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Enhancement of *Bik* Antitumor Effect by *Bik* Mutants

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Abstract

Bik was initially identified as a BH3-domain-only protein that interacts with E1B 19K. Although systemically administered wild-type *Bik* significantly inhibited tumor growth and metastasis in an orthotopic nude mouse model, the proapoptotic potency of Bik can be modulated by posttranslational phosphorylation. Here, we found that Bik mutants, in which threonine 33 and/or serine 35 were changed to aspartic acid to mimic the phosphorylation at these two residues, enhanced their binding affinity with the antiapoptotic proteins Bcl-X_L and Bcl-2 and were more potent than wild-type Bik in inducing apoptosis and inhibiting cell proliferation in various human cancer cells. *Bik* mutants also suppressed tumorigenicity and tumor-taking rate in a mouse *ex vivo* model. Moreover, *Bik* mutant-liposome complexes inhibited tumor growth and prolonged life span more effectively than the wild-type *Bik*-liposome complex in an *in vivo* orthotopic animal model. Thus, our results demonstrate that *Bik* mutant genes, more potent than wild-type *Bik*, induce cell death and suggest that their inhibition on the growth of various cancers should be explored further.

Introduction

Bik, also known as *nbk*, is a proapoptotic gene that contains only one of the Bcl-2 homology regions, the BH3[3] domain, and has been recognized recently as an essential initiator of apoptosis (1, 2). *Bik*-mediated apoptosis requires the *BAX* gene (3). In addition, *Bik* is implicated in the development of human breast and colorectal cancers by studies showing loss of informative alleles on chromosome 22q, where the *Bik* gene is located (4). The 18-kDa Bik protein interacts with E1B 19K and forms heterodimers with various antiapoptotic proteins, such as Bcl-2 and Bcl-X_L, and thus inhibits their antiapoptotic function (1). Bik is also a downstream apoptotic effecter of p53 in response to a physiological p53-mediated death stimulus provided by *E1A* (5, 6). Moreover, *Bik* can sensitize tumor cells to apoptosis mediated by certain chemotherapeutic agents (7), such as doxorubicin (8). All of these findings suggest that *Bik* is a potential therapeutic gene for human cancer. We demonstrated previously that the *Bik* gene, complexed with a nonviral gene delivery system when delivered i.v., significantly inhibited the growth and metastasis of human breast cancer cells implanted in nude mice and prolonged the life span of the treated animals (9). Bik is one of the BH3-only proteins, and many BH3 proteins differ in their expression patterns and activation (10, 11). For example, the apoptotic activity of Bik protein is regulated by phosphorylation (12). Phosphorylation at residues threonine 33 and serine 35 in Bik, possibly by a casein kinase II-related enzyme, is required for its full apoptotic activity. Furthermore, mutation of the phosphorylation sites whereby the threonine and serine residues were changed to alanine residues reduced the apoptotic activity of Bik. However, the molecular mechanism of this modification is currently unknown (12). In the light of these findings and because of our continuous effort to search for a more potent gene for cancer therapy, we have mutated the residues threonine 33 and serine 35 of *Bik* to aspartic acid to mimic phosphorylation to create potentially constitutively active forms. We found that these *Bik* mutants enhanced their association with Bcl-2 and Bcl-X_L. Moreover, these mutants exhibited a greater apoptotic activity *in vitro* and in antitumor activity in an *in vivo* animal model than wild-type *Bik*. Therefore, these *Bik* mutants are more potent than wild-type *Bik* in a gene therapy setting.

Materials and Methods

Cell Lines. Human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-468, ovarian cancer cell SKOV-ip1, prostate carcinoma cell PC-3, non-small cell H1299, pancreatic cancer cell PANC1, and 293T cells were purchased from American Type Culture Collection (Rockville, MD) and cultured according to the vendor's instructions.

Plasmid Construction and Site-directed Mutagenesis. *Bik*, *Luc*, and *GFP*-expressing plasmids were constructed by inserting the cDNAs of *Bik*, *Luc*, and *GFP*, respectively, into the pcDNA3 vector containing a CMV promoter. Site-directed mutagenesis was performed according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA). Threonine 33 and serine 35 of *Bik* were changed to aspartic acid by using the following primers: for T33D, 5′-GTTCTTGGCATGGACGACTCTGAAGAGG-3′ and 5′-CCTCTTCAGAGTCGTCCATGCCAAGAAC-3′; for S35D, 5′-GGCATGACTGACGACGAAGAGGACCTG-3′ and 5′-CAGGTCCTCTTCGTCGTCAGTCATGCC-3′; and for T33DS35D (DD), 5′-GTTCTTGGCATGGACGACGATGAAGAGGACC-3′ and 5′-GGTCCTCTTCATCGTCGTCCATGCCAAGAAC-3′. The sequences of the three *Bik* mutant constructs were confirmed by automated sequencing.

Formulation. The nonviral gene delivery system, SN, was essentially a cationic liposome, which is formulated as described elsewhere (9).

Transfection. Cell transfections were performed as described previously (9).

Western Blot Analysis. Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 10 μg/ml leupeptin. The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with primary goat anti-Bik polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The immunoblots were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ).

***In Vitro* Direct Protein-binding Assay.** For fusion protein-binding assays, GST-Bcl-2 and GST-Bcl-X_L plasmid DNAs were transformed into BL21 DE3 cells and induced with 0.5 mM isopropyl-β-D-thiogalactosidase. A 10-ml aliquot of culture was analyzed by SDS-PAGE to evaluate fusion protein induction. The remaining culture was sonicated on ice with 10 short bursts at 10 s each and clarified by centrifugation, and the supernatant was resuspended

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[3] The abbreviations used are: BH3, Bcl-2 homology region 3; CMV, cytomegalovirus; GST, glutathione *S*-transferase; wt, wild-type; Luc, luciferase; MFP, mammary fat pad; GFP, green fluorescence protein.

in 50% (vol/vol) glutathione-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). An aliquot of the protein-bound beads was analyzed by SDS-PAGE to ensure that equal amounts of pure fusion protein were present.

In vitro binding assays were performed by incubating equal amounts of GST, GST-Bcl-2, and GST-Bcl-X_L, immobilized onto glutathione-Sepharose beads, with *in vitro*-translated Bik wt or mutant (T33S35D), which was labeled with [^{35}S]methionine using the TNT coupled reticulocyte lysate system (Promega Corp., Madison, WI). The mixture, which was diluted in 0.5 ml of the RIPA-B cell lysis buffer, was incubated for 3 h at 4°C under gentle agitation and washed five times with lysis buffer, and the binding complexes were dissolved in SDS-PAGE buffer. All samples were boiled for 5 min and were resolved by 15% SDS-PAGE. The gels were dried and exposed to X-ray films.

Luc Assay. To determine the Bik dose effect on cell proliferation, different cancer cells were cotransfected with 50 ng of CMV-*Luc* and increasing amounts (0, 0.5, or 2 μg) of CMV-*Bik*. The total amount of DNA transfected at each dose was kept constant (2.05 μg) by adding an appropriate amount of pcDNA3 vector. Forty-eight h after transfection, cells were harvested, and *Luc* activity was measured by using the Luc assay system (Promega) according to the manufacturer's protocol. The relative activities were calculated by setting the *Luc* activities obtained from transfections without CMV-*Bik* (0 μg) at 100%. The data represent mean ± SD of three independent experiments.

Apoptosis Assay. For *in vitro* studies, standard fluorescence-activated cell sorter analysis was used to identify apoptotic cells. Briefly, cells were transfected with SN-*Bik* or other agents. Twelve or 24 h after transfection, the apoptotic cells were assessed by flow cytometric detection of sub-G_1 DNA content after being stained with propidium iodide.

***Ex Vivo* Tumor Inhibition Assay.** An *ex vivo* tumor inhibition assay was performed as described elsewhere (9). Female nude mice were inoculated with 4×10^6 breast cancer MCF-7 cells into the MFP, and male nude mice were inoculated with 1×10^6 prostate cancer PC-3 cells into s.c. connective tissue.

Antitumor Activity Assay. The antitumor activity assay was done as described previously to study tumor growth inhibition by *BikDD* (9).

Statistical Analysis. All data analyses used two-sided log-rank statistical tests.

Results and Discussion

To search for forms of *Bik* that would be more potent than wt *Bik* in cancer gene therapy, three *Bik* mutants were constructed by site-directed mutagenesis. We replaced *Bik* residues threonine 33, serine 35, or both with aspartic acid (T33D, S35D, and T33DS35D) to mimic constitutive phosphorylation. Expression efficiencies of these mutants were similar to that of wt *Bik* (Fig. 1*A*). Because the BH3-domain-only proapoptotic protein Bik can bind to the antiapoptotic protein Bcl-2 and its relatives, such as Bcl-X_L, to initiate apoptosis, we examined the binding affinity of mimicking phopsphorylation of *Bik* with Bcl-2 and Bcl-X_L. After using the purified GST Bcl-2 and Bcl-X_L fusion protein to pull down the *in vitro* translated Bik wt and Bik T33S35D (Bik DD) mutant proteins labeled with [^{35}S]methionine, we found that the double mutant, which mimics constitutive phosphorylation, enhanced the binding affinity with the antiapoptotic proteins Bcl-2 and Bcl-X_L by about 2–3-fold (Fig. 1*B*). This result indicated that the BH3-only protein Bik was regulated by posttranslational phosphorylation, and that mimicking phosphorylation might result in conformational changes from an inactive form to the constitutive active form, and thus the BikDD mutant increased its affinity or accessibility to antiapoptotic Bcl-2 homologs. We also measured the mutants' abilities to induce apoptosis by fluorescence-activated cell sorter analysis; after transfection in MCF-7 and PC-3 cells, the *Bik* mutants induced more apoptosis (40–80%) than wt *Bik* (Fig. 1*C*). The onset of apoptosis occurred earlier in cells transfected with *Bik* mutants than in those transfected with wt *Bik*, as early as 8 h after transfection. These results indicate that *Bik* mutants can induce significant cancer cell apoptosis, with both greater potency and speed than wt *Bik*. Opposite to the circumstance of *Bad* (13), phosphorylation increases the proapoptotic potency of *Bik*.

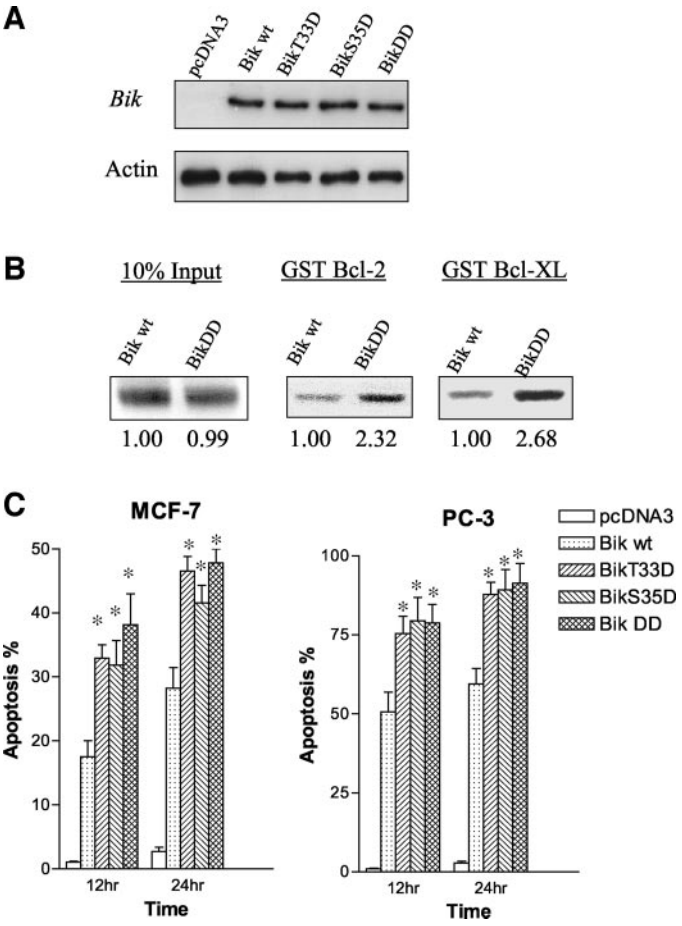

Fig. 1. The *Bik* mutants exhibited stronger *in vitro* binding affinity with the antiapoptotic proteins Bcl-2 and Bcl-X_L and enhanced the cell-killing effect, compared with wt *Bik* in two different human cancer cell lines. *A*, Western blot analysis of wt and mutant Bik protein expression after transient transfection with wt or mutant Bik in 293T cells. Actin was used as an equal loading control. *B*, *in vitro* binding assay showed that the Bik mutant T33S35D (*BikDD*) exhibited stronger *in vitro* binding affinity with the antiapoptotic proteins Bcl-2 and Bcl-X_L. The GST-Bcl-2 and GST-Bcl-X_L fusion proteins were incubated with an *in vitro*-translated wt Bik and double-mutant BikDD labeled with [^{35S}]methionine in immunoprecipitation buffer, then recovered on glutathione beads, and analyzed by SDS-PAGE. The intensity of double mutant, relative to the wt is shown. *C*, the human breast cancer cell line MCF-7 and the prostate cancer cell line PC-3 were cotransfected with 100 ng of CMV-*GFP* and 2 μg of CMV-*Bik* wt or mutants. pcDNA3-transfected cells were used as a control. Twelve or twenty-four h after transfection, the apoptotic cells were harvested and assessed by flow cytometric detection of sub-G_1 DNA content after being stained with propidium iodide. The data represent means of three independent experiments; *bars*, SD. ∗, significant difference compared with wt ($P < 0.05$).

We then tested the ability of the *Bik* mutants to inhibit the growth of human cancer cell lines *in vitro*. Using a luciferase assay as an index for cell survival, the *Bik* mutants were shown to be more potent than wt *Bik* in multiple cancer cell lines including lines from cancers of breast, ovary, prostate, lung, and pancreas (Fig. 2). In general, all three *Bik* mutants appeared to exert stronger growth-inhibitory effects than wt *Bik*; the *BikDD* seemed to be more potent. During site-direct mutagenesis, an unexpected triple mutant T33DS35DD102E(DDE) formed, in which Asp-102 was changed to Glu, in addition to the double mutation of Thr-33 and Ser-35. This *BikDD* activity was found to be similar to that of *BikDD* and stronger than the wt *Bik* in multiple cancer cell lines (data not shown), suggesting that the Asp to Glu mutation does not alter its activity and further supports that the double mutations in Thr-33 and Ser-35 result in higher activity.

To test whether the *Bik* mutants also had greater antitumor activity than wt *Bik*, we first performed an *ex vivo* tumorigenicity assay in nude mice. The human breast cancer cell line MCF-7 and the prostate

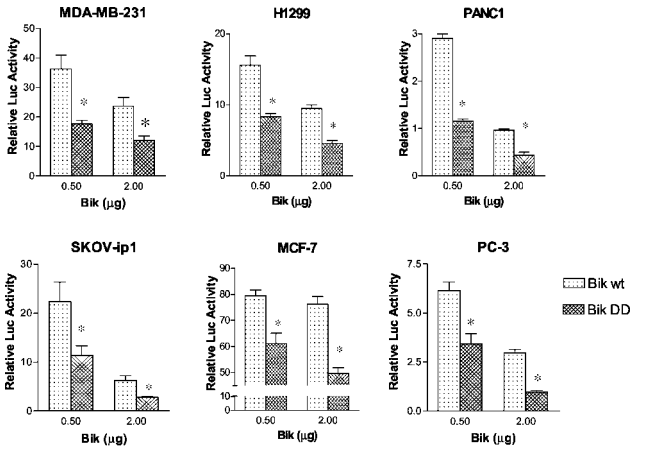

Fig. 2. The expression of the *Bik* mutant exhibited stronger growth-inhibitory effects than wt *Bik* in different human cancer cell lines. *A*, comparison of the growth-inhibitory effects between the *Bik* wt and *BikDD* mutant in different human cancer cell lines. Human breast cancer cell lines MDA-MB-231 and MCF-7, the ovarian cancer cell line SKOV-ip1, the small lung cancer cell line H1299, the pancreatic cancer cell line PANC1, and the prostate cancer cell line PC-3 were cotransfected with 50 ng of CMV-*Luc* and increasing amounts (0, 0.5, or 2 μg) of CMV-*Bik*, wt or mutants. The relative activities were calculated by setting the *Luc* activities obtained from transfections without CMV-*Bik* (0 μg) at 100%. The data represent means of three independent experiments; *bars*, SD. *, significant difference compared with wt ($P < 0.05$).

cancer cell line PC-3 were transfected with either CMV-*Bik* wt or the *Bik* mutants in culture plates. Cells transfected with pcDNA3 vector were used as a control. Twenty-four h later, the treated cells were harvested and then inoculated into the MFP of female nude mice (for MCF-7) or s.c. connective tissue of male nude mice (for PC-3). We found that the *Bik* mutant transfectants had less tumor growth potential *ex vivo* (Fig. 3*A*) and lower tumor-taking rates (Fig. 3*B*) than wt *Bik* transfectants. Tumor growth from *Bik* mutants' transfectants in nude mice was delayed by at least 3 weeks compared with that from the pcDNA3 control transfectants. The tumor volume ratios of wt *Bik* mice and *Bik* mutant mice during week 7 ranged from 2.1 to 3.7 for MCF-7 cells and from 2.2 to 4.1 for PC-3 cells. The average tumor weight in mice treated with wt *Bik* was about 2- to 4-fold greater than that of mice treated with *Bik* mutant (Fig. 3*B*).

These results show that the three *Bik* mutants inhibited tumor cell growth better than wt *Bik in vitro* and *ex vivo* and, of the three *Bik* mutants, *BikDD* appeared to be the most potent one. To further compare the antitumor activities in a gene therapy setting, we tested the *BikDD* and wt *Bik* genes in a previously established animal tumor model (9). Mice with established tumors were treated with wt *Bik*, *BikDD*, or *pcDNA3* control by using the SN nonviral delivery system. Treatment with *BikDD* inhibited tumor growth in mice to a significantly greater extent than that of wt *Bik* or *pcDNA*. By weeks 3–5, the mean tumor volume of wt *Bik*-treated mice was larger than that of *BikDD*-treated mice in both models. The most significant tumor suppression effect was observed by weeks 8 and 9, with a 2–3-fold difference in tumor volumes between the wt and mutant treatment groups ($P < 0.05$; Fig. 4*A*). Treatment with *BikDD* yielded a significantly higher survival rate among the treated mice than treatment with wt *Bik* ($P < 0.05$; Fig. 4*B*). These median survival times were 175 days for *BikDD*-treated mice and 112 days for wt *Bik*-treated mice with MCF-7 tumors; the median survival times were 140 days for

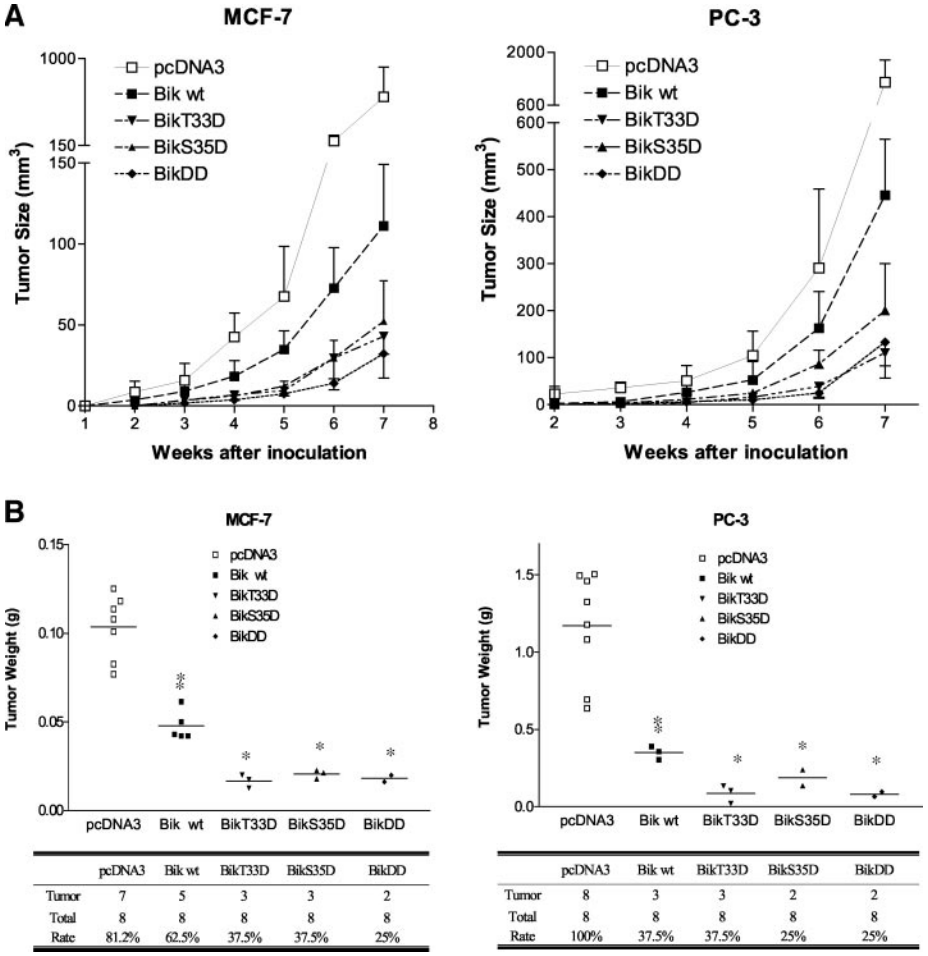

Fig. 3. The *Bik* mutants exhibited stronger tumor-suppressive effects in an *ex vivo* assay than wt *Bik*. The human breast cancer cell line MCF-7 (4×10^6 cells) and the prostate cancer cell line PC-3 (1×10^6 cells) were transfected with CMV-*Bik*, wt or mutants, delivered by SN liposome in culture plates. Mice inoculated with MCF-7 cells were implanted with a 0.72-mg 17-β-estradiol pellet 2 weeks before inoculation. pcDNA3-transfected cells were used as a control. Twenty-four h later, the treated cells were harvested and inoculated in MFP (for MCF-7) or s.c. connective tissue (for PC-3) of nude mice, with eight mice in each group. Tumor sizes were measured weekly, as shown (*A*). After 7 weeks, the mice were sacrificed, and the tumor weight and tumor-forming rate were measured as shown (*B*). *Bars*, SD. *, mutant tumors whose sizes were significantly different than wt tumors ($P < 0.05$); **, wt *Bik* whose sizes were significantly different than pcDNA3 control tumors ($P < 0.05$).

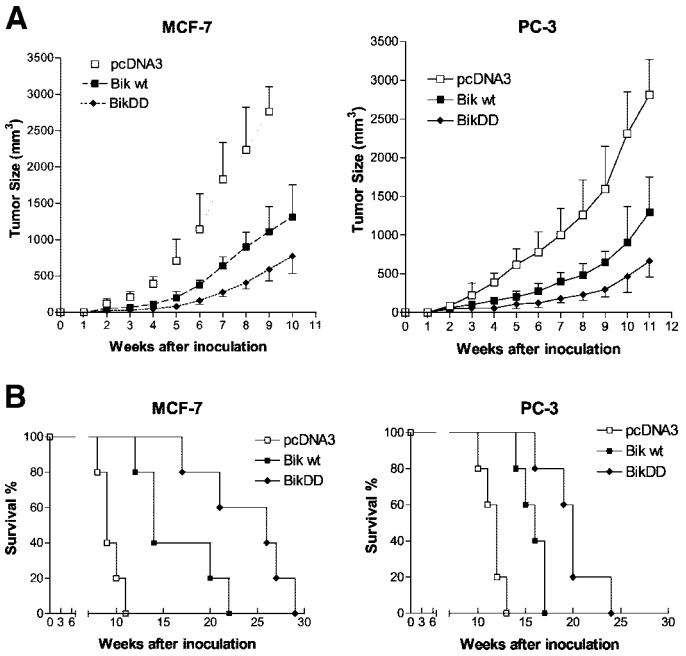

Fig. 4. The *BikDD* mutant gene delivered by SN significantly inhibited the growth of human tumors in mice. *A*, models used were orthotopic human breast cancer MCF-7 cells (injected into the MFP, 4×10^6 cells/mouse), where each mouse was implanted s.c. with a 0.72-mg 17-β-estradiol pellet 2 weeks before inoculation, and the model of ectopic human prostate cancer PC-3 cells (injected s.c., 1×10^6 cells/mouse) in mice. Two weeks later, the mice bearing tumors were randomly divided into three groups with five mice in each. One group received multiple injections of SN-*BikDD* DNA (15 μg of DNA/mouse), three times a week for a total of 12 treatments, orthotopic breast cancer by i.v. injection, and ectopic prostate cancer by intratumor injection; the other group received the same doses of SN-*pcDNA3*. Tumor volumes were measured weekly. *Bars*, SD. *B*, the mutant *Bik* gene delivered by SN significantly prolonged the life of mice with orthotopic or ectopic human cancers ($P < 0.05$). The data shown represent the mean ± SD from five individual mice.

BikDD-treated mice and 105 days for wt *Bik*-treated mice with PC-3 tumors. The results indicate that *BikDD* inhibited ~50% of the tumor growth and significantly increased survival rates, compared with wt *Bik*.

Taken together, we found that the *BikDD* mutant enhanced the binding affinity with antiapoptotic proteins Bcl-X_L and Bcl-2 and is more potent than wt *Bik* in inducing apoptosis and inhibiting cell proliferation. We also demonstrated that the three *Bik* mutants are more potent than wt *Bik* in both i.v. (MCF-7, mammary tumor model) and intratumor (PC-3, s.c. tumor model) treatments. The *BikDD* mutant was further shown to be more effective than wt *Bik* in inhibiting tumor development and prolonging survival in a gene therapy setting. Thus, the *BikDD* mutant may serve as a more effective agent than wt *Bik* for cancer gene therapy.

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