

Primary research

BRCA1 Zinc RING Finger Domain Disruption Alters Caspase Response in Ovarian Surface Epithelial Cells

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Published: 5 July 2002

Received: 28 January 2002

Cancer Cell International 2002, **2**:7

Accepted: 5 July 2002

This article is available from: <http://www.cancerci.com/content/2/1/7>

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Abstract

Background: The frequently occurring 185delAG mutation occurs in the amino-terminal zinc RING domain of the breast and ovarian cancer susceptibility gene, BRCA1. We sought to determine differential cell viability and apoptotic response of human ovarian surface epithelial cells with and without the 185delAG mutation.

Results: BRCA1wt and BRCA1+ cells were treated with staurosporine. Cell proliferation assays showed BRCA1wt cells grew to a greater extent compared to BRCA1+ cells. Trypan blue exclusion assays confirmed this observation. Western immunoblot analysis revealed that caspase 3 levels were higher after staurosporine treatment in BRCA1+ cells than in wild type cells, while full length DNA Fragmentation Factor 45 levels were lower in BRCA1+ cells. While there was no significant difference in levels of excision repair cross complementing protein1 (ERCC1) with BRCA1 status, BRCA1+ cells demonstrated cleavage of polyribose ADP polymerase (PARP) before wild type cells.

Conclusions: Disruption of the BRCA1 RING domain caused altered cell viability and caspase-dependent apoptotic response after chemotoxic stress.

Background

The breast and ovarian cancer susceptibility gene, BRCA1, is located at 17q21, and encodes a 1863 amino acid protein. Mutations in this gene account for 60% of hereditary ovarian cancers [1]. Loss of heterozygosity in this gene occurs in 30–70% of sporadic ovarian carcinomas [2]. Species homology studies have shown that while the entire 22 exon gene is poorly conserved, the terminal ends maintain over an 80% homology between rat, human and mouse [3]. BRCA1 has long been known to function in DNA repair. Studies have shown BRCA1 is upregulated in cells treated by DNA damaging agents such as cisplatin

[4]. BRCA1 has been shown to interact with DNA repair proteins such as Rad50 and Rad51, the tumor suppressor genes RB and BRCA2, transcriptional factors (RNA pol II, histone deacetylase complex, ctIP) as well as influence numerous cyclins and cyclin dependent kinases contributing to cell cycle regulation [5–12]. More recently, BRCA1 has been shown to influence apoptosis in a p53 independent manner [13]. This apoptotic response involved the c-jun kinase (JNK) pathway, though the details of this mechanism remain unclear [14].

The highly acidic carboxy-terminal (BRCT) region of BRCA1 has been suggested to play a role in transactivation [11]. BRCT interacts with BRCA2, Rad51, other tumor suppressing elements, as well as numerous transcription factors, such as RNA helicase A and STAT1 [15,16]. Recently, it has been discovered that truncation of this region resulted in suppression of apoptosis following proapoptotic stimuli [17]. Further, these studies also suggested that the BRCT region facilitates apoptotic functions within the caspase pathway.

The amino terminal (BRNT) of BRCA1 contains a highly conserved zinc binding or RING finger domain also involved in multiple functions within the cell. Molecular modeling has shown that this domain contains two zinc finger-like motifs connected through linking C₃HC₄ regions [18]. Naturally occurring splice variants of the gene suggest at least two transcription initiation points above and below the coding region for the RING domain [19]. Truncation studies have shown that the RING domain may function in direct protein binding of ER- α , ATF1, and BARD1, a ubiquitin ligase [20–22]. While zinc RING domains are common motifs in several protein families such as oncoproteins and regulatory proteins, the actual function of the domain differs among these proteins. For example, inhibitors of apoptosis proteins, (IAPs), contain one to three tandem baculovirus inverted repeat (BIR) domains as well as a carboxy terminal RING domain. Previous studies have shown this RING domain essential in the anti-apoptotic function of some IAPs [23].

The most common mode a cell uses to undergo apoptosis is the cysteine-aspartate specific protease (caspase) pathway. This proteolytic cascade may be triggered by a wide variety of stimuli and employs numerous initiation routes within the cell. While there is extensive crosstalk between the caspases, the two most common initiator pathways are the Fas/Fas ligand pathway, involving caspase 8 and caspase 10, and the mitochondrial pathway, triggering caspase 9 [24,25]. Caspase 3, a pivotal downstream protease, functions in virtually every caspase pathway and serves as an executioner in the cells by cleavage of downstream targets which lead to irreversible chromosomal degradation. Perhaps the most prominent caspase 3 substrate is DNA Fragmentation Factor 45 (DFF45), an inhibitor of caspase-activated DNase [26]. Following caspase 3-mediated cleavage, DFF45 releases DFF40, the DNase responsible for DNA fragmentation into the characteristic apoptotic DNA ladder. Caspase 3 also deactivates vital DNA repair enzymes such as poly ribose ADP polymerase (PARP) [27]. Cleavage of PARP has been regarded as a hallmark of caspase-dependent apoptosis [28].

No study to date has explored the possible involvement of the BRCA1 amino-terminal RING domain in caspase-me-

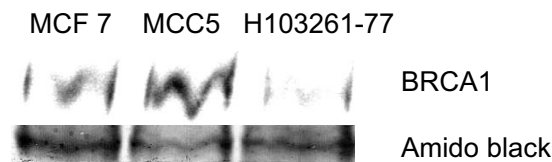


Figure 1
BRCA1 expression is decreased in the H103261-77 cells. One hundred thousand cells were electrophoresed in a 5% SDS-polyacrylamide gel and probed for BRCA1 expression (216 kDa). Loading accuracy was measured by Amido black staining. Figure is representative of at least 3 independent trials.

diated apoptosis. Therefore, ovarian surface epithelial cell lines with and without the 185delAG BRCA1 mutation were used to ascertain whether the RING domain of the amino-terminal affected apoptosis. This mutation, common among families with hereditary ovarian cancer, is a frameshift mutation occurring at the beginning of the C₃HC₄ region of exon 2 that essentially interrupts RING domain function [19,29,30]. The results showed that disruption of the BRCA1 amino-terminal RING domain altered caspase 3 activation and subsequent DFF45 and PARP cleavage, resulting in accelerated STS-induced apoptosis.

Results

Loss of BRCA1 expression resulted in increased cell death when exposed to 1 μ M staurosporine treatment

SV-40 large T antigen transfected ovarian surface epithelial cell lines from women with and without an amino-terminal BRCA1 mutation were employed to ascertain the function of the amino-terminal RING domain in apoptosis [31]. To confirm BRCA1 status in these cell lines, whole cell lysates were western blotted using a monoclonal anti-BRCA1 antibody against the amino-terminal (Figure 1). Using the MCF7 breast carcinoma cell line as a positive control, MCC5 cells expressed the full length 216 kDa BRCA1 protein and were confirmed BRCA1wt. In contrast, the H103261-77 cells were found to have significantly reduced levels of full length BRCA1 than the wild type cell line, confirming the mutated amino-terminal BRCA1 (BRCA1+) in these cells. Due to the high molecular weight of BRCA1, actin could not be used as a loading control. Thus, the membranes were stained with 7% Amido black with the protein front used as a loading control.

Having confirmed the BRCA1 status of these cell lines, cell viability was then assayed under cytotoxic stress. Cells were treated with 1 μ M STS for 3 h and subjected to MTS

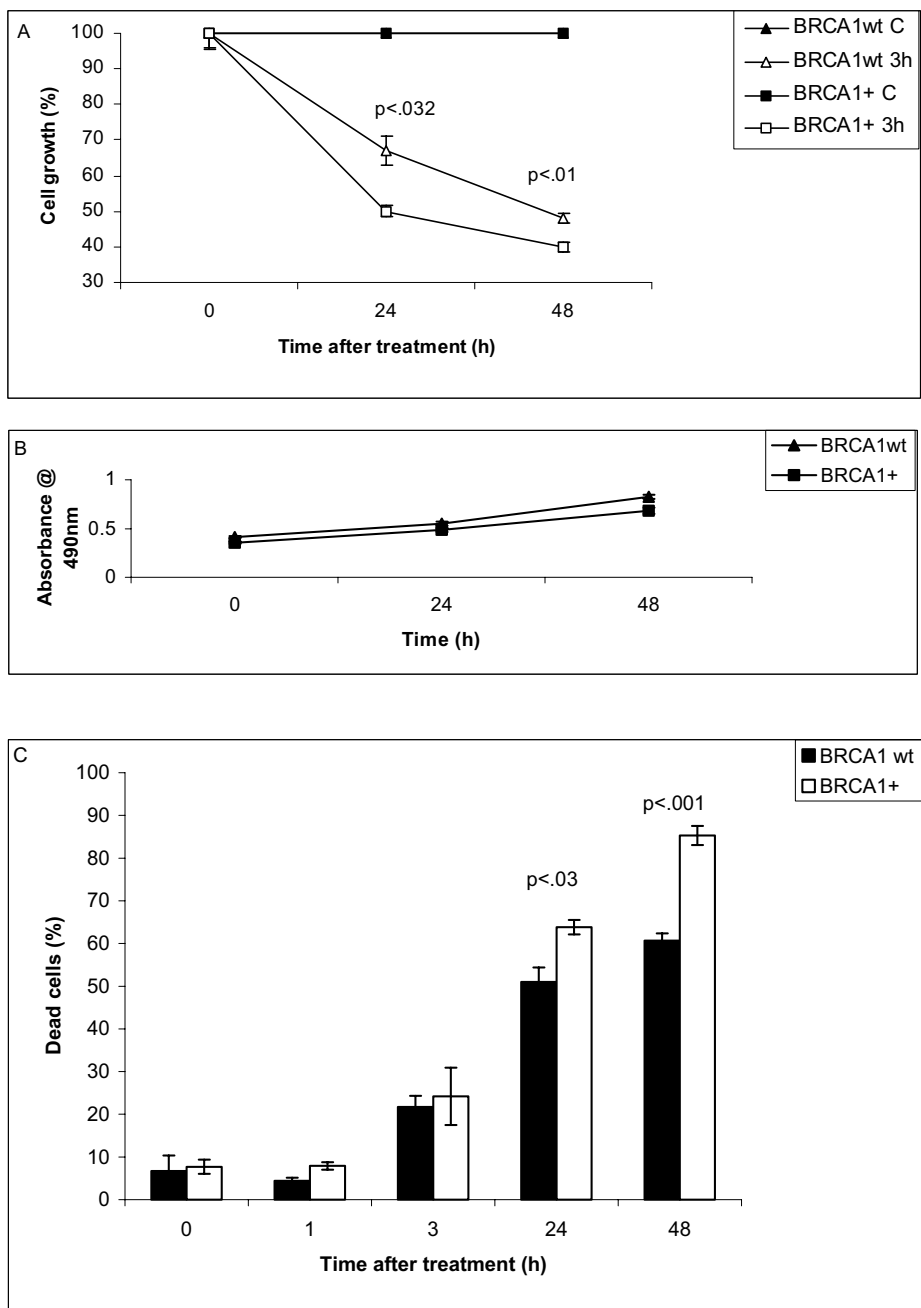


Figure 2

Reduced HIO3261-77 cell viability after 1 μ M STS treatment. A) Triplicate samples of cells were treated with 1 μ M STS for 3 h and assayed for cell growth by MTS at 0, 24, and 48 h. Results shown were percent growth of untreated cells. B) Triplicate samples of untreated cultures were grown in the absence of STS and results were shown as the linear regression of BRCA1wt and BRCA1+ cells. Both figures were generated from samples counted in triplicate. C) Trypan blue analysis was performed on STS-treated cultures to assay for cell death at 0, 1, 3, 24, and 48 h. Both adherent and suspended cell cultures were counted, and the results were reported as the percent of dead cells from the combined counts. Error bars and p values were generated from 3 independent treatments for each cell line.

assay at 0, 24, and 48 h (Figure 2A). Results were reported as percent growth of respective untreated cells allowed to grow in serum containing media. BRCA1wt cells grew 17% greater at 24 h and 8% greater at 48 h than BRCA1+ cells. This difference proved to be statistically significant ($p < .03$ and $p < .01$, respectively). BRCA1wt cells appeared to recover at 72 h while BRCA1+ cells continued to decline in growth (data not shown).

To confirm that the difference in cell viability was due to alterations in survival response after STS treatment and not an intrinsic property of the individual cell lines, growth of both cell lines was examined by MTS assay under the same conditions in the absence of STS (Figure 2B). Linear regression analysis of cell growth revealed that the slopes of the BRCA1wt cells, (0.12) and that of the BRCA1+ cells, (0.14) were essentially the same.

To ensure the survival difference after STS treatment seen between the BRCA1wt and BRCA1+ cells was associated with cell death, a trypan blue exclusion assay was conducted (Figure 2C). Cells were plated in triplicate and both adherent and suspended cell populations were assayed with results reported as percent of total population dead. No appreciable difference was observed in the amount of death between the cell lines at 0, 1, or 3 h (1%, 3.5%, and 2.5%, respectively). There was, however, a significantly higher percentage of dead cells in the BRCA1+ cell line at 24 h (12.8%; $p < .027$) and 48 h (24.6%; $p < .001$).

Amino-terminal BRCA1 mutation was associated with elevated caspase 3 activation following STS treatment

To investigate the role of amino-terminal of BRCA1 in apoptosis, the effect of STS was examined on elements of the caspase pathway (Figure 3). First, to determine whether a mutation in amino-terminal RING domain of BRCA1 preferentially targeted either the mitochondrial [25] or Fas/Fas ligand apoptotic pathway [24], levels of the respective initiator caspases 9 and 8 were determined. Both cell lines produced activated caspases 8 and 9 by 1 h after treatment with equivalent levels at 3 h.

Next, levels of the executioner caspase 3 were examined. Once more, both cell lines produced activated caspase 3 by 1 h after treatment. However, BRCA1+ cells showed significantly more active caspase 3 by 3 h after treatment than the wild type. To quantify the difference in caspase activation, the immunoblots were scanned and analyzed via Imagen densitometry (Table 1). Densitometric analysis revealed that although BRCA1+ cells initially had lower levels of caspase 3, after 3 h STS treatment, caspase 3 levels were 72% higher. Levels of STS-induced caspase 7, a structural and functional homolog of caspase 3 [32] were also evaluated (Figure 3). Procaspase 7 began to be cleaved at 1 h of treatment and was completely processed

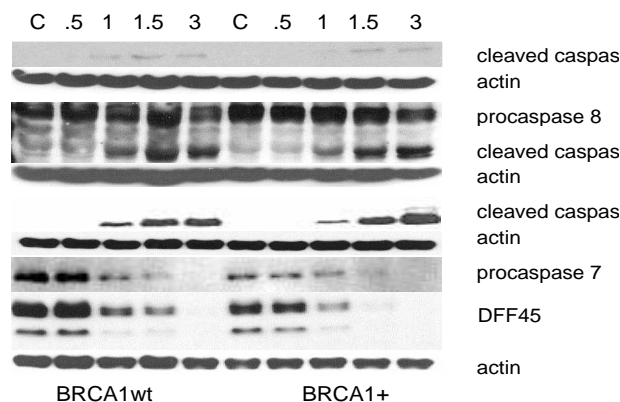


Figure 3

Effect of amino-terminal BRCA1 mutation in caspase-dependent apoptosis. BRCA1wt and BRCA1+ cells were treated with 1 μ M STS and adherent populations were collected at 0, .5, 1, 1.5, and 3 h. Cells were lysed and separated via 12.5% SDS-PAGE for western immunoblot analysis. Membranes were probed with antibodies for activated caspase 9 (37 kDa), caspase 8 (43 kDa), caspase 3 (19 kDa), procaspase 7 (36 kDa), and full length DFF45 (45 kDa). Membranes were then reprobed with anti-actin to ensure proper loading (shown immediately under respective caspases). Membranes are representative of at least 3 independent treatments.

by 3 h of treatment in both BRCA1 wt and BRCA1+ cells. Although caspase 7 plays a subsidiary role in DNA fragmentation and apoptosis morphology [33], densitometric analysis illustrated that BRCA1+ cells contained substantially reduced levels of procaspase 7 in untreated cells (39% of that seen in BRCA1wt), and during initial STS treatment (31% of that seen in BRCA1wt at 0.5 h).

To determine whether elevated levels of cleaved caspase 3 resulted in increased cleavage of caspase 3 substrates, DFF45 cleavage was studied (Figure 3). Degradation of full length DFF45 was used to indicate caspase 3 activity. In both cell lines, DFF45 began to significantly degrade by 1 h after treatment. In BRCA1wt cells, the levels of full length DFF45 were 95% of control at 0.5 h, 40% of control at 1 h, and 22% of control at 1.5 h. In contrast, in BRCA1+ cells, full length DFF45 was only 71% of control at 0.5 h, 16% of control at 1 h, and 10% of control at 1.5 h (Table 1).

Amino-terminal BRCA1 mutation caused increased degradation of caspase-linked DNA repair proteins

To ascertain whether increased caspase 3 activity in BRCA1+ cells could also affect DNA repair pathways, the DNA repair enzymes PARP, a known substrate of caspase

Table 1: Densitometric analysis of selected members of the caspase pathway.

	BRCA1wt			BRCA1+		
	Caspase 3	Procaspase 7	DFF45	Caspase 3	Procaspase 7	DFF45
Control	nd	7.44	21.7	nd	2.9	21.0
0.5 h	nd	5.4	20.52	nd	1.66	14.94
1.0 h	1.4	1.68	8.71	0.61	1.04	3.44
1.5 h	2.4	.35	4.86	2.08	.66	2.06
3.0 h	2.63	nd	nd	3.64	nd	nd

Densitometric analysis of selected members of caspase pathway. Representative immunoblots for activated caspase 3, procaspase 7, and full length DFF45 were scanned and analyzed via the ImaqQuant software application. Values reported were normalized to the immunoblots' respective actin levels. An entry of 'nd' represents a value not detectable by the application.

3, [27] and ERCC1, a repair protein not dependent on caspase 3 [35] were examined (Figure 4). Interestingly, cleavage and inactivation of PARP was noted only at 3 h after STS treatment in BRCA1wt cells. In contrast, accelerated cleavage and inactivation of PARP was seen in BRCA1+ cells as early as 1 h after STS treatment. Levels of ERCC1 were not significantly different between BRCA1wt and BRCA1+ cells.

Discussion

A frameshift mutation in the amino-terminal of the BRCA1 gene disrupts the RING domain and all domains after it. Our data showed that a BRCA1 mutation interrupting the RING domain altered apoptosis in ovarian surface epithelial cells. While there was no difference in overall growth between BRCA1+ and BRCA1wt cells, BRCA1+ cells showed a marked reduction in survival following STS treatment. Reduced cellular survival in BRCA1+ cells was associated with increased cell death due to alterations in apoptosis. No difference was detected in the levels of caspase 9 or caspase 8 among the BRCA1+ or BRCA1wt cells, suggesting that the reduced cell survival in BRCA1+ cells was not associated with a difference in initiation of either the mitochondrial or the Fas/FasL apoptotic pathways. In contrast, STS induced 72% greater caspase 3 activity in BRCA1+ compared to BRCA1wt cells. The enhanced caspase 3 activity in BRCA1+ cells was clearly functional and resulted in increased proteolysis of downstream targets of caspase 3. That is, we found 40% less full length DFF45 at 1 h and 42% less at 1.5 h in BRCA1+ cells compared with BRCA1wt cells. The cleavage and subsequent deactivation of this caspase 3-dependent DNase inhibitor suggested that amino-terminal BRCA1 mutations enhance cellular apoptosis contributing to poor cell survival.

With BRCA1's extensive connection to DNA repair already established [35], we also examined whether an amino-terminal BRCA1 mutation reduced cellular survival by enhanced caspase 3-dependent cleavage and subsequent inactivation of the caspase 3-dependent DNA repair enzyme PARP. As with DFF45, PARP cleavage was significantly enhanced in BRCA1+ cells suggesting that decreased DNA repair capacity in BRCA1+ cells may, in part, be due to premature inactivation of PARP. This pattern was not seen with ERCC1 and may be due to the choice of apoptotic stimulus used. While the exact mechanism remains unclear, STS has been shown to initiate caspase driven apoptosis in a manner distinct from chemotherapeutic agents such as cisplatin, etoposide, and tamoxifen, which directly cause DNA damage and tend to favor ERCC1 activation [37,38].

Previous studies have shown that truncation of the highly acidic carboxy-region BRCT resulted in resistance to caspase induced apoptosis [17]. Further, this failure of apoptosis was traced specifically to caspase 8 and the Fas/FasL pathway. In contrast, our data showed that the 185delAG mutation, affecting the amino-terminal domain of BRCA1, conferred an increased apoptotic response with no caspase pathway preferentially selected. Instead, this amino-terminal mutation favored elevated caspase 3 levels that subsequently facilitated enhanced apoptosis by inactivating the caspase-dependent DNA repair proteins PARP and by inactivating DFF45, an inhibitor of the caspase-dependent DNase, DFF40. The nature of the 185delAG frameshift makes it difficult to determine if the apoptotic alterations seen are directly caused by the mutation or by downstream effects of it and certainly deserves further scrutiny. In this way, carboxy- and amino-terminal BRCA1 mutations may target two distinct anti-apoptotic pathways. Therefore, disruptions in either or both BRCA1 terminals effect apoptotic response.

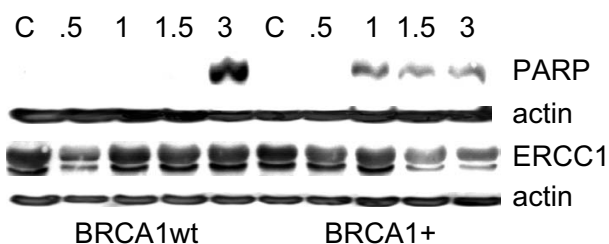


Figure 4
Effect of amino-terminal BRCA1 mutation on DNA repair protein expression. BRCA1wt and BRCA1+ cells were treated and lysed as in Figure 3 and probed for cleaved PARP (86 kDa) and ERCC1 (36 kDa). Membranes were then probed for actin for loading consistency. Images are representative of at least 3 independent treatments blotted twice.

In summary, our findings suggest a possible novel mechanism by which the amino-terminal of BRCA1 suppresses apoptosis and facilitates DNA repair in human ovarian surface epithelial cells.

Conclusions

The 185delAG mutation in the BRCA1 gene disrupts the zinc linker region of the amino-terminal RING domain. Disruption of this domain triggered an elevated caspase 3-dependent apoptotic response and affected downstream proteins such as DFF45 and PARP.

Materials and Methods

Cell Culture

SV-40 large T-antigen transfected human ovarian surface epithelial cell lines, MCC5 and HIO3261-77, were derived from women with and without a family history of breast/ovarian cancer, respectively [31]. While MCC5 cells were derived from a patient denoted as wild type BRCA1 status, HIO3261-77 cells were derived from a patient characterized as 185delAG mutated [39]. Dr. W. Bai (USF) kindly provided the MCF7 breast cancer carcinoma line. Cells were maintained in Medium 199/MCDB 105 (1:1) (Sigma, St. Louis, MO) with 5% fetal bovine serum (Gibco-BRL, Rockville, MD) and 10 µg/ml gentamicin (GibcoBRL) in 5% CO₂/95% air at 37°C as described previously [31,40].

Induction of Apoptosis and Cell Viability Assessment

Cells were grown in 100 mm tissue culture disks until confluent. Cultures were treated with 1 µM staurosporine (STS) (Sigma) in serum containing medium until collected. Control samples were rinsed in DPBS, drained, and fresh medium was added.

Cell growth was determined by the MTS (Promega, Madison, WI) colorimetric assay following STS treatment. The

assay was performed in 96 well microtiter plates according to manufacturer's instructions and is based on soluble formazan production by dehydrogenase enzymes found in metabolically active cells. Samples were seeded in six wells per time point at 2.5×10^3 cells per well. Absorbance was determined at 490 nm using a Dynex MRX plate reader (Dynex Technologies, Chantilly, VA) and the results expressed as the mean absorbance of triplicate experiments \pm SE.

SDS-PAGE and Western Blot Analysis

In order to observe changes at the onset of apoptosis, only adherent cell populations were trypsinized, pelleted 5 minutes at $500 \times g$, and lysed in ice cold lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 5 mM β -mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 30 minutes at 4°C. Lysates were then centrifuged at $100,000 \times g$ for 1 h at 4°C. Protein concentrations of the lysates were determined using the DC Protein Assay (Biorad, Hercules, CA) according to the manufacturer's instructions. Fifteen micrograms of protein were added to 4X loading buffer (250 mM Tris pH 6.8, 8% SDS, 20% glycerol, 0.012% bromophenol blue, 4% β -mercaptoethanol), heated to 95°C for 5 minutes, electrophoresed in 12.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane (Amersham Piscataway, NJ) via semi-dry transfer. Due to the high molecular weight of PARP, SDS-PAGE was performed using 7% polyacrylamide gels, and proteins were then transferred to PVDF membrane (Biorad) via wet transfer. All membranes were blocked for 1 hour with 5% non-fat milk Tris Buffered Saline plus 0.1% Tween-20 (T-TBS) and incubated at least overnight at 4°C in primary antibody.

Polyclonal antibodies to caspase 3, caspase 9, and PARP were purchased from Cell Signaling (Beverly, MA), a monoclonal antibody to caspase 7 and a polyclonal antibody to DFF45 were obtained from BD Transduction (San Diego, CA). Polyclonal anti-caspase 9, and monoclonal actin antibodies were purchased from Alexis (San Diego, CA) and Sigma, respectively. Membranes were incubated and developed according to the Enhanced Chemiluminescent Protocol, according to manufacturer's instructions (Amersham). After initial blotting, membranes were reprobed for actin to ensure even loading.

BRCA1 status of the cell lines used in this study was confirmed via western immunoblotting. Cells were mixed with equal volume of 2X loading buffer, vortexed and boiled for 5 minutes. One hundred thousand cells were separated by 5% SDS-PAGE, transferred by wet-transfer to PVDF membrane, and blotted as described above using monoclonal antibody specific for the N-terminus of BRCA1 (Oncogene Boston, MA). After blotting, the PVDF membrane was stained with 2% amido black in 7% gla-

cial acetic acid and the protein fronts of all lanes were compared for loading accuracy.

Statistical Analysis

Samples for MTS and trypan blue exclusion assays were performed in triplicate and the data subjected to the Student's paired *t*-test analysis for determination of statistical significance between BRCA1+ and BRCA1wt samples. Two-tailed results are reported as P values within the corresponding figures.

Acknowledgements

This work was supported by a New Investigator Award from the H. Lee Moffitt Cancer Center to P.A.K.

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