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Inhibition of Survivin by Adenovirus Vector Enhanced Paclitaxel-induced Apoptosis in Breast Cancer Cells

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Abstract. *Background/Aim: Survivin expression has been shown to be associated with cancer progression, poor prognosis, and drug resistance. The aim of this study was to examine whether survivin knock-down could enhance paclitaxel-induced apoptosis in breast cancer cells in vitro. Materials and Methods: MCF-7 cells were infected with an siRNA-expressing adenovirus vector against survivin (Adv-siSurv) or Renilla luciferase as a control (Adv-siRL). After treatment with paclitaxel, cells were analyzed by apoptotic, cell cycle and immunoblotting assays. Results: Of cells treated with paclitaxel alone, only 20.2±2.08% showed apoptotic features. An increase in the paclitaxel dose was associated with increased survivin expression. In contrast, Adv-siSurv infection resulted in a marked increase in apoptotic cell death in paclitaxel-treated MCF-7 cells (49.9±7.70%). The percentage of cells in the G_2M phase was lower (23.9±1.64%) in Adv-siSurv-infected cells than that of Adv-siRL–treated cells (40.0±2.43%). Adv-siSurv infection reduced survivin, procaspase-9, and procaspase-3 levels in paclitaxel-treated MCF-7 cells. Conclusion: Loss of survivin expression enhanced paclitaxel-induced apoptosis in MCF-7 breast cancer cells in vitro.*

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is characterized by a unique structure that distinguishes it from other members of the IAP family. To date, eight IAP members have been identified (1), all of which contain one to three copies of the baculovirus IAP repeat (BIR) domains in the N-terminus. In some IAP members, such domains prevent apoptosis to promote growth of tumor cells through inhibition of caspase-3 and -7 activations (2). Survivin, which has only one BIR domain and consists of 142 amino acids, is the smallest of the IAP family members. Survivin expression has been shown to be associated with cancer progression, poor prognosis, and drug resistance.

Paclitaxel is one of the most active cancer chemotherapeutic agents in breast cancer. Paclitaxel induces mitotic arrest through polymerisation and stabilization of microtubules and induces cell death by apoptosis. However, acquired resistance to paclitaxel is one of the most significant reasons for its eventual failure in chemotherapy (3). Previous studies have demonstrated that paclitaxel resistance is attributable to various mechanisms: First, the presence of elevated drug efflux that results from the up-regulation of membrane transporters such as P-glycoprotein (4). Second, alterations in the expression of β-tubulin isotypes (5). Third, changes in apoptotic regulatory proteins such as survivin (6). We had previously constructed a replication-deficient adenovirus that expressed survivin siRNA (7). In this study, it was examined whether an adenovirus vector that expressed survivin siRNA could enhance paclitaxel-induced apoptosis in breast cancer cells *in vitro*.

Materials and Methods

Cell culture. MCF-7 cells, a human breast cancer cell line, were maintained in MEM medium containing 10% FBS, 0.2% sodium bicarbonate, penicillin (100 units/ml) and streptomycin (0.1 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Adenovirus vectors expressing siRNA. Recombinant adenovirus vectors used were based on E1- and E3-deleted Ad5 with a modified fiber F/RGD harboring an integrin-binding RGD motif within the HI loop of its knob protein. The construction of the siRNA expression unit was of a tandem type, in which sense and antisense strands composing the siRNA duplex were independently transcribed by two human U6 promoters (Figure 1). Adv-siSurv is a vector expressing siRNA against survivin, as previously described (7), inserted immediately downstream of the U6 promoter as follows: 5'-GAAAGTGCGCCGTGCCATC-3'. Adv-siRL is a

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control vector, expressing siRNA against *Renilla* luciferase, as previously described (8).

Real-time polymerase chain reaction. MCF-7 cells were infected with Adv-siSurv or Adv-siRL for three days. Total RNA was reverse transcribed to cDNA at 48˚C for 30 min using TaqMan Reverse Transcription Reagents (N808-0234; Applied Biosystems, Foster City, CA, USA). Survivin primers were designed by an assay-by-design service (#185137684; Applied Biosystems). The primers for polymerase chain reaction (PCR) amplification used in this study were as follows: survivin, forward primer 5'-CTACATTCAAGAACTGGC CCTTCT-3', reverse primer 5'-CAAGTCTGGCTCGTTCTCAGT-3'; and GAPDH (internal control), forward primer 5'-CCACCCATGG CAAATTCCAT GGCA-3', reverse primer 5'-TCTAGACGGCAGG TCAGG TCCACC-3'. This system was used for real-time monitoring of PCR amplification of the cDNA following a TaqMan Universal PCR Master Mix protocol (Perkin–Elmer, Waltham, MA, USA).

Cell cycle analysis. Cell cycle analysis was performed by flow cytometry following propidium iodide (PI) staining. MCF-7 cells were infected with Adv-siSurv or Adv-siRL for three days and treated with varying doses (ranging from 0 to 30 nM) of paclitaxel. From 24 h to 120 h later, cells were removed by trypsinisation, washed with phosphate buffered saline (PBS) and fixed with 70% ethanol. Fixed samples were centrifuged, treated with RNase (0.25 mg/ml), and resuspended in PI (50 μg/ml). PI-stained cells were analysed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Immunoblot analysis. MCF-7 cells were infected with Adv-siSurv or Adv-siRL for three days, and then treated with varying doses (ranging from 0 to 30 nM) of paclitaxel for five days. Total cell lysates from treated cells were extracted with lysis buffer (10 mM Tris-HCL [pH 8.0], 0.2% NP-40, 1 mM EDTA, and 2% SDS). Thirty μg of protein from each extract were separated using 5-20% gradient polyacrylamide gels and were transferred onto nitrocellulose membranes. After blocking with 5% dry milk in TBS (10 mM Tris-HCl (pH 7.5), 150 mM sodium chloride), the membranes were incubated with the primary antibodies for 1 h at 25˚C. The primary antibody was either mouse anti-survivin monoclonal antibody (#2802; Cell Signaling Technology, Danvers, MA, USA), rabbit anti–caspase-9 polyclonal antibody (#9502; Cell Signaling Technology), mouse anti–caspase-3/CPP32 monoclonal antibody (C31720; Transduction Laboratories), or mouse anti–β-actin monoclonal antibody (A5441; Sigma–Aldrich, St Louis, MI, USA). The secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse IgG+A+M (H+L; #61-6420; Zymed Laboratories, San Francisco, CA, USA) or donkey anti-rabbit IgG, peroxidase-linked species-specific F(ab') fragment (Amersham, Little Chalfont, USA). Staining was carried out using an ECL kit (Amersham) according to the manufacturer's instructions.

Phase contrast microscopy. MCF-7 cells were infected with Adv-siSurv or Adv-siRL for three days, and then treated with varying doses (ranging from 0 to 30 nM) of paclitaxel for five days. Cellular morphological change was observed by phase contrast microscopy.

Statistical evaluation. The significance of differences between groups was assessed by a Student's *t*-test using JMP. Statistical significance was defined as $p<0.05$.

Results

Silencing of survivin with siRNA in MCF-7 cells. In an attempt to down-regulate the expression of survivin, MCF-7 cells were infected with Adv-siSurv or Adv-siRL for 72 h. The expression levels of survivin and GAPDH genes in MCF-7 cells were evaluated based on their quantitative qRT–PCR threshold cycle values (Ct). As shown in Figure 2, the average Ct value of survivin was 26.55±0.15 cycles for Adv-siSurv–transfected cells compared to 23.86±0.40 cycles for Adv-siRL–transfected cells. For the $2^{-\Delta Ct}$ value method, adenovirus-mediated siRNA resulted in a 6.43-fold reduction in survivin expression. In contrast, average Ct values for GAPDH were not different, with 21.03±0.15 cycles for Adv-siSurv–transfected cells and 21.01±0.03 cycles for Adv-siRL–transfected cells. Thus, survivin expression was successfully reduced in MCF-7 cells.

Enhanced apoptosis in MCF-7 cells by siRNA against survivin. The changes in the apoptosis-inducing potential of Adv-siSurv transfected cells were next examined *via* flow cytometric analysis. MCF-7 cells were stained with PI, treated with adenovirus-mediated siRNA for 3 days and then exposed to various concentrations of paclitaxel for 24, 72 and 120 h. Adv-siSurv–mediated survivin knock-down treatment alone slightly increased the percentage of cells in the sub-G1 fraction compared with Adv-siRL treatment during the time course (Figure 3). Adv-siRL–transfected MCF-7 cells showed an increased population of cells in G_2M arrest after exposure to paclitaxel in a dose-dependent manner for 120 h (Figure 4A: white columns). The percentage of cells arrested in the G_2M phase at paclitaxel concentrations of 0, 3, and 30 nM was 16.4±0.81, 21.2±0.83, and 40.0±2.43, respectively. Only a small proportion of the cell population had apoptotic features (sub-G_1 population: 6.12±0.79, 18.07±1.56, and 20.2±2.08% respectively; Figure 4B: white columns). In contrast, paclitaxel treatment of Adv-siSurv–transfected MCF-7 cells suppressed any increase in paclitaxel-mediated G_2M arrest compared to the control treated group: The percentage of cells arrested in the G_2M phase at concentrations of 0, 3, and 30 nM of paclitaxel was 17.7±0.58, 19.2±0.58, and 23.9±1.64, respectively ($p<0.05$; Figure 4A: black columns). Thus, Adv-siSurv infection resulted in a marked increase in apoptotic cell death in paclitaxel-treated MCF-7 cells compared to the control group over time (sub-G_1 population: 7.26±0.29, 30.14±5.15, and 49.9±7.70% respectively; Figure 4B black columns; $p<0.05$).

To confirm that exposure to paclitaxel affected the expression of survivin and to show that paclitaxel-induced apoptosis enhanced by survivin knock-down was mediated by the activation of caspases, western blot analysis was performed for procaspase-9, procaspase-3 and survivin proteins as shown in Figure 5. Surprisingly, in Adv-siRL–

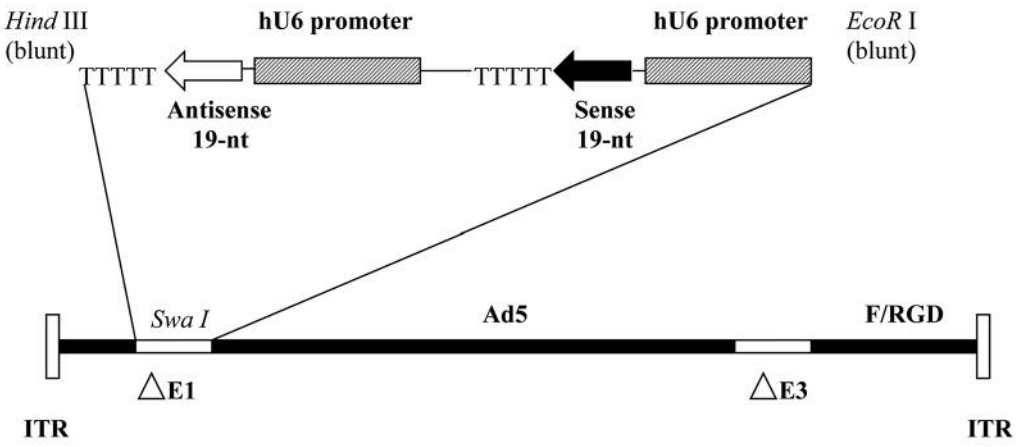

Figure 1. *A schematic presentation of the structure of an adenovirus vector encoding U6 promoter-driven siRNA. The backbone of the adenoviral vectors used in this study was an E1- and E3-deleted serotype 5 adenovirus with a modified fiber F/RGD, harbouring an integrin-binding RGD motif within the HI loop of its knob protein. The construction of this siRNA expression unit is of a tandem type, in which sense and antisense strands composing the siRNA duplex were independently transcribed by two human U6 promoters.*

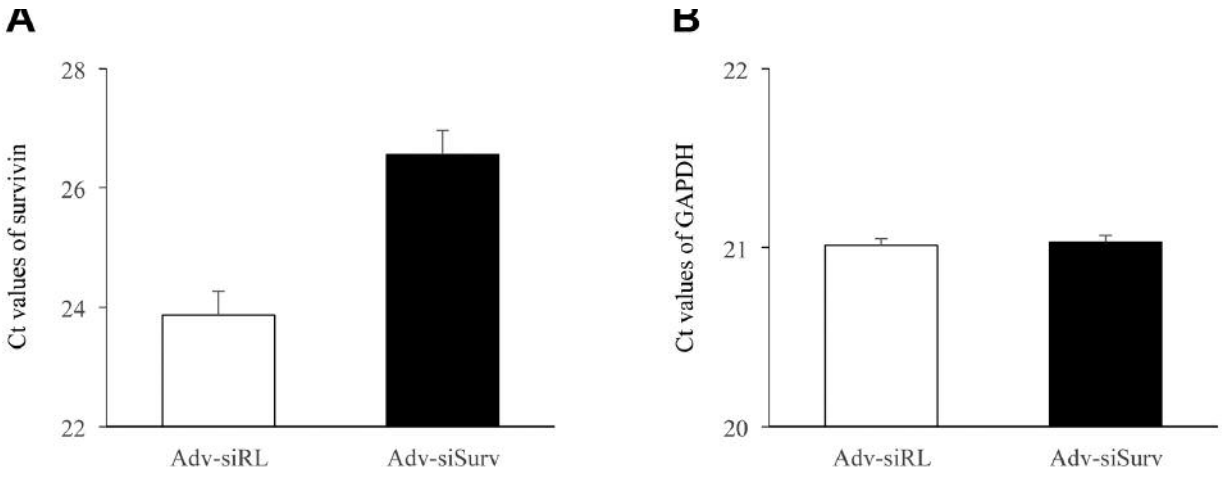

Figure 2. *The effects of adenovirus-mediated siRNA against survivin in MCF-7 cells. The RNA levels of survivin (A) and GAPDH (B) after infection with Adv-siRL or Adv-siSurv at 3,000 VP/cell for 72 h were evaluated by quantitative real-time (qRT)–polymerase chain reaction (PCR). Data are presented as mean±standard deviation (SD). n=9. Threshold cycle value (Ct)=qRT–PCR.*

transfected MCF-7 cells exposed to paclitaxel (0-30 nM) for 120 h, survivin expression increased in a paclitaxel concentration-dependent manner. In contrast, MCF-7 cells transfected with Adv-siSurv and exposed to paclitaxel did not show an increase in paclitaxel-mediated survivin expression. As a result, despite paclitaxel exposure, the amount of pro-caspase-9 and pro-caspase-3 proteins decreased in MCF-7 cells after infection with Adv-siSurv. Infection with Adv-siRL did not affect the levels of procaspase-9 and the procaspase-3. siRNA for survivin and paclitaxel treatment strikingly increased cell death in MCF-7 cells.

Phase-contrast microscopic analysis of the cell morphology showed that Adv-siSurv–transfected cells treated with paclitaxel appeared rounded-up and in fragmented form at concentrations above 10 nM (Figure 6: lower panels). In contrast, the Adv-siRL–transfected cell line showed few floating cells, even when the concentration of paclitaxel was increased to 30 nM (Figure 6: upper panels). These results indicated that the inhibition of paclitaxel-mediated survivin induction by adenoviral transduction of siRNA against survivin significantly increased paclitaxel-induced cell death.

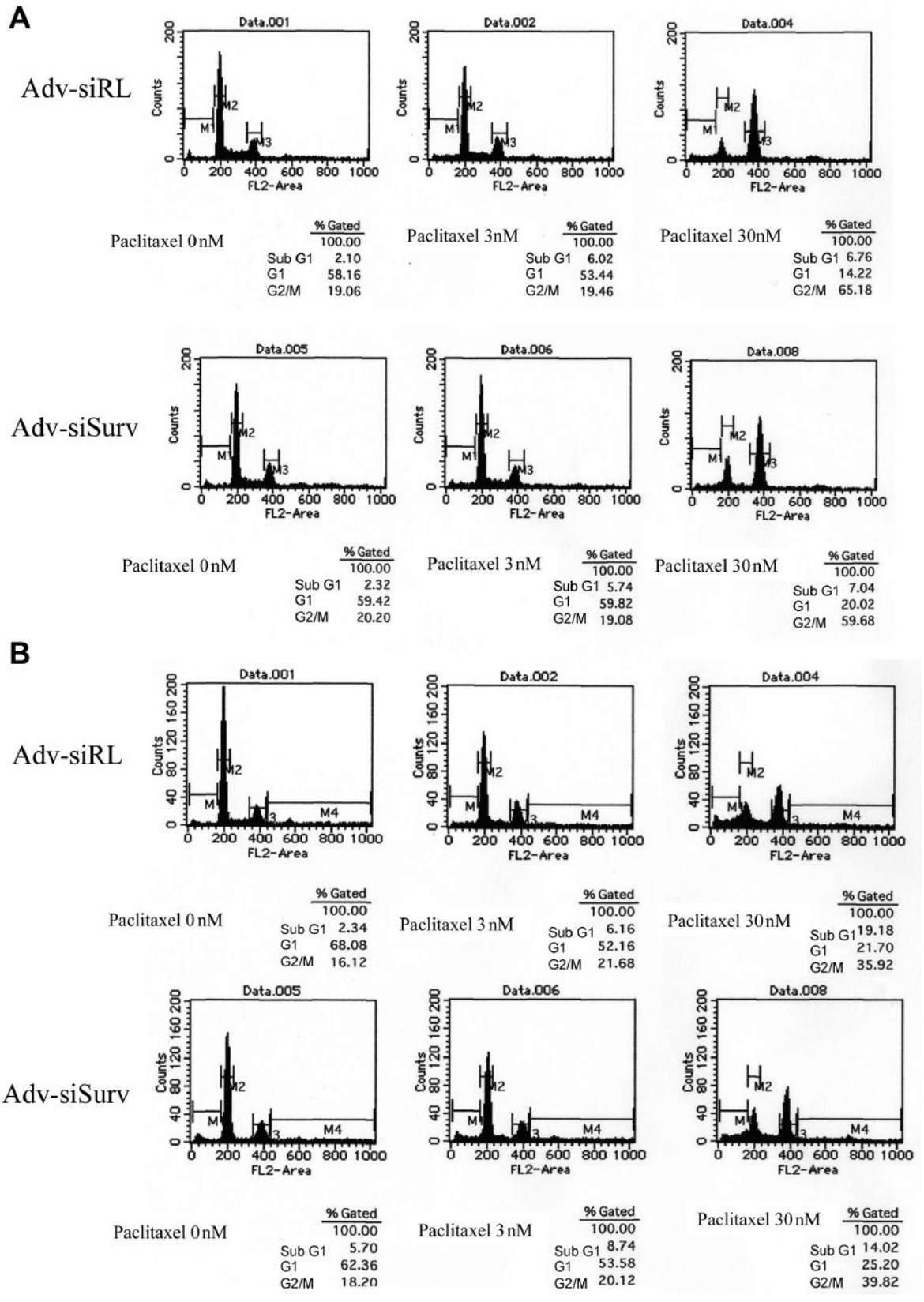

Figure 3. *Continued*

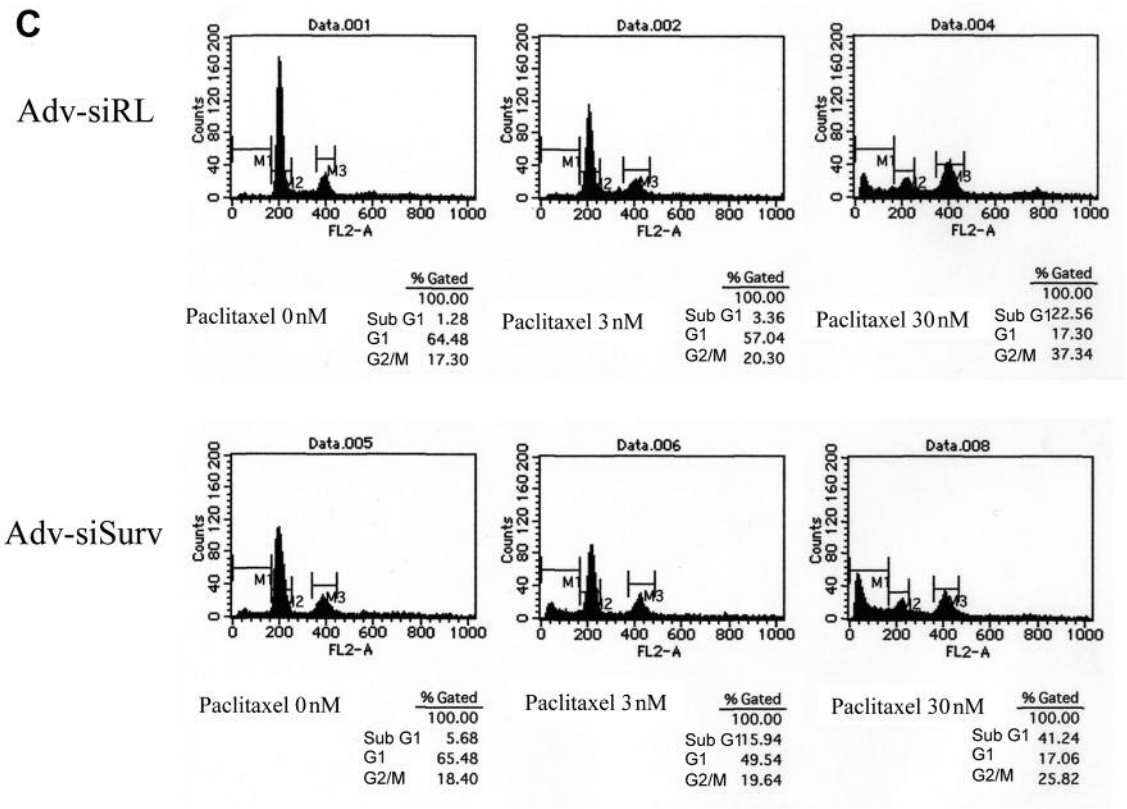

Figure 3. *Flow cytometric analysis of cell cycle phases in MCF-7 cells treated with adenovirus-mediated siRNA (Renilla luciferase as a control [Adv-siRL]) or siRNA-expressing adenovirus vector of survivin [Adv-siSurv] for 72 h and exposed to paclitaxel for 24 to 120 h. Cells were collected after treatment with paclitaxel at 24 h (A), 72 h (B), or 120 h (C) and stained with propidium iodide (PI) for flow cytometric analysis Each histogram image shows representative results obtained from three independent experiments.*

Discussion

Many cancer cases that were once responsive to anti-cancer drugs acquire resistance through expression of protective gene products or mutations induced by exposure to such drugs. Resistance to a single agent eventually develops into a multidrug resistance and the disease's ability to counteract all available chemotherapies. Therefore, elucidating the mechanism of resistance to anti-cancer drugs and using this knowledge to overcome this resistance is very important. One of the causes of resistance to anti-cancer drugs is the induction of a wide range of stress-related genes involved in cell death and apoptosis. This mechanism involves systems activated by conditions associated with microenvironments surrounding tumors, such as hypoxia, low glucose levels, low pH, the elevation or absence of a DNA repair system, and inhibition of cell death through elevation of activation signals of anti-apoptotic factors such as Akt, Bcl-2 and survivin. IAP family proteins are crucial factors involved in the regulation of apoptosis.

The anti-apoptotic mechanism of survivin has not yet been completely elucidated. Unlike other IAP family members, survivin lacks the BIR2 domain structure necessary for binding caspase-3 and -7; consistent with this, certain studies have shown that survivin does not directly inhibit the activity of these two caspases (9). Survivin most likely inhibits the activity of caspase-9, not through direct binding but, rather, by interacting with cofactors such as XIAP (10). In the present study, caspase-3 activity was induced by caspase-9 activation triggered by survivin knock-down in MCF-7 breast cancer cells (Figure 5), which supports the results of the above described studies.

Survivin also plays an essential role as a mitotic regulatory factor ensuring the appropriate completion of cell division. Expression of survivin is strictly regulated during the cell cycle and peaks during G_2M phase (11). In the metaphase of the cell cycle, survivin is localized in the centromere, interacts with the chromosomal passenger proteins, Aurora B kinase, inner centromere protein, and Borealin/Dasra, and is involved in the dynamics and stabilization of microtubules

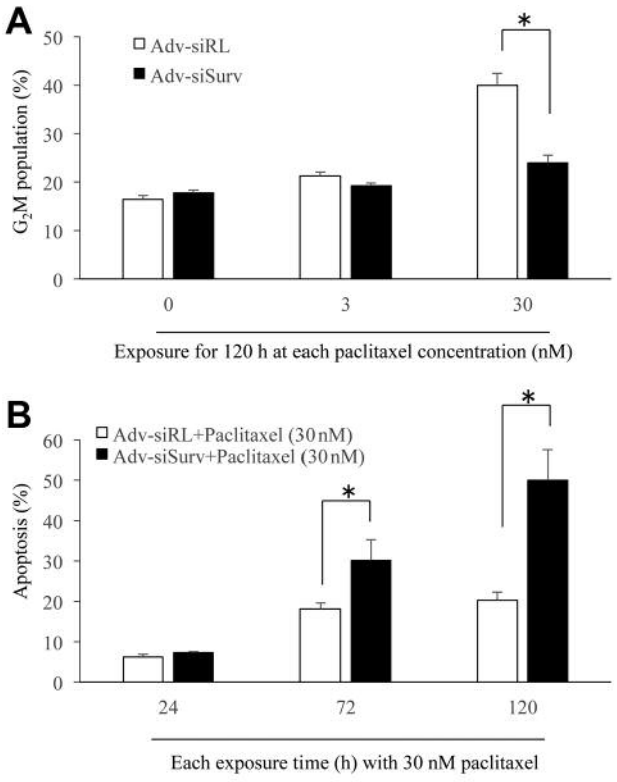

Figure 4. *Percentage of cells arrested in the G_2M phase after exposure to various doses of paclitaxel for 120 h (A). Percentage of induced apoptotic cells from 24 to 120 h after paclitaxel treatment (B). An siRNA-expressing adenovirus vector of survivin (Adv-siSurv) suppressed the G_2M population of the cell cycle, while paclitaxel treatment induced apoptosis. Values are percentages and represent means±standard deviation (SD) of three experimental replicates as shown in Figure 3. Renilla luciferase was used as a control (Adv-siRL). *p<0.05, Student's t-test.*

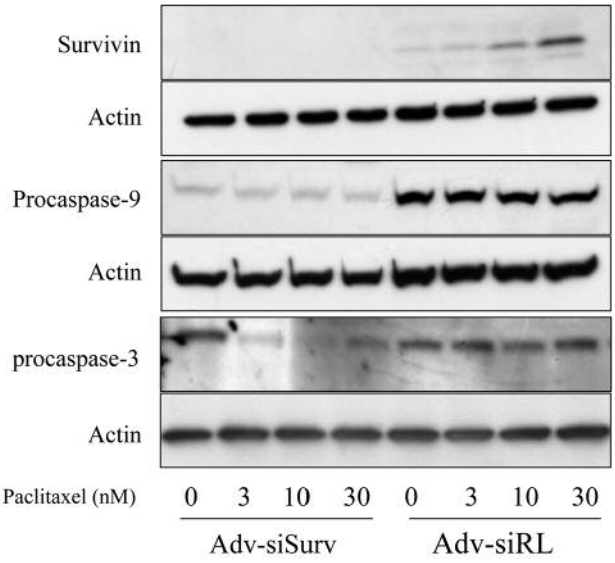

Figure 5. *Immunoblot analysis of apoptosis-related proteins in MCF-7 cells treated with adenovirus-mediated siRNA and exposed to paclitaxel for 120 h. Renilla luciferase was used as a control (Adv-siRL). siRNA-expressing adenovirus vector of survivin (Adv-siSurv).*

(12). Furthermore, survivin expression is increased in cancer cells resistant to many available cytotoxic anti-cancer drugs, including taxanes (13) and radiotherapy (14). Similarly, in our study, cell populations arrested in the G_2M phase also displayed increased expression of survivin in a paclitaxel concentration-dependent manner (Figure 4A).

Several approaches, including the use of the transcriptional inhibitor sepantronium bromide (YM155), have been designed to target survivin (15-17). However, recent studies have revealed that an improvement in the response rate was not observed when YM155 was added to the combination therapy of carboplatin and paclitaxel for progressive non-small cell lung cancer in Phase I/II trials (18). This failure is probably due to the lack of specificity of YM155 and its inadequate inhibition of survivin in patients (19). It is believed that a novel strategy that can effectively down-regulate survivin is required to increase chemotherapeutic efficacy, thereby improving the survival of cancer patients. We have developed genetically modified adenoviral vectors to introduce therapeutic genes into cancer cells and have reported that they are effective for *in vitro* (20) and *in vivo* (21, 22) cancer treatments. In this study, survivin expression was successfully inhibited using a tandem siRNA expressed in an adenovirus system under a U6 promoter, which increased the antitumor activity of paclitaxel (Figures 4B and 6).

In summary, the sensitivity of paclitaxel towards a breast cancer cell line was investigated after treatment with a recombinant adenovirus vector encoding siRNA targeting survivin. Strong inhibition of survivin by specific siRNA enhanced paclitaxel-induced apoptosis in cultured breast cancer cells, suggesting that this system is an attractive strategy for treating breast cancer from the perspective of conquering drug resistance.

Conflicts of Interest

The Authors declare that they have no potential conflicts of interest to disclose.

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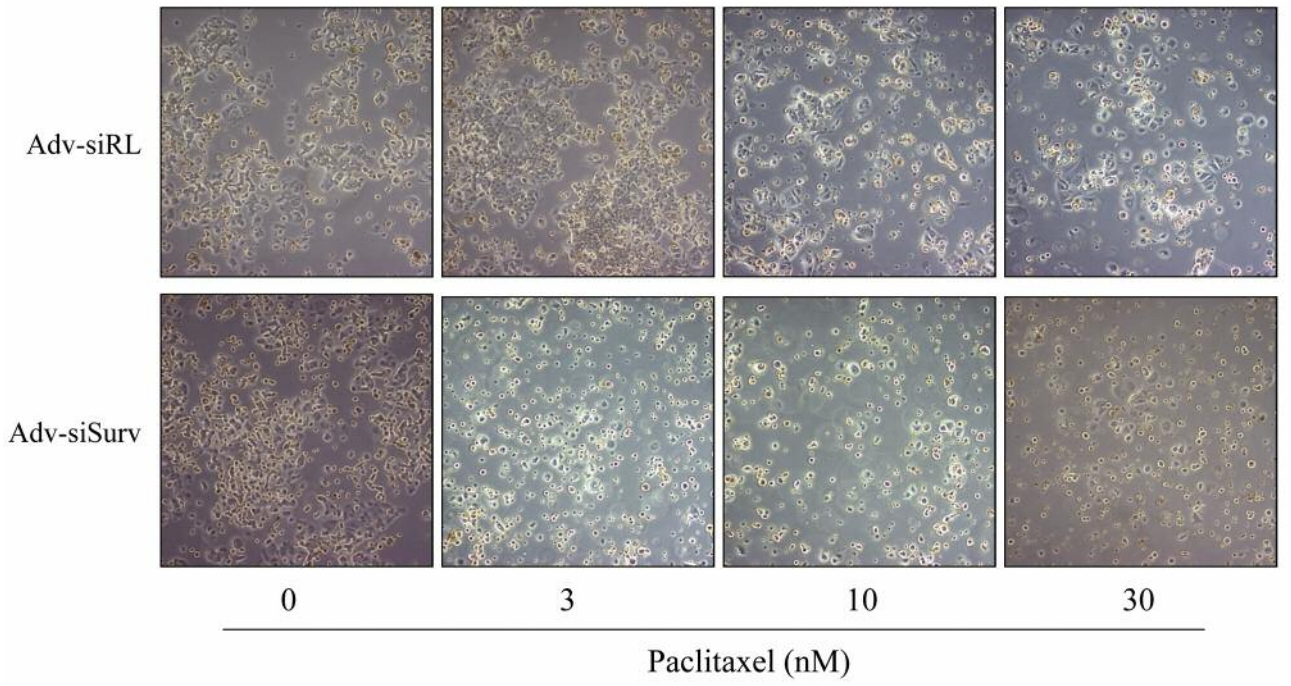

Figure 6. *MCF-7 cells treated with adenovirus-mediated siRNA and exposed to paclitaxel at various concentrations for 120 h were photographed by phase-contrast microscopy. The photographs represent a comparison of cell morphology and proliferation after survivin knock-down (lower panels) or under control conditions (upper panels).*

References

1 Altieri DC: Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene *22*: 8581-8589, 2003.

2 Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T and Reed JC: IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res *58*: 5315-5320, 1998.

3 Yusuf RZ, Duan Z, Lamendola DE, Penson RT and Seiden MV: Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. Curr Cancer Drug Targets *3*: 1-19, 2003.

4 Jang SH, Wientjes MG and Au JL: Kinetics of P-glycoprotein-mediated efflux of paclitaxel. J Pharmacol Exp Ther *298*: 1236-1242, 2001.

5 Mozzetti S, Ferlini C, Concolino P, Filippetti F, Raspaglio G, Prislei S, Gallo D, Martinelli E, Ranelletti FO, Ferrandina G and Scambia G: Class III beta-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. Clin Cancer Res *11*: 298-305, 2005.

6 Ling X, Bernacki RJ, Brattain MG and Li F: Induction of survivin expression by taxol (paclitaxel) is an early event, which is independent of taxol-mediated G_2/M arrest. J Biol Chem *279*: 15196-15203, 2004.

7 Uchida H, Tanaka T, Sasaki K, Kato K, Dehari H, Ito Y, Kobune M, Miyagishi M, Taira K, Tahara H and Hamada H: Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth *in vitro* and *in vivo*. Mol Ther *10*: 162-71, 2004.

8 Miyagishi M and Taira K: U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol *20*: 497-500, 2002.

9 Banks DP, Plescia J, Altieri DC, Chen J, Rosenberg SH, Zhang H and Ng SC: Survivin does not inhibit caspase-3 activity. Blood *96*: 4002-4003, 2000.

10 Dohi T, Beltrami E, Wall NR, Plescia J and Altieri DC: Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. J Clin Invest *114*: 1117-1127, 2004.

11 Zhao J, Tenev T, Martins LM, Downward J and Lemoine NR: The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner. J Cell Sci *113*: 4363-4371, 2000.

12 Vader G, Kauw JJ, Medema RH and Lens SM: Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody. EMBO Rep *7*: 85-92, 2006.

13 Ghanbari P, Mohseni M, Tabasinezhad M, Yousefi B, Saei AA, Sharifi S, Rashidi MR and Samadi N: Inhibition of survivin restores the sensitivity of breast cancer cells to docetaxel and vinblastine. Appl Biochem Biotechnol *174*: 667-681, 2014.

14 Grdina DJ, Murley JS, Miller RC, Mauceri HJ, Sutton HG, Li JJ, Woloschak GE and Weichselbaum RR: A survivin-associated adaptive response in radiation therapy. Cancer Res *73*: 4418-4428, 2013.

15 Nitta T, Koike H, Miyao T, Miyazawa Y, Kato H, Furuya Y, Sekine Y and Suzuki K: YM155 reverses statin resistance in renal cancer by reducing expression of survivin. Anticancer Res *37*: 75-80, 2017.

16 Peery RC, Liu JY and Zhang JT: Targeting survivin for therapeutic discovery: past, present, and future promises. Drug Discov Today *22*: 1466-1477, 2017.
17 Sanomachi T, Suzuki S, Kuramoto K, Takeda H, Sakaki H, Togashi K, Seino S, Yoshioka T, Okada M and Kitanaka C: Olanzapine, an atypical antipsychotic, inhibits survivin expression and sensitizes cancer cells to chemotherapeutic agents. Anticancer Res *37*: 6177-6188, 2017.
18 Kelly RJ, Thomas A, Rajan A, Chun G, Lopez-Chavez A, Szabo E, Spencer S, Carter CA, Guha U, Khozin S, Poondru S, Van Sant C, Keating A, Steinberg SM, Figg W and Giaccone G: A phase I/II study of sepantronium bromide (YM155, survivin suppressor) with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer. Ann Oncol *24*: 2601-2606, 2013.
19 Rauch A, Hennig D, Schäfer C, Wirth M, Marx C, Heinzel T, Schneider G and Krämer OH: Survivin and YM155: how faithful is the liaison? Biochim Biophys Acta *1845*: 202-220, 2014.
20 Tanaka T, Yamada H, Kuroki Ma, Kodama S, Tamura K and Takamatsu Y: A modified adenovirus vector-mediated antibody screening method identifies EphA2 as a cancer Target. Transl Oncol *10*: 476-484, 2017.
21 Tanaka T, Shimura H, Sasaki T, Narumi K, Maemondo M, Nukiwa T, Matsumoto K, Nakamura T and Ikeda S: Gallbladder cancer treatment using adenovirus expressing the HGF/NK4 gene in a peritoneal implantation model. Cancer Gene Ther *11*: 431-440, 2004.
22 Tanaka T, Huang J, Hirai S, Kuroki Mo, Kuroki Ma, Watanabe N, Tomihara K, Kato K and Hamada H: Carcinoembryonic antigen-targeted selective gene therapy for gastric cancer through FZ33 fiber-modified adenovirus vectors. Clin Cancer Res *12*: 3803-3813, 2006.

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