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p73-dependent induction of 14-3-3σ increases the chemo-sensitivity of drug-resistant human breast cancers

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Abstract

It has been well documented that tumor suppressor *p53* is mutated in about 50% of all human tumors. *p53* status might be one of the critical determinants for the chemo-sensitivity of human tumors. In the present study, we have found that p53 family member p73 as well as 14-3-3σ is down-regulated in response to adriamycin (ADR) in ADR-resistant human breast cancer-derived MBA-MD-436 cells which carry *p53* mutation. Like p53, *14-3-3σ* was transactivated by p73 and, in turn, stabilized p73. Luciferase reporter analysis and colony formation assays demonstrated that 14-3-3σ has an ability to enhance the p73-mediated transcriptional activity as well as its pro-apoptotic function. Furthermore, enforced expression of 14-3-3σ increased the ADR sensitivity of MBA-MD-436 cells. Taken together, our present results strongly suggest that p73-dependent induction of 14-3-3σ plays an important role in the regulation of chemo-sensitivity of breast cancers bearing *p53* mutation.

Keywords: Adriamycin; Breast cancer; 14-3-3σ; p53; p73

The tumor suppressor *p53* which encodes a sequence-specific nuclear transcription factor is the most frequently mutated gene in human cancers [1]. Mounting evidence showed that p53 is induced to be accumulated in response to various cellular stresses such as DNA damage, and thereby exerts its pro-apoptotic function through the transactivation of the p53-target genes implicated in the regulation of the apoptotic cell death including *Bax*, *Noxa*, *Puma,* and *p53AIP1* [2–5]. Newly identified p53 family members, p73 and p63, promote cell cycle arrest and/or apoptosis similarly to p53 [6–10]. Although *p73* and *p63* are rarely mutated in human cancers [11], it has been shown that they are required for the p53-mediated apoptosis in response to DNA damage [12]. Since cancer cells which carry *p53* mutation are more resistant to chemotherapeutic agents than those with wild-type p53 [13,14], it is likely that p73 and/or p63 might contribute to sensitize p53-deficient cancer cells to chemotherapeutic agents.

About 30–50% of human breast cancers express mutant forms of p53 [15,16]. Additionally, Moll et al. found that the pro-apoptotic function of wild-type p53 expressed in about 30% of human breast cancers is inhibited due to its aberrant cytoplasmic localization [17]. 14-3-3σ, which is one of the 14-3-3 family members, has been initially identified as a human mammary epithelium-specific marker 1 [18]. Intriguingly, the expression of 14-3-3σ is undetectable in most breast cancers due to the hypermethylation of CpG islands in the *14-3-3σ* gene [19]. Enforced expression of 14-3-3σ suppresses the anchorage-independent growth of several breast cancer cell lines [20]. Alternatively, it has been shown that 14-3-3σ is strongly induced in response to DNA damage, and its expression is directly regulated by

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p53 [21]. Yang et al. described that 14-3-3σ stabilizes p53 and enhances its transcriptional activity through the interaction with p53, suggesting that 14-3-3σ has a positive feedback effect on p53 [22].

In the present study, we have found that the adriamycin (ADR)-resistant phenotype of certain human breast cancer cells with *p53* mutation is significantly associated with the down-regulation of 14-3-3σ as well as p73. Of note, *14-3-3σ* was transactivated by p73, and enhanced its transcriptional and pro-apoptotic activity. Thus, it is likely that p73/14-3-3σ pathway plays an important role in the regulation of DNA damage-induced apoptosis in certain breast cancer cells bearing *p53* mutation.

Materials and methods

Cell culture and transfection. COS7 and human breast cancer-derived cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and antibiotic mixture. p53-deficient human lung carcinoma H1299 cells were grown in RPMI 1640 medium plus 10% heat-inactivated FBS and antibiotic mixture. Cultures were grown at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. For transfection, cells were transfected with the indicated combinations of the expression plasmids using LipofectAMINE 2000 transfection reagent according to the manufacturer's recommendations (Invitrogen).

MTT assays. Cells were seeded at a cell density of 5000 cells/well in 96-well tissue culture plates. After attachment overnight, cells were exposed to adriamycin (ADR) at a final concentration of 1 μM for the indicated time periods. MTT assays were performed as described previously [23]. In brief, 10 μl of MTT solution was added to each well. After one hour of incubation at 37 °C, the absorbance readings for each well were carried out at 570 nm using the microplate reader (Bio-Rad).

TUNEL assays. Transfected cells were exposed to ADR at a final concentration of 1 μM for 24 h. Apoptotic cells were identified using an *in situ* cell detection, peroxidase kit (Roche Applied Science). In brief, cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 1 h. Cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice and washed with PBS. The labeling reaction was performed using TMR red-labeled dUTP together with other nucleotides by terminal deoxynucleotidyl transferase for 1 h in the dark at 37 °C in a humidified chamber. Then cells were washed with PBS, mounted, and the incorporated TMR red-labeled dUTP was analyzed using a Fluoview laser scanning confocal microscope (Olympus).

RT-PCR. Total RNA was extracted from the indicated cell lines by using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was generated from 1 μg of total RNA using random primers and SuperScript II reverse transcriptase (Invitrogen). PCR-based amplification was performed using the cDNA as a template with the following primers: *p73* forward, 5′-TGGAACCAGACAGCACCTACTTCG-3′ and *p73* reverse, 5′-TGCTGGAAAGTGACCTCAAAGTGG-3′; $p21^{WAF1}$ forward, 5′-ATGAAATTCACCCCCTTTCC-3′ and $p21^{WAF1}$ reverse, 5′-CCCTAATCACCTGCCTGACCATTCCACCAAGG-3′; *erbB2* forward, 5′-GGGCTGGCCCGATGTATTTGAT-3′ and *erbB2* reverse, 5′-ATA GAGGTTGTCGAAGGCTGGGC3′; *Noxa* forward, 5′-CTGGAAGTC GAGTGTGCTACT-3′ and *Noxa* reverse, 5′-TCAGGTTCCTGAGCA GAAGAG-3′; *14-3-3σ* forward, 5′-GAGCGAAACCTGCTCTCAGT-3′ and *14-3-3σ* reverse, 5′-CTCCTTGATGAGGTGGCTGT-3′; *GAPDH* forward, 5′-ACCTGACCTGCCGTCTAGAA-3′ and *GAPDH* reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunoblotting. Whole cell lysates were separated on 10% SDS–PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with TBS (50 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.1% Tween 20) containing 5% nonfat dried milk, and then probed with the monoclonal anti-p73 (Ab-4, NeoMarkers), monoclonal anti-erbB2 (3B5, Calbiochem), polyclonal anti-14-3-3σ (C-18, Santa Cruz Biotechnology), polyclonal anti-Noxa (Zymed), or with polyclonal anti-actin (20-33, Sigma) antibody. The immunoreactive bands were visualized by using HRP-conjugated anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories) and ECL (Amersham Biosciences).

Immunoprecipitation. Whole cell lysates prepared from MBA-MD-436 cells treated with or without ADR were immunoprecipitated with the monoclonal anti-p73 followed by immunoblotting with the anti-p73 antibody.

Protein decay rate analysis. COS7 cells were transiently transfected with the indicated combinations of the expression plasmids. Twenty-four hours after transfection, cells were exposed to cycloheximide (Sigma) at a final concentration of 100 μg/ml. At the indicated time points after the treatment with cycloheximide, cells were harvested and subjected to immunoblotting with the anti-p73 or with the anti-actin antibody.

Luciferase reporter assay. p53-deficient H1299 cells were transiently transfected with 100 ng of the p53/p73-responsive luciferase reporter plasmid ($p21^{WAF1}$ or *BAX*), 10 ng of pRL-TK *Renilla* luciferase cDNA, and 25 ng of the expression plasmid for HA-p73α together with or without the increasing amounts of the expression plasmid encoding 14-3-3σ. Total amounts of plasmid DNA were kept constant (510 ng) with pcDNA3 (Invitrogen) per transfection. Forty-eight hours after transfection, both firefly and *Renilla* luciferase activities were measured by the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. The results were obtained from at least three sets of transfection and presented as means ± SD.

Colony formation assay. H1299 cells were seeded at a cell density of 2×10^5 cells/well in 6-well tissue culture plates and transfected with the indicated combinations of the expression plasmids. Forty-eight hours after transfection, cells were selected with G418 (at a final concentration of 400 μg/ml) for 2 weeks. The surviving colonies were fixed in methanol and stained with Giemsa's solution.

Results

ADR sensitivity and expression of p53-related genes in human breast cancer-derived cell lines

To examine the adriamycin (ADR) sensitivity of the human breast cancer-derived cell lines including MCF-7, MDA-MB-231, and MDA-MB-436, these cells were treated with 1 μM of ADR for the indicated time periods, and their viabilities were analyzed by the standard MTT assay. ADR has been considered as a mandatory agent in breast cancer chemotherapy [24]. As described previously [25], MCF-7 cells carry wild-type p53, whereas MDA-MB-231 and MDA-MB-436 cells express mutant form of p53. As shown in Fig. 1A, MCF-7 and MDA-MB-231 cells underwent apoptotic cell death in response to ADR, whereas MDA-MB-436 cells showed an ADR-resistant phenotype, which was also supported by FACS analysis (data not shown). Consistent with the previous observations [1], p53 was induced to be accumulated at protein level in MCF-7 cells exposed to ADR in association with the up-

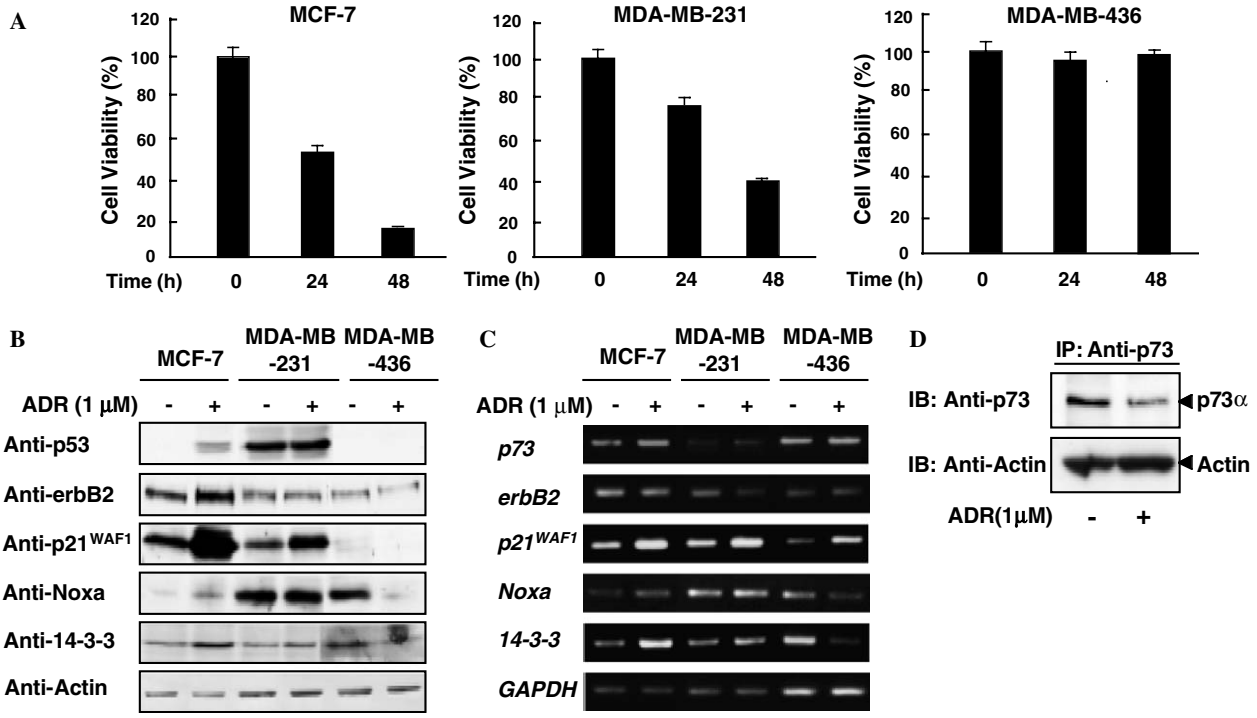

Fig. 1. The expression of *p53*-related genes in breast cancer cells in response to ADR. (A) Cell survival assays. Human breast cancer-derived MCF-7, MDA-MB-231, and MDA-MB-436 cells were exposed to ADR (at a final concentration of 1 μM). At the indicated time periods after the treatment with ADR, cell viability was examined by standard MTT assay. (B) Immunoblotting. MCF-7, MDA-MB-231, and MDA-MB-436 cells were treated with ADR (at a final concentration of 1 μM) or left untreated. Whole cell lysates were prepared and processed for immunoblotting with the indicated antibodies. Immunoblotting for actin is shown as control for protein loading. (C) RT-PCR analysis. MCF-7, MDA-MB-231, and MDA-MB-436 cells were treated with ADR as in (B). Twenty-four hours after the treatment, total RNA was prepared and subjected to RT-PCR for the expression of *p73*, *erbB2*, $p21^{WAF1}$, *Noxa* and *14-3-3σ*. Amplification of *GAPDH* served as an internal control. (D) Immunoprecipitation. MDA-MB-436 cells were treated with 1 μM ADR or left untreated. Twenty-four hours after the treatment, whole cell lysates were immunoprecipitated with the monoclonal anti-p73 antibody followed by immunoblotting with the anti-p73 antibody.

regulation of its direct targets such as $p21^{WAF1}$, Noxa, and 14-3-3σ (Fig. 1B). In ADR-sensitive MDA-MB-231 cells, ADR-mediated increase in the amounts of $p21^{WAF1}$ was detectable, however, the expression levels of p53, Noxa, and 14-3-3σ remained unchanged regardless of ADR treatment, suggesting that ADR-mediated apoptotic cell death in MDA-MB-231 cells might be regulated in a p53-independent manner. In a sharp contrast to MCF-7 and MDA-MB-231 cells, p53 was undetectable under our experimental conditions, and ADR-dependent down-regulation of Noxa and 14-3-3σ was observed in ADR-resistant MDA-MB-436 cells. Similar results were also obtained in RT-PCR analysis (Fig. 1C). *p73* as well as *erbB2* mRNA level remained almost constant regardless of ADR treatment. In addition, ADR had no detectable effects of the expression levels of *p53* mRNA (data not shown). Of note, immunoprecipitation experiments demonstrated that p73 is reduced in MDA-MB-436 cells exposed to ADR (Fig. 1D), whereas p73 levels remained unchanged in MCF-7 and MBA-MD-231 cells regardless of ADR treatment (data not shown), indicating that ADR-mediated down-regulation of Noxa and 14-3-3σ could be due to the down-regulation of p73, and also suggesting that p73 could play an important role in the regulation of ADR sensitivity in certain breast cancer cells bearing *p53* mutation.

14-3-3σ is a direct target of p73 and increases its stability

Since Noxa has been shown to be a direct transcriptional target of p53 as well as p73, and play a critical role in the regulation of p53/p73-mediated apoptotic cell death [9], we focused our attention on the possible link between p73 and 14-3-3σ. Recently, Yang et al. demonstrated that 14-3-3σ is a direct transcriptional target of p53 and increases its stability [22]. Thus, we sought to examine whether p73 could transactivate *14-3-3σ* in cells. COS7 cells were transiently transfected with the expression plasmid for HA-p73α or p53. Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR analysis. As shown in Fig. 2A, HA-p73α as well as p53 enhanced the transcription of the endogenous *14-3-3σ*. Similar results were also obtained in immunoblotting (Fig. 2B). Next, we examined the possible effect of 14-3-3σ on the stability of p73α. To this end, p53-deficient H1299 cells were transiently co-transfected with the constant amount of the HA-p73α expression plasmid along with or without the increasing amounts of the expression plasmid for 14-3-3σ. As seen

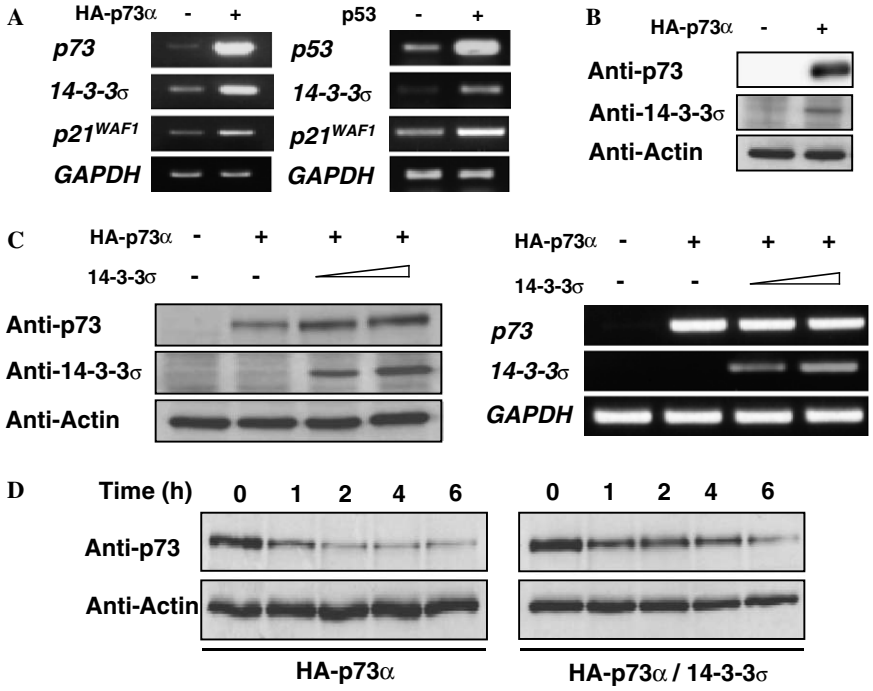

Fig. 2. *14-3-3σ* is a transcriptional target of p73 and increases its stability. (A) p73-mediated up-regulation of 14-3-3σ. COS7 cells were transiently transfected with the expression plasmid for HA-p73α (left panel) or p53 (right panel). Forty-eight hours after transfection, total RNA was prepared and subjected to RT-PCR. (B) Immunoblotting. p53-deficient H1299 cells were transiently transfected with the empty plasmid or with the expression plasmid encoding HA-p73α. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. (C) 14-3-3σ stabilizes p73. H1299 cells were transiently co-transfected with the constant amount of HA-p73α expression plasmid together with or without the increasing amounts of the expression plasmid for 14-3-3σ. Forty-eight hours after transfection, whole cell lysates and total RNA were analyzed by immunoblotting with the indicated antibodies and RT-PCR, respectively. (D) A half-life of p73 in the presence of 14-3-3σ. H1299 cells were transiently transfected with the HA-p73α expression plasmid alone or with the HA-p73α expression plasmid plus 14-3-3σ expression plasmid. Twenty-four hours after transfection, cells were exposed to cycloheximide (at a final concentration of 100 μg/ml). At the indicated time points after the addition of cycloheximide, whole cell lysates were analyzed for HA-p73α. Actin was used for equal protein loading.

in Fig. 2C, 14-3-3σ increased the amounts of HA-p73α in a dose-dependent manner, whereas 14-3-3σ had undetectable effect on the *p73α* mRNA level, suggesting that 14-3-3σ has an ability to stabilize p73 in cells. To further confirm this notion, we measured a half-life of p73α in the presence or absence of the exogenous 14-3-3σ using cycloheximide blockade. As seen in Fig. 2D, a half-life of p73α was prolonged in the presence of 14-3-3σ as compared with that of p73α alone. Taken together, our present results strongly suggest that, like p53, 14-3-3σ is a direct transcriptional target of p73 and stabilizes p73.

14-3-3σ enhances the p73-mediated transcriptional activity as well as pro-apoptotic function

To address whether 14-3-3σ could affect the p73 function, we performed the luciferase reporter assays. For this purpose, H1299 cells were transiently co-transfected with the constant amount of the p73α expression plasmid and the p53/p73-responsive luciferase reporter construct carrying the $p21^{WAF1}$ or *Bax* promoter together with or without the increasing amounts of the expression plasmid for 14-3-3σ. Forty-eight hours after transfection, cells were harvested and their luciferase activities were measured. As expected, 14-3-3σ enhanced the p73α-mediated transactivation toward $p21^{WAF1}$ and *Bax* promoters in a dose-dependent manner (Fig. 3A). Next, we examined whether 14-3-3σ could enhance the p73-mediated apoptosis. H1299 cells were transfected with the empty plasmid, HA-p73α expression plasmid, or with the HA-p73α expression plasmid plus 14-3-3σ expression plasmid. Forty-eight hours after transfection, cells were transferred to the fresh medium containing G418. After 2 weeks of selection, G418-resistant colonies were stained with Giemsa's solution. As shown in Fig. 3B, the number of G418-resistant colonies was significantly reduced in cells co-expressing HA-p73α and 14-3-3σ as compared with that in cells expressing HA-p73α alone. Thus, we concluded that 14-3-3σ has an ability to enhance the p73 function through the stabilization of p73.

Enforced expression of 14-3-3σ increases the ADR sensitivity of ADR-resistant MDA-MB-436 cells

To ask whether 14-3-3σ could affect the ADR sensitivity in ADR-resistant MDA-MB-436 cells, MDA-MB-436 cells were transfected with the empty plasmid (436-C) or with the expression plasmid for 14-3-3σ (436-14), and maintained in the presence of G418. Two weeks of selection,

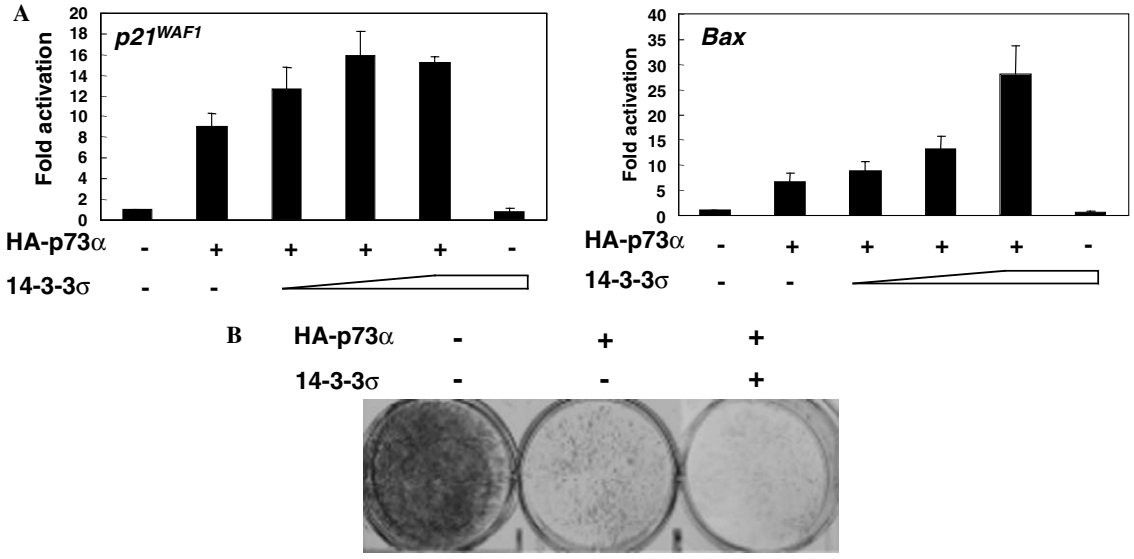

Fig. 3. *14-3-3σ* enhances the p73-mediated transcription as well as apoptosis. (A) Luciferase reporter assay. H1299 cells were transiently co-transfected with the constant amounts of the expression plasmid for HA-p73α, p53/p73-responsive luciferase reporter construct containing the $p21^{WAF1}$ (left panel) or *Bax* (right panel) promoter, *Renilla* luciferase reporter together with or without the increasing amounts of the expression plasmid for 14-3-3σ. Forty-eight hours after transfection, luciferase activities were measured as described under "Materials and methods". (B) Colony formation assay. H1299 cells were transfected with empty plasmid, the expression plasmid for HA-p73α or with the expression plasmid for HA-p73α plus 14-3-3σ expression plasmid. Forty-eight hours after transfection, cells were transferred to the fresh medium containing G418 (at a final concentration of 400 μg/ml). After 2 weeks of selection, drug-resistant colonies were fixed and stained with Giemsa's solution.

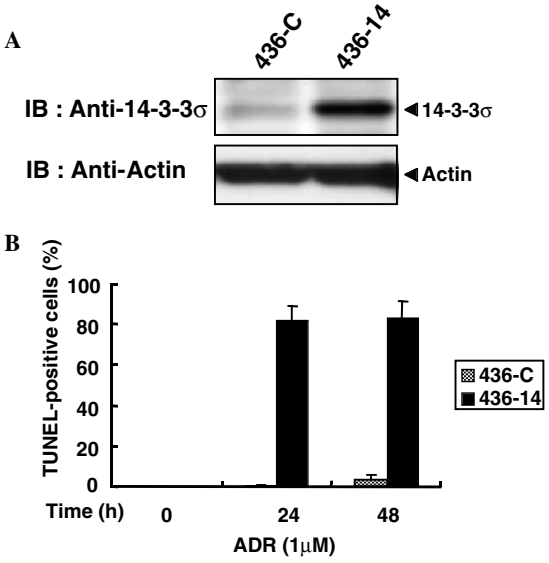

Fig. 4. Effects of 14-3-3σ on the ADR sensitivity in MDA-MB-436 cells. (A) Enforced expression of 14-3-3σ in MDA-MB-436 cells. MDA-MB-436 cells were transfected with the empty plasmid (436-C) or with the expression plasmid for 14-3-3σ (436-14). Forty-eight hours after transfection, cells were transferred to the fresh medium containing 400 μg/ml G418. After 2 weeks of selection, surviving cells were harvested and then whole cell lysates were subjected to immunoblotting with the indicated antibodies. (B) TUNEL assay. Control MDA-MB-436 cells (436-C) and MDA-MB-436 cells expressing 14-3-3σ (436-14) were exposed to ADR (at a final concentration of 1 μg/ml) or left untreated. Twenty-four and Forty-eight hours after the treatment with ADR, apoptotic cell death was examined by TUNEL assay.

whole cell lysates were prepared from the surviving cells and subjected to immunoblotting. As shown in Fig. 4A, the increased expression of 14-3-3σ was observed in cells transfected with 14-3-3σ expression plasmid. We then examined the ADR sensitivity by TUNEL assays. As seen in Fig. 4B, the number of MDA-MB-436 cells expressing 14-3-3σ with apoptotic nuclei was significantly increased in response to ADR as compared with that of the control MDA-MB-436 cells. Collectively, our present results strongly suggest that p73/14-3-3σ pathway plays an important role in the regulation of the chemo-sensitivity of certain breast cancer cells bearing *p53* mutation.

Discussion

Mammalian 14-3-3 protein family comprises at least seven isoforms. Among them, *14-3-3σ* is mapped at human chromosome 1p35, a region which shows frequent loss of heterozygosity in a wide variety of human cancers including breast cancers [21,26]. Indeed, the expression levels of *14-3-3σ* were significantly lower in breast cancers than those in normal breast epithelium, which might be due to the hypermethylation of CpG islands in the *14-3-3σ* gene locus [19], and an enforced expression of 14-3-3σ inhibited the oncogene-activated tumorigenesis [27]. Intriguingly, 14-3-3σ was transactivated by p53 in response to DNA damage [21], and positively regulated its activity [22], indicating that there exists a positive feedback regulation of p53 by its target 14-3-3σ. In the present study, we have found that 14-3-3σ is a direct

transcriptional target of p73 and enhances the p73-mediated transcriptional as well as pro-apoptotic activity. Furthermore, an enforced expression of 14-3-3σ in ADR-resistant human breast cancer MDA-MB-436 cells bearing *p53* mutation resulted in an increased cell killing by ADR. Collectively, our present results strongly suggest that p73/14-3-3σ pathway plays an important role in the regulation of chemo-sensitivity in certain breast cancer cells bearing *p53* mutation.

According to the results described by Yang et al. 14-3-3σ had an ability to increase the stability and the transcriptional activity of p53 through the physical interaction with p53 [22]. They also demonstrated that 14-3-3σ inhibits the MDM2-mediated p53 ubiquitination and nuclear export. In spite of our extensive efforts, we could not detect the physical interaction between p73α and 14-3-3σ under our experimental conditions (data not shown). Although it might be due to the different cell systems used in our immunoprecipitation experiments, it is likely that 14-3-3σ-mediated increase in the stability and activity of p73α could be regulated by a still unknown indirect mechanism(s). Recently, Rossi et al. reported that a HECT-type E3 ubiquitin protein ligase Itch binds to and ubiquitinates p73, and thereby promoting its rapid proteasome-dependent degradation [28]. They also described that the expression levels of Itch are down-regulated in cells in response to DNA damage. Since 14-3-3σ induced the degradation of MDM2 [22], it is tempting to speculate that E3 ubiqitin protein ligase activity of Itch could be blocked by 14-3-3σ. This issue is currently under investigation.

According to the previous results, p73 was induced to be accumulated at protein level in response to ADR treatment and thereby promoting apoptotic cell death [29,30]. In a sharp contrast, p53/p73-target genes such as *Noxa* and *14-3-3σ* were significantly down-regulated following ADR treatment in ADR-resistant MDA-MB-436 cells. MCF-7 cells carrying wild-type p53 underwent apoptosis in response to ADR in a p53-dependent manner. Since MDA-MB-436 cells express nonfunctional p53 [25], it is possible that the ADR-mediated upstream activation pathways including the post-translational modifications such as phosphorylation and acetylation which regulate p73 function [9,10] might be disrupted in these cells. Based on our present results, enforced expression of 14-3-3σ in p53-deficient H1299 cells enhanced the p73-mediated apoptotic cell death as examined by colony formation assay. Furthermore, the ADR sensitivity of MDA-MB-436 cells was increased in the presence of exogenously expressed 14-3-3σ. Thus, it is likely that p73/14-3-3σ-mediated apoptotic pathway might be one of the critical determinants of cell fate in response to ADR in certain breast cancer cells bearing *p53* mutation, and that p73 might be effective in p53-deficient breast cancer cells which are resistant to anti-cancer drug. In this connection, further work is required to determine how the ADR-mediated activation of p73 is blocked in MDA-MB-436 cells.

Acknowledgments

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