKT enhanced tamoxifen-induced cell apoptosis in malignant gliomas [17]. In our previous study, tamoxifen combined with erlotinib resulted in cytotoxicity and cell growth inhibition synergistically in NSCLC cells, accompanied with reduced activation of phospho-AKT protein levels [18]. In this study, inhibition of AKT activity and MSH2 expression increased the tamoxifen-mediated cytotoxicity in A549 and H1703 cells.

ERβ is expressed in a major part of NSCLC cells [5]. In this study, knockdown of ERβ expression decreased the tamoxifen-elicited MSH2 expression in A549 and H1703 cells. Previous studies indicated that estrogen contributes greatly to both the genesis and

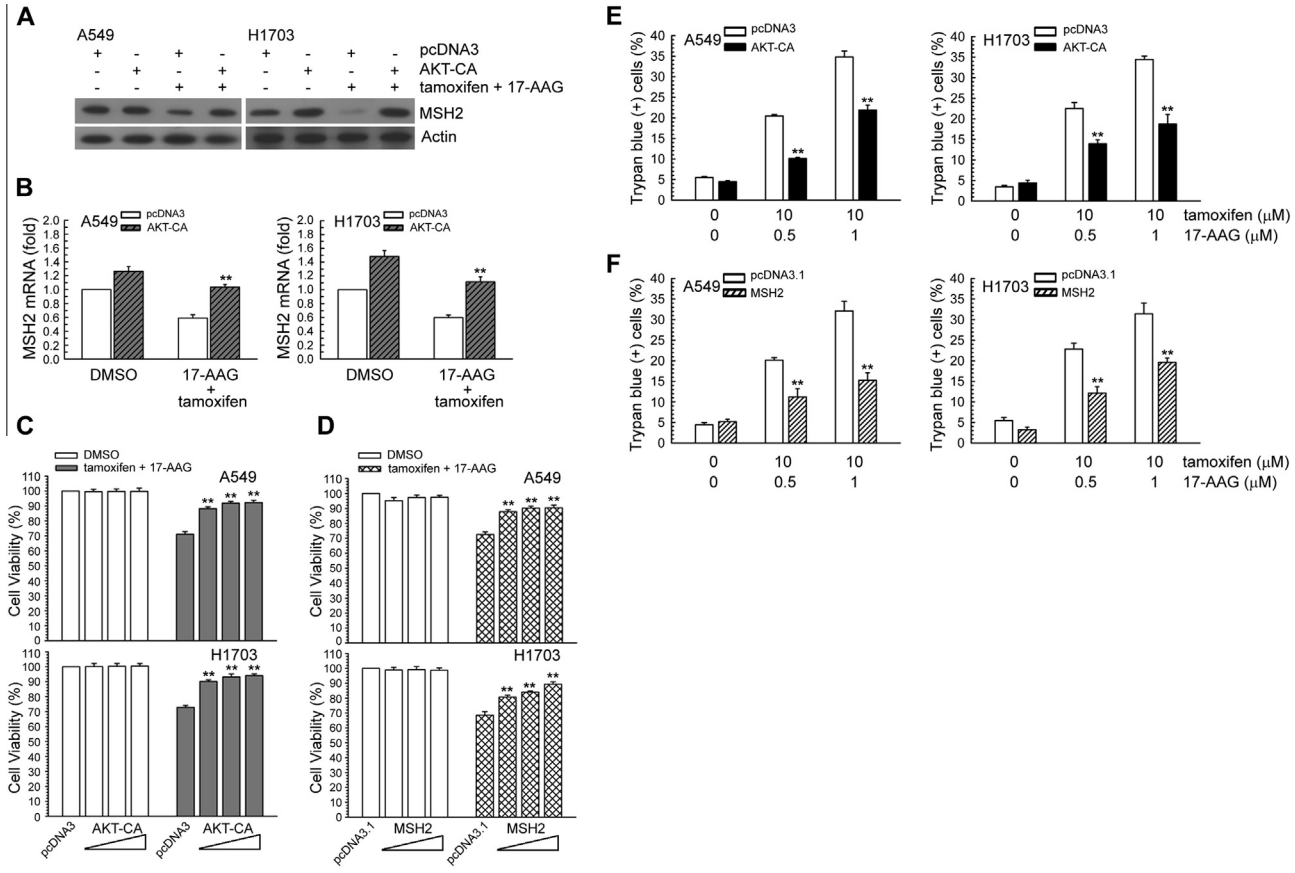

Fig. 4. Overexpression of AKT-CA or MSH2 vectors restored 17-AAG and tamoxifen-suppressed cell survival in NSCLC cells. (A) AKT-CA (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 h, the cells were treated with tamoxifen (1 μM) and 17-AAG (1 μM) for an additional 24 h, and whole-cell extracts were collected for Western blot analysis. (B) After treatment as the above, total RNA was isolated and subjected to real-time PCR for MSH2 mRNA expression. The means ± standard deviation (SD) from 4 independent experiments. **Denotes $p < 0.01$, respectively, using Student's *t*-test to compare cells treated with tamoxifen and 17-AAG in AKT-CA vs. pcDNA3-transfected cells. (C–F) After AKT-CA (1, 3, 5 μg) or MSH2 (1, 3, 5 μg) expression plasmids transfection, cells were treated with tamoxifen (2 μM) and 17-AAG (0.1 μM) for 24 h. Cytotoxicity was determined by assessment with the MTS assay (C and D) and trypan blue exclusion assay (E and F). **$p < 0.01$ by Student's *t*-test comparing cells treated with tamoxifen and 17-AAG in AKT-CA/MSH2 vs. pcDNA3-transfected A549 or H1703 cells.

development of NSCLC by either triggering cell proliferation or inhibiting apoptosis [6,7]. The specific antiestrogen fulvestrant was shown to exhibit significant antitumor activity in NSCLC cells *in vitro* and *in vivo*, and the drug showed evidence of significant interactions with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) [19,20]. Furthermore, Stabile et al. have targeted both the ER and the EGFR in lung cancer cells with fulvestrant and gefitinib, respectively, to achieve greater antiproliferative effects over either agent alone [21]. Moreover, our results suggested that down-regulation of AKT-mediated MSH2 expression involved in tamoxifen and the Hsp90 inhibitor induced synergistic cytotoxic effect in A549 and H1703 cells. However, the detailed mechanism of how estrogen and ERβ link to MSH2 expression in tamoxifen-exposed NSCLC cells needs further clarification.

Hsp90 inhibitors have been shown to increase sensitivity of cancer cells to chemotherapeutic agents [22]. In the present study, combination treatment with 17-AAG promoted the cytotoxic effect and growth inhibition of tamoxifen. A previous study indicated that estrogen-induced transcriptional activation is inhibited by 17-AAG in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells [23]. In this study, combination treatment with 17-AAG significantly decreased the expression of MSH2 through enhancing mRNA and protein instability in tamoxifen-exposed A549 and H1703 cells. However, the detail mechanisms of estrogen and Hsp90 involve in MSH2 transcription need to further be examined. Together, this study contributes to a proposed rationale to combine Hsp90 inhibition with anti-estrogen therapy for lung cancer treatment.

Conflict of interest statement

None declared.

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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.2014.11.116.

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