Inhibition of Cancer Cell Growth by BRCA2

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Abstract

The breast cancer susceptibility gene BRCA2 has been suggested to function as a “caretaker” of the genome. Cells without wild-type BRCA2 are deficient in repairing DNA damage. However, whether BRCA2 can also suppress oncogenesis by regulating cell proliferation remains to be determined. To address this question, the expression of wild-type BRCA2 protein was reconstituted, in an either constitutive or regulated manner, in the pancreatic cancer cell line Capan-1, which expresses only a mutant BRCA2. Expression of wild-type BRCA2 inhibited cell proliferation in culture and suppressed tumor growth in animals. Our results showed that, in addition to the DNA repair function, BRCA2 also suppresses tumor development by inhibiting cancer cell growth.

Introduction

People carrying germ-line mutations of the breast cancer susceptibility gene BRCA2 have increased risk for breast, ovarian, pancreatic, and other types of cancer (1–3). Tumors developed in heterozygous BRCA2 mutation carriers are frequently associated with loss of heterozygosity at the BRCA2 locus, a result consistent with a critical function of BRCA2 in tumor suppression. BRCA2 has been suggested to be a “caretaker” and to play an important role in maintaining genomic integrity (4). Cells without a wild-type BRCA2 gene are deficient in repairing the DNA damage caused by genotoxic agents, such as ionizing radiation (5–11). We and others have shown that the expression of BRCA2 is tightly regulated in a cell cycle-dependent manner, with an expression level low in G1 phase and peaked in S-G2 phases of cell cycle. Because the signaling of DNA damage repair is usually coupled with cell cycle progression, the question of whether BRCA2 can also regulate cell proliferation is intriguing and remains to be determined (12, 13). To address this question, we expressed the wild-type BRCA2 protein in Capan-1 cells. Capan-1 is a human pancreatic-cancer cell line that expresses only a COOH-terminal truncated BRCA2 protein (14, 15). We established and characterized stable transfec tants of Capan-1 cells that expressed wild-type BRCA2 either constitutively or through a tetracycline-regulated expression system. Our results showed that, in addition to the DNA repair function reported previously, BRCA2 also involved in the negative regulation of cell proliferation in vitro and tumor growth in vivo.

Materials and Methods

BRCA2 Expression Plasmids. We isolated the cDNA for the entire coding region of BRCA2 by RT-PCR. Because of its large size, the BRCA2 coding region was divided into four fragments for RT-PCR. Four to 10 clones of each amplified fragment were sequenced to identify those that did not contain any mutation resulting from the PCR reaction. These fragments were ligated sequentially together to obtain the full-length cDNA for BRCA2. The XhoI restriction site was engineered at both ends of the assembled BRCA2 cDNA. To facilitate the assembly of the full-length cDNA of BRCA2, codon 798 was changed from CTC to CCT to create a HindIII restriction site; however, this change does not alter the encoded amino acid. To construct pCINBRCA2, the BRCA2 cDNA was inserted at the XhoI site of an expression vector pCIN (16). To construct p236BRCA2, the pcDNA3 vector (Invitrogen, Calsbad, CA) was first modified by inserting a 236-bp fragment of the 5′ untranslated region of BRCA2 between the KpnI and NotI sites. The assembled full-length BRCA2 cDNA was then inserted at the XhoI site of this plasmid. The 5′ untranslated region of BRCA2 was obtained by RT-PCR using primers 5′-GGTACGGTGGCCGAGTTCTCTGA-3′ and 3′-GGGGCGCCCAACTACGATATTCCTCCAAT-3′.

Generation of Wild-type BRCA2-expressing Capan-1 Derivatives. The stable cell line CINBRCA2 was generated by transfecting Capan-1 cells with pCINBRCA2, and the cell clone 236BRCA2 was generated by transfecting Capan-1 cells with p236BRCA2. Plasmid DNA (10 μg) was mixed with the cationic liposome DC-Chol at a ratio of 1 μg DNA:13 nmol of DC-Chol (17). The DNA/liposome complex was then added to the cell culture dish and incubated for 16 h. Transfected cells were cultured for 3 days before subjected to G418 (500 μg/mL) selection. BRCA2TN, neoTN-1, and neoTN-2 were obtained by transfecting Capan-1 cells with pTA-IRES-Neo (18) together with a derivative of pUHD10–3 (19) that expressed BRCA2 at 1:9 ratio using Lipofectamine Plus (Life Technologies, Inc.), then selected with G418. Resulting clones were screened for BRCA2 protein expression using anti-BRCA2 antibodies. Both neoTN-1 and neoTN-2 expressed the tetracycline-controlled transactivator but did not express wild-type BRCA2 protein (data not shown).

Detection of the Expression of Exogenous BRCA2. To detect the expression of the exogenous BRCA2 RNA, a 524-bp BRCA2 cDNA fragment containing codon 798 was amplified by RT-PCR and was digested with the restriction enzyme HindIII. The RT-PCR product of the exogenous BRCA2 could be digested by HindIII to generate two fragments of 255 bp and 265 bp because of the presence of an engineered HindIII site. The RT-PCR product of the endogenous BRCA2 RNA lacked this HindIII site and remained intact as a 524-bp fragment.

Immunoblotting was used to detect BRCA2 protein. Cells were harvested and lysed in ice-cold NETN [150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.5% NP40] or radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP40] buffer. Total cell protein extracts were quantified by the Bradford assay (Bio-Rad, Hercules, CA.). Equal amounts of lysate protein (60–120 μg) were separated by SDS-PAGE and blotted to polyvinylidene fluoride membrane (Bio-Rad). Proteins were then identified using BRCA2 antibodies N61 (15) or Ab-2 (Oncogene Science, Cambridge, MA).

Determination of Cell Growth Rate. Thymidine incorporation assay was used to measure DNA synthesis. Cells (250) were plated in each well of a 96-well plate and cultured for 48 h, then incubated with 1 μCi of [methyl-3H] thymidine (Amersham, Arlington Heights, IL.) in the presence of serum for 16 h. Cells were harvested, and the amount of incorporated [methyl-3H] thymidine was measured with a scintillation counter (Beckman). To measure the rate of cell number increasing, 2000 cells were plated in each well of a 96-well plate. Cell number of the culture at different days after plating was determined by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Clonogenic assay was also used to measure the growth rate of cells. Cells were plated in a six-well plate at a density of 1000 cells/well and allowed to grow for 2.5 weeks with media changed every 2 days. For tetracycline-regulated Capan-1 derivatives, cells were cultured in the tetracy-
cline-containing media all of the time or in the tetracycline-free media beginning 2 days after plating.

**Tumorigenicity Assay and Culture of Tumor Cells.** Cells were harvested by trypsinization, washed with PBS, then suspended in PBS at the density of 1 × 10^7 cells/ml. Cell suspension was injected s.c. into both flanks of female nude mice of 6-8 weeks of age. Tumor volumes were determined by external measurement in two dimensions and calculated using the equation

\[ V = \frac{L 	imes W^2}{2} \times 0.5, \]

where \( V \) is volume, \( L \) is length, and \( W \) is width. To recover the cells from the 236BRCA2-derived tumor, the tumor was resected, chopped, and digested with trypsin, then plated for culturing. Individual colonies and a pool of the tumor cells were obtained. Only cells of the early passages (two to six passages) were used for molecular characterization. Animal care was performed in accord with institution guidelines.

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**Fig. 3. Inhibition of Capan-1 growth by inducible wild-type BRCA2 expression.**

A. tetracycline-regulated BRCA2 expression in BRCA2TN. Lysates were prepared from BRCA2TN cells grown in the presence (Uninduced) or absence (Induced) of tetracycline for 1 or 2 days as indicated. The lysate of MCF-7, a breast cancer cell line that expresses wild-type BRCA2 protein (15), was included as a positive control. BRCA2 was detected by immunoblotting using N61 antibody. Arrow and *, the full-length BRCA2 and endogenous truncated BRCA2 in Capan-1 cells, respectively. B, suppression of BRCA2TN growth by the wild-type BRCA2. Cells (1000) from each of the indicated Capan-1 derivatives were plated in each well of six-well plates in medium containing tetracycline. Cells were grown for 2.5 weeks, either in media containing tetracycline (U) or in tetracycline-free media (I) beginning 2 days after plating. The resulting colonies were stained with crystal violet. The results shown are from a triplicate experiment. C, flow cytometry analysis of the BRCA2 inducible, as well as the control cell lines. Numbers, the percentage of cells in each cell cycle stage. Data shown are derived from a representative experiment. Data shown are derived from a representative experiment. The percentage of each cell cycle stage added up to be 100% and was determined independently from the subG0 measurement. U, uninduced; I, induced.

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**Fig. 1. Generation of constitutive wild-type BRCA2-expressing Capan-1 derivatives.**

A, expression of wild-type BRCA2 protein. Proteins isolated from a positive control cell line (U-2 OS) that expressed endogenous wild-type BRCA2, two negative control cell lines (Capan-1 and Capan-1/neo), and the two BRCA2-expressing clones (CINBRCA2 and 236BRCA2) were separated using SDS-PAGE on a 6% gel. BRCA2 proteins were detected by the monoclonal antibody N61 that recognized the NH2-terminal region of the BRCA2 (15). Arrows, the full-length BRCA2; *, the endogenous truncated BRCA2 in Capan-1 cells. B, expression of exogenous BRCA2 mRNA. BRCA2 RNA isolated from the parental cells (Capan-1), the vector-transfected cells (Capan-1/neo), and the two BRCA2-expressing derivatives (236BRCA2 and CINBRCA2) was amplified using RT-PCR. The RT-PCR products were digested with HindIII and resolved on an agarose gel. The RT-PCR product of the exogenous BRCA2 could be digested by HindIII to generate two fragments of 255 and 265 bp because of the presence of an engineered HindIII site and remained intact as a 524-bp fragment. The HindIII-digested PCR product from a plasmid carrying the BRCA2 cDNA was included as a positive control. Arrow, the digested small fragments, which were not resolved in this gel. RT, reverse transcriptase.

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**Fig. 2. Inhibition of Capan-1 cell growth in vitro by constitutive wild-type BRCA2 expression.**

A, cell growth assay using [3H]thymidine incorporation. The results shown are from a quadruplet assay. B, determination of cell growth by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. The results shown are from a quadruplet assay. C, clonogenic assay for cell growth. Cells (1000) from the indicated Capan-1 derivatives were plated in each well of six-well plates. The cells were allowed to grow for 2.5 weeks, and the resulting colonies were stained with crystal violet. The results shown are from a triplicate experiment. D, flow cytometry analysis of BRCA2 transfectants. Numbers, the percentage of cells in each cell cycle stage. Data shown are derived from a representative experiment. The percentage of each cell cycle stage added up to be 100% and was determined independently from the subG0 measurement.

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Results and Discussion

To investigate the possible role of wild-type BRCA2 in regulating cell growth, we generated Capan-1 derivatives that express wild-type BRCA2. We did not use approaches that transiently express BRCA2 in Capan-1 cells, such as transient transfection or virus-mediated gene transfer, because these approaches would not allow us to study the phenotypes of BRCA2-expressing Capan-1 cells for a long period of time. In addition, the Capan-1 cell line is known to have very low transfection efficiency (7), and the size of the expression vector (e.g., recombinant viral vectors) is beyond the packaging capacity of virus when using commonly used recombinant viral vectors.

We first obtained two Capan-1 derivatives (236BRCA2 and CINBRCA2) that constitutively expressed wild-type BRCA2 after screening ~400 G418-resistant clones by immunoblotting (Fig. 1A). The level of full-length BRCA2 protein in clone 236BRCA2 was higher than that of CINBRCA2. The expression of wild-type BRCA2 protein was confirmed by using immunoprecipitation followed by immunoblotting with different BRCA2 antibodies (data not shown). The expression of the exogenous BRCA2 RNA in these two clones was also demonstrated by RT-PCR followed by HindIII digestion (Fig. 1B). The growth rate of these two BRCA2-expressing clones correlated with the expression levels of wild-type BRCA2 protein, and both clones grew slower than the parental Capan-1 cells and a control Capan-1 derivative (Capan-1/neo), which was transfected with an empty vector (Fig. 2). These results suggested that high-level expression of BRCA2 in the Capan-1 cells was not compatible with cell growth and might partially explain the difficulty in obtaining Capan-1 derivatives that constitutively expressed wild-type BRCA2 protein.

To rule out the possibility that the reduced growth rate of BRCA2-expressing derivatives was simply because of clonal variation and confirm that the cell growth suppression was the consequence of expressing wild-type BRCA2, we established a Capan-1 derivative (BRCA2TN) that expressed wild-type BRCA2 protein under the regulation of tetracycline (Fig. 3A). The growth inhibition was unlikely simply because of the activation of the tetracycline-controlled transactivator, because the growth of two control cell lines (neoTN-1 and neoTN-2) that did not express BRCA2 but expressed functional tetracycline-controlled transactivator (data not shown) was not affected by the removal of tetracycline (Fig. 3B). Capan-1 cells are highly tumorigenic in nude mice. In a preliminary experiment, we found that expression of wild-type BRCA2 suppressed tumorigenesis, and the level of suppression correlated with the level of BRCA2 (data not shown). This observation was confirmed in a subsequent experiment. Of 10 inoculations, 236BRCA2 cells re-

![Fig. 4. Inhibition of Capan-1 cell growth in vivo by the wild-type BRCA2 gene. A, Capan-1/neo or 236BRCA2 cells (1 × 10^5) were inoculated s.c. into both flanks of each mouse (five mice in each group), and the tumor number and volume were determined starting 6 weeks after inoculation. The number of tumors resulting from each cell line is shown at each time point (Capan-1/neo/236BRCA2). One tumor in each group was lost for unknown reasons, and both tumors were very small and grew slowly. +, the time of the loss. Additionally, the number of Capan-1/neo tumors was reduced later in the experiment because of the death of animal. B, survival curves of the mice bearing Capan-1/neo- or 236BRCA2-derived tumors as described in A. The only remaining 236BRCA2-derived tumor was dissected at week 28, and tumor cells were recovered. Insert, the expression of wild-type BRCA2 protein was decreased in the tumor-derived cells (Lane 2) compared with that in the original 236BRCA2 cells (Lane 1). Arrow and *, the full-length BRCA2 and endogenous truncated BRCA2 proteins in Capan-1 cells, respectively. The truncated BRCA2 protein confirmed the Capan-1 origin of the recovered cells. C, induction of BRCA2 expression inhibited the growth of the Capan-1-derived tumors. Ten nude mice were s.c. inoculated with 1 × 10^5 of the indicated cells on each side of their flanks. Five mice in each group had been fed with doxycycline (0.2 mg/ml) in drinking water for 5 days before the inoculation. The mice were fed continuously with water containing doxycycline (■) or without doxycycline (□). The tumor volume was measured on the indicated days.
sulted in only two slow-growing tumors, whereas Capan-1/neo cells resulted in only two slow-growing tumors, whereas Capan-1/neo-derived tumors died in 2\(\leq\)28 weeks after the inoculation, whereas all of the mice bearing Capan-1/neo-derived tumors died in 2\(\leq\)28 weeks of the inoculation (Fig. 4B). The expression of wild-type BRCA2 protein was undetectable in cells recovered from the 236BRCA2-derived tumor. This result suggested that loss of wild-type BRCA2 protein expression might be necessary for the growth of such tumors (Fig. 4B, inset).

The inhibition of tumor growth by BRCA2 was confirmed when BRCA2TN cells were studied. The growth rate of these cells was reduced significantly in mice that the wild-type BRCA2 protein was induced to express than in mice that the expression was repressed. The inhibition of tumor growth was not because of the activation of tetracycline-regulated transactivator or lack of doxycycline because there was no difference in the growth of control cell lines (Capan-1, neoTN-1, and neoTN2) between the two groups of mice (Fig. 4C).

By reconstituting wild-type BRCA2 expression in the BRCA2 mutant cell line Capan-1, we demonstrated that the expression of wild-type BRCA2 protein suppressed the growth of Capan-1 cells in vitro and in vivo. Our results strongly suggest that, in addition to guarding the genomic integrity as reported previously (5–11), regulation of cell proliferation contributes to the tumor suppression function of BRCA2. Because the p53 gene is mutated in Capan-1 cells (20), this cell-growth inhibition likely occurs through a p53-independent mechanism. The Rb pathway is also not required for growth suppression by BRCA2 because the Rb cell cycle regulation pathway is not functional in Capan-1 cells (21). Flow cytometry analysis of the constitutive BRCA2 transfectants, as well as the inducible BRCA2 clone, did not show an increased sub-G\(_1\) - G\(_1\) cell population, nor significant abnormality of cell cycle distribution (Fig. 2D and 3C). Therefore, the decreased growth rate of these two clones was unlikely to have resulted from increased cell death or arrest of cell cycle progression at certain stages. Additional investigations will be necessary to understand the mechanism of regulating cell proliferation by BRCA2.

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References


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