Original Article

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Levels of effectiveness of gene therapies targeting survivin and its splice variants in human breast cancer cells

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ABSTRACT: In order to develop an effective strategy of breast cancer therapy targeting survivin and its splice variants survivin-ΔEx3 and survivin-2B, the present study constructed four expression vectors by fusing the survivin antisense gene, the survivin (T34A) gene, the survivin-ΔEx3 antisense gene, and the survivin-2B gene with the enhanced green fluorescent protein (eGFP) gene. Each of these vectors was transiently transfected into the B-Cap-37 human breast cancer cell line. The effects of these four vectors with diverse genes on the proliferation and apoptosis of B-Cap-37 breast cancer cells were examined and compared *in vitro* using MTT and flow cytometry assays. Results of the MTT assay indicated that all four gene therapy plasmids were most effective at inhibiting the proliferation of B-Cap-37 cells 72 h after transfection. However, the four gene therapies had different rates of cell inhibition. pcDNA3.1(+)-egfp-anti-survivin and pcDNA3.1(+)-survivin (T34A)-egfp had almost equivalent or better effectiveness at suppressing cell growth. pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 moderately inhibited the growth of B-Cap-37 cells. In contrast, pcDNA3.1(+)-survivin-2B-egfp had limited inhibition of cell growth. Similar profile of effectiveness of four gene therapies in soliciting cell apoptosis was also observed. These results suggest the relative importance of targeting survivin and its splice variant survivin-ΔEx3 in breast cancer treatment.

Keywords: Survivin, splice variant, growth inhibition, apoptosis, breast cancer

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1. Introduction

Breast cancer is one of the most frequent female malignancies worldwide. Existing treatment modalities such as chemotherapy, radiotherapy, and chemotherapy are not always effective and curative and even cause significant side effects. In recent years, gene therapy has been incorporated in breast carcinoma treatment as a novel anti-tumor strategy and has had considerable success (*1,2*). Selecting the appropriate target and therapeutic strategy are key factors for an effective gene therapy.

Survivin, a member of the inhibitor of apoptosis (IAP) family, has attracted considerable attention as an ideal target for cancer treatment because it is highly and uniquely expressed in most human tumors and plays a critical role in both control of cell division and inhibition of apoptosis (*3-7*). A high level of survivin expression has been observed in breast carcinoma and has been found to be strongly associated with poor patient prognosis (*8-10*). This fact suggests that survivin may represent a promising target for breast cancer gene therapy. However, several survivin splice variants, such as survivin-ΔEx3 and survivin-2B, have also been identified in breast cancer thus far. Survivin-ΔEx3 is presumed to play a positive role in inhibiting apoptosis while survivin-2B might act as a proapoptotic factor in breast cancer and a high level of its expression is thought to be associated with a good prognosis (*11*). Targeting either, whether by decreasing the expression of survivin-ΔEx3 or increasing the expression of survivin-2B, may induce apoptosis and suppress proliferation in breast cancer cells, but their potential importance, especially when compared to survivin, to novel gene drug research and development remains unclear.

Several studies reported that inhibiting or blocking survivin activity by strategies like siRNA, antisense RNA, and dominant negative mutants promoted cell apoptosis in many cancers (*12-16*). In order to examine the effectiveness of targeting survivin and its splice variants and different gene therapy strategies in treating

breast cancer, four gene therapy plasmids expressing survivin (T34A) (the dominant negative mutant of survivin), antisense survivin, antisense survivin-ΔEx3, and survivin-2B were constructed and transiently transfected into the B-Cap-37 breast cancer cell line. The present study examined their inhibition of proliferation and induction of apoptosis in breast cancer cells.

2. Materials and Methods

2.1. *Cell line and cell culture*

Human breast carcinoma cell line B-Cap-37 is kindly provided by Dr. Yanhong Zhang (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). They were cultivated in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (100 IU/mL-100 μg/mL) and incubated at 37°C in a humid atmosphere (5% CO_2, 95% air).

2.2. *Vector construction and identification*

Total RNA was isolated from the B-Cap-37 breast carcinoma cell line with Trizol reagent (Promega, Madison, WI, USA). The cDNAs of survivin and two splice variants were amplified by RT-PCR using 5 μg total RNA with the RT-PCR kit (Promega) and primers P1 and P2 (Table 1). The amplified PCR products of survivin and its splice variants survivin-2B and survivin-ΔEx3 were introduced into a pGEM-T vector (Promega) after clean-up and A-tailing reaction with Pfu/An Amp Tailing Kit (Takara Bio, Otsu, Shiga, Japan), and the resultant plasmid was used to transform *E. coli* DH5α cells (EMD Chemicals Inc., Darmstadt, Germany). The transformants were cultured on an Luria broth (LB) plate with IPTG/X-gal and ampicillin and white clones were selected. The recombinant plasmids pGEM-T-survivin, pGEM-T-survivin-2B, and pGEM-T-survivin-ΔEx3 were identified with restriction endonuclease cleavage of *Eco*R I and *Xho* I. PCR amplification and sequence confirmation were performed (Invitrogen). Similarly, a fluorescent reported gene in the form of the enhanced green fluorescent protein (eGFP) gene was cloned from the pcDNA-eGFP vector (Clontech, Mountain View, CA, USA) by PCR with Pfu (a thermostable DNA Polymerase isolated from *Pyrococcus furiosus*) (Takara Bio) and then inserted into the pGEM-T vector. The primer pairs used were Pegf1a/Pegf1b and Pegf1a/Pegf2 (Table 1). pGEM-T-egfp was then selected and sequenced for confirmation.

2.3. *Gene therapy vectors construction and identification*

Survivin (T34A), a nonphosphorylated mimic of survivin generated though site-directed mutagenesis (Thr34→Ala), was obtained by overlapping PCR as described in a previous report (*17*). Survivin (T34A), antisense survivin, antisense survivin-ΔEx3, and survivin-2B were each subcloned with eGFP into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen) through multiple cloning sites. These vectors were then subjected to DNA sequence analysis for sequence confirmation.

2.4. *Establishment of cancer cells containing constructed vectors*

B-Cap-37 cells were seeded in 24-well plates (1×10^5 cells/mL, 500 μL per well) and grown to 50-60% confluence after growing overnight. Cells were transiently transfected with the four gene therapy vectors of pcDNA3.1(+)-survivin (T34A)-egfp, pcDNA3.1(+)-egfp-anti-survivin, pcDNA3.1(+)-egfp-anti-survivin-ΔEx3, and pcDNA3.1(+)-survivin-2B-egfp with FuGENE® 6 (Roche, Basel, Switzerland) under suitable conditions according to respective manuals. The pcDNA-eGFP vector was transfected into cells to serve as the control. Transfection efficiency was determined by fluorescence microscopy.

2.5. *MTT assay*

The established cells containing different vectors were seeded into 96-well plates and incubated normally. At the indicated times, cells were treated with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL and continuously incubated for 4 h at 37°C in a 5% CO_2 incubator. After the medium was carefully removed and DMSO 500 μL was added to each well, the cells were incubated at 37°C for 10 min. The cell absorbance of each well was determined at 570 nm with an Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) based on absorbance intensity at 485 nm. All experiments were repeated at least three times.

Table 1. Primers for plasmid design and construction

Target	Primer	Sequence (5'-3')	Restriction enzyme cutting site
Subcloning of survivin and its splice variants	P1	gaattcggccggctgcggggcattcgc	*Eco*R I
	P2	gtcgaattctcacaggtctgagcagcgatcctgcttgct	*Xho* I
Subcloning and identification of eGFP	Pegf1a	gacctcgagatggtgagcaagggcgaggagctg	*Xho* I
	Pegf1b	cgaggatccatggtgagcaagggcgaggagctg	*Bam*H I
	Pegf2	atgcggccgcgggccccttgtacagctcgtccatgccgag	*Not* I

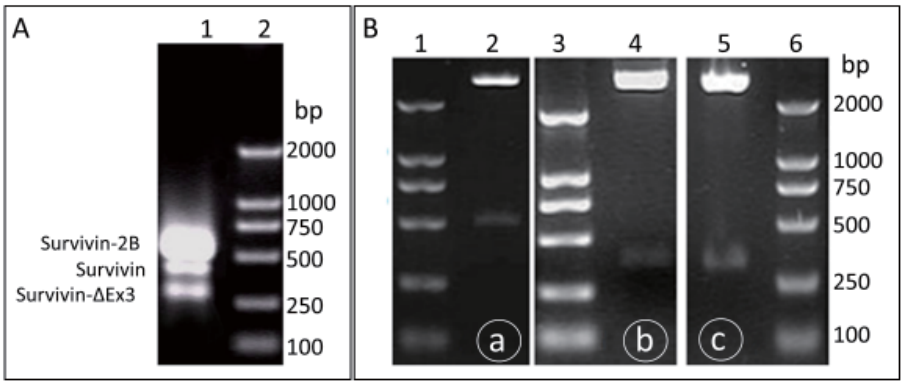

Figure 1. Agarose-gel electrophoresis of PCR products of survivin and splice variants gene (A) and target fragments in pGEM-T cleaved by restriction endonuclease *Eco*R I and *Xho* (B). (A) Lane 1: survivin-ΔEx3 with an MW of 320 bp, survivin with an MW of 429 bp, and survivin-2B with an MW of 510 bp. **Lane 2**: marker. **(B) a**, pGEM-T-survivin-2B; **b**, pGEM-T-survivin, **c**, pGEM-T-survivin-ΔEx3.

2.6. *Flow cytometry assay*

Flow cytometry was performed to examine the induction of apoptosis by various gene therapies in B-Cap-37 cells. Cells stained with propidium iodide (PI) (CycleTEST™ PLUS DNA Reagent Kit, Becton, Dickinson and Company, San Jose, CA, USA) were analyzed with a FACScan (Becton, Dickinson and Company) using Cell Fit software. All experiments were repeated at least three times. Apoptotic hypodiploid cells have less DNA than diploid cells in the G1 phase due to apoptosis-induced DNA fragmentation.

2.7. *Statistical analysis*

Data are shown as the mean ± S.D. and were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test (DMCT). The level of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. *Cloning and sequencing of cDNA of survivin, its splice variants, and eGFP*

cDNA of survivin and its splice variants survivin-2B and survivin-ΔEx3 was obtained from the B-Cap-37 breast carcinoma cell line using RT-PCR with Pyrobest DNA polymerase and total RNA as a template. Three amplified fragments of different sizes, approximately 500 bp, 400 bp, and 300 bp, are shown in Figure 1A. The PCR products were ligated into pGEM-T after clean-up and A-tailing reaction. The results of restriction enzyme digestion are shown in Figure 1B. The resulting pGEM-T were sequenced and the results revealed that the DNA fragment of about 500 bp was survivin-2B (510 bp), the fragment of about 400 bp was survivin (429 bp, GenBank accession No. CR541740), and the fragment of about 300 bp was survivin-ΔEx3 (320 bp). The nucleotide sequences were consistent with those published in the literature (*18*).

The eGFP gene with *Eco*R I/*Xho* I or *Not* I/*Xho* I restriction site was amplified by PCR and inserted into the pGEM-T vector. The process of identification was similar to that for survivin and its splice variants. Sequence analysis indicated that the 717 bp of eGFP were consistent with the known sequence and indicated that eGFP was ready for use in subsequent steps.

3.2. *Construction and verification of expression vectors*

To examine the effectiveness of targeting survivin and its splice variants in cancer treatment, four gene therapy plasmids, *i.e.* pcDNA3.1(+)-survivin (T34A)-egfp, pcDNA3.1(+)-egfp-anti-survivin, pcDNA3.1(+)-egfp-anti-survivin-ΔEx3, and pcDNA3.1(+)-survivin-2B-egfp, were successfully constructed *via* different connection strategies following a series of steps as shown in Figure 2. Restriction cleavage and sequencing revealed that the recombinant expression plasmids had the correct sequences and reading frames.

3.3. *Growth inhibition by four gene therapy plasmids in B-Cap-37 cells*

Four gene therapy vectors were transiently transfected into B-Cap-37 cells using the cationic liposome method. The expression of the target genes and reporter gene in the cells had already begun after 48 h of transfection. An MTT assay revealed almost no inhibition of cell growth 24 h after transfection, slight inhibition of cell growth 48 h after transfection, and maximum inhibition 72 h after transfection (Figure 3). However, the growth inhibition rates had decreased at 96 h compared to those at 72 h. This may be due to decreasing activity of the transfected expression vectors while untransfected cells continued to proliferate. The ratio of liposomes to DNA had no evident effect on transfection efficiency, regardless of whether it was 3:1 or 6:1.

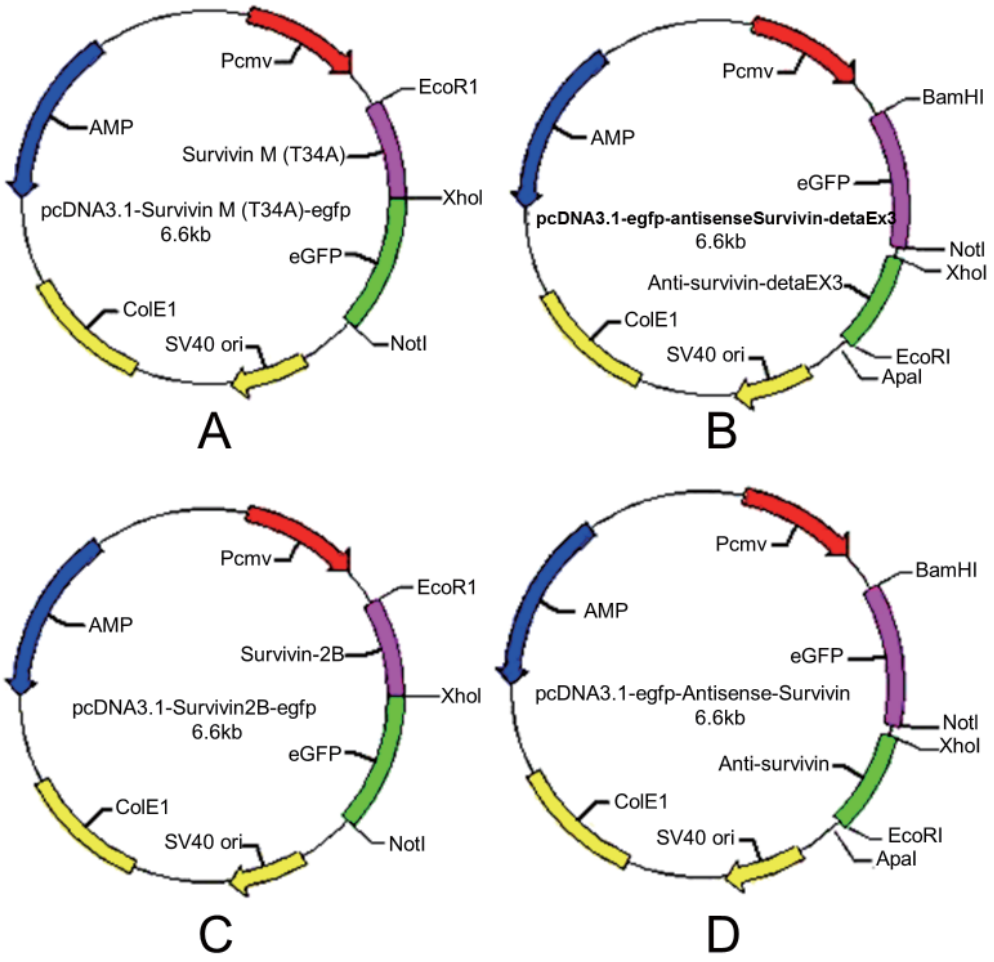

Figure 2. Construction of four gene therapy vectors simultaneously expressing target genes and the eGFP gene. (A) pcDNA3.1(+)-survivin (T34A)-egfp; **(B)** pcDNA3.1(+)-egfp-anti-survivin-ΔEx3; **(C)** pcDNA3.1(+)-survivin-2B-egfp; **(D)** pcDNA3.1(+)-egfp-anti-survivin.

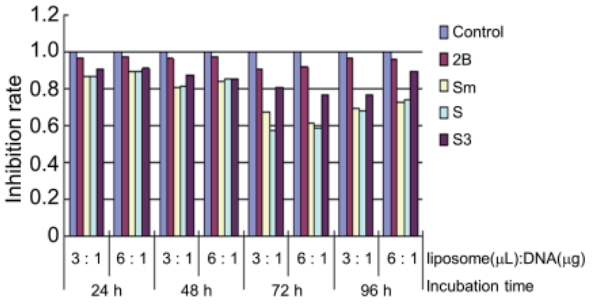

Figure 3. Effects of four gene therapy plasmids on cell proliferation. Cells were transiently transfected with four gene therapy plasmids and incubated for 24 h, 48 h, 72 h, and 96 h. Cell growth was evaluated with an MTT assay and the inhibition rate was calculated by comparison to the control in which cells were transfected with pcDNA-egfp.

Results of the MTT assay revealed that the four gene therapy plasmids had different levels of effectiveness at suppressing the growth of B-Cap-37 cells. pcDNA3.1(+)-egfp-anti-survivin and pcDNA3.1(+)-survivin (T34A)-egfp were most effective at inhibiting cell growth, with cell inhibition rates of 42% ($p < 0.01$ *vs.* control) and 39% ($p < 0.01$ *vs.* control), as were determined 72 h after transfection. pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 moderately inhibited growth in B-Cap-37 cells, as indicated by an inhibition rate of 34% ($p < 0.05$ *vs.* control). In contrast, pcDNA3.1(+)-survivin-2B-egfp had an inhibition rate of only 9.5% ($p > 0.05$) in B-Cap-37 cells 72 h after transfection (Figure 3).

3.4. *Apoptosis induction by four gene therapy plasmids in B-Cap-37 cells*

Cells transfected with various constructed plasmids were incubated for 72 h. Then, flow cytometric analysis was performed to determine the proportion of apoptotic cells by detecting hypodiploid cells. As shown in Figure 4, the sub-G0/1 population of B-Cap-37 cells transfected with pcDNA3.1(+)-egfp-anti-survivin increased significantly in comparison to the control and the percentage of hypodiploid cells reached 30.06 ± 3.25%. Similar results were obtained in B-Cap-37 cells transfected with pcDNA3.1(+)-survivin (T34A)-egfp, as indicated by an inhibition rate of 28.68 ± 3.25%. When

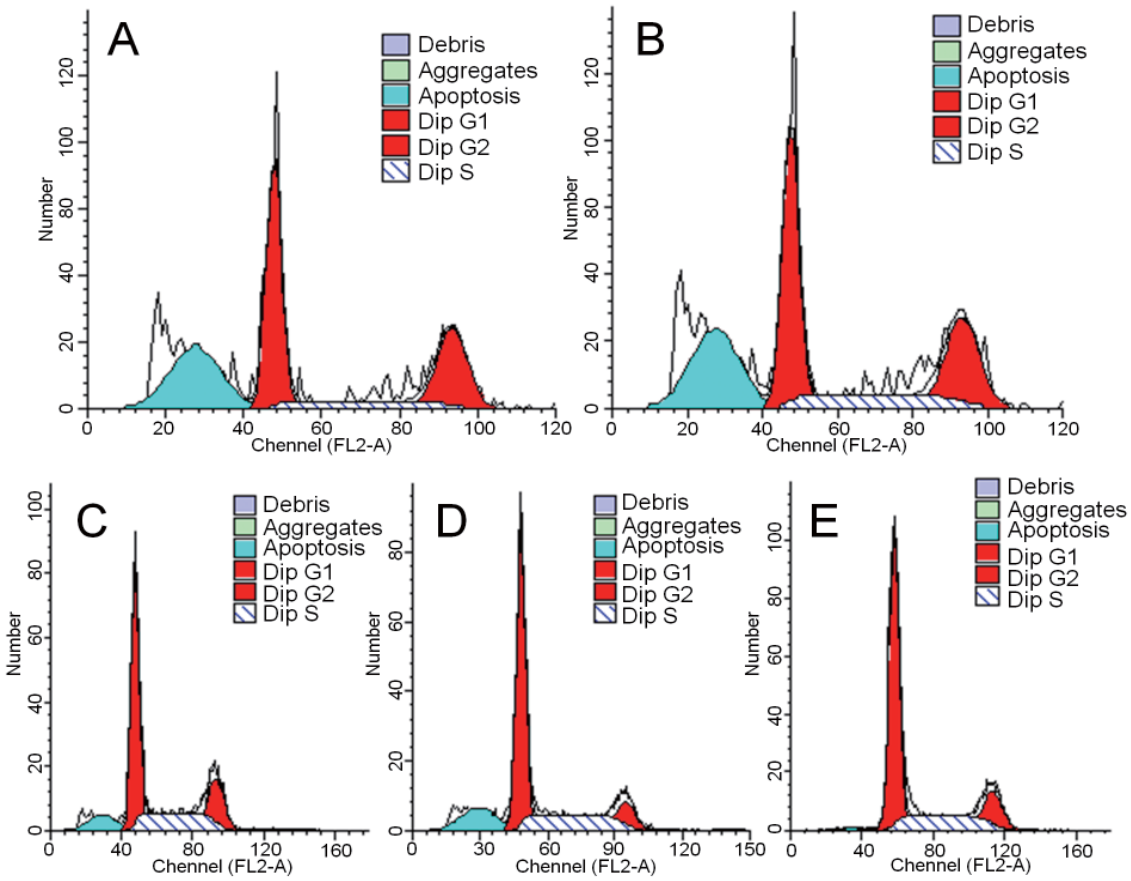

Figure 4. Induction of B-Cap-37 cell apoptosis by four gene therapy plasmids. Cells were transfected with four gene therapy plasmids and incubated for 72 h. The ratio of liposomes to DNA is 3:1. **(A)** pcDNA3.1(+)-survivin (T34A)-egfp; **(B)** pcDNA3.1(+)-egfp-anti-survivin-ΔEx3; **(C)** pcDNA3.1(+)-survivin-2B-egfp; **(D)** pcDNA3.1(+)-egfp-anti-survivin; **(E)** pcDNA-egfp.

cells were transfected with pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 and pcDNA3.1(+)-survivin-2B-egfp, the percentage of hypodiploid cells was found to be 14.66 ± 3.25% and 9.37 ± 3.25%. These results suggest that antisense survivin and survivin (T34A) were most effective at inducing cell apoptosis in B-Cap-37 cells.

4. Discussion

The present study constructed four plasmids expressing survivin (T34A), antisense survivin, antisense survivin-ΔEx3, and survivin-2B and transiently transfected each of these plasmids into the B-Cap-37 human breast cancer cell line. The inhibition of growth and induction of apoptosis by these gene therapy strategies was then investigated in this cell line. Results indicated that targeting survivin, by using either antisense survivin or the dominant negative mutant survivin (T34A), was most effective at suppressing cell growth and inducing cell apoptosis. Targeting survivin-ΔEx3 *via* use of its antisense RNA resulted in moderate anti-tumor action. In contrast, transfecting cells with plasmids expressing survivin-2B resulted in limited inhibition of growth and induction of apoptosis. These results suggest the feasibility of targeting survivin and/or its splice variant survivin-ΔEx3 in treating breast carcinoma.

Survivin and its splice variants, which are involved in multiple signal transduction pathways, have potential value in cancer diagnosis and treatment (*6,19*). In recent years, many strategies of targeting survivin for therapeutic purposes have been explored. The present research revealed that targeting survivin by using antisense RNA or its dominant negative mutant survivin (T34A) was almost equally effective at inhibiting cell growth and inducing cell apoptosis in B-Cap-37 cells. Like antisense survivin, the survivin mutant survivin (T34A) is also clearly a potential agent for breast cancer treatment. The effectiveness of survivin (T34A) may be because the dominant negative mutant competed with survivin, thus leading to phosphorylation-defective survivin (*14*). These results indicate that inhibiting or blocking survivin may be the first step for drugs designed to treat breast carcinoma.

In order to further define the roles of survivin splice variants survivin-ΔEx3 and survivin-2B and their biological significance in breast cancer, the present study constructed plasmids that expressed antisense survivin-ΔEx3 and survivin-2B for use as possible gene therapy agents. Compared to the control, antisense survivin-ΔEx3 significantly inhibited the proliferation and promoted the apoptosis of breast cancer cells *in vitro*. That said, splice variant survivin-2B had limited

anti-tumor action in B-Cap-37 cells, which was consistent with previous reports (*19-22*). These results suggest that survivin-ΔEx3 may also serve as a target for drugs to treat breast cancer.

In conclusion, this research has provided clues on the effectiveness of targeting survivin and its splice variants in treating breast carcinoma. Although the best strategy appears to be blocking survivin, designing treatments that inhibit both survivin and survivin-ΔEx3 may be a rational and comprehensive strategy for treating breast carcinoma.

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