p53-dependent BRCA1 nuclear export controls cellular susceptibility to DNA damage


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Running title: p53 regulates BRCA1 shuttling and cytotoxicity

Key words: BRCA1, DNA damage, DNA repair, p53, radiation, sporadic breast cancer, nuclear export, subcellular distribution

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Abstract

Subcellular localization regulates BRCA1 function, and BRCA1 is exported to the cytoplasm following DNA damage in a p53-dependent manner. Because more than 50% of solid tumors harbor p53 mutations, it is possible that genetically wildtype BRCA1 is functionally abnormal through compromised nuclear-cytoplasmic shuttling in sporadic breast cancer patients with dysfunctional p53. In this study, we have investigated the mechanisms of p53-dependent BRCA1 subcellular distribution and DNA damage-induced nuclear export, as well as the impact on the resulting cytotoxic response to therapy in human breast cancer. We first show that p53 mediates BRCA1 nuclear export via protein-protein binding, rather than by modulation of its transcription. Furthermore, it is the C-terminal BRCT region of BRCA1 that is critical for its interaction with p53, and p53 may promote BRCA1 nuclear export by interrupting the association of BRCA1 with BARD1. In sporadic breast cancer specimens, dysfunctional p53 strongly correlates with nuclear retention of sequence-verified wild-type BRCA1. This p53-dependent BRCA1 shuttling determines cellular susceptibility to DNA damage as augmentation of cytosolic BRCA1 significantly enhances cancer cell susceptibility to ionizing radiation (IR). Taken together, our data suggests that p53 dysfunction compromises nuclear export of wild-type BRCA1 as a mechanism to increase cellular resistance to DNA damage in sporadic breast cancer. We propose that targeting nuclear BRCA1 to the cytoplasm may offer a unique strategy to sensitize p53-deficient sporadic breast cancers to DNA damage-based therapy.
Introduction

BRCA1 serves critical roles in multiple cellular processes, such as chromatin remodeling, DNA replication, DNA repair, transcriptional regulation, cell cycle checkpoint, and apoptosis (1, 2). These BRCA1 functions are controlled by multiple mechanisms, including phosphorylation, protein-protein interactions, and subcellular localization.

BRCA1 is a nucleocytoplasmic shuttling protein (3). Nuclear BRCA1 functions in DNA repair (4, 5) and cell cycle checkpoints (1), while cytoplasmic BRCA1 regulates centrosome function (2) and p53-independent apoptosis (6). BARD1 has been shown to promote BRCA1 nuclear import and inhibit BRCA1 NE (7). We have reported that p53 regulates BRCA1 shuttling (8). Specifically, p53 dysfunction leads to increased nuclear retention of BRCA1 and diminished DNA damage-induced BRCA1 NE (8). Furthermore, DNA damage repair depends on nuclear BRCA1, whereas DNA damage-induced cytotoxicity depends on BRCA1 cytosolic translocation (6, 9). Thus, p53 status may play a critical role in regulating BRCA1 shuttling and cellular susceptibility to DNA damage.

p53 is the most commonly mutated tumor suppressor gene and is dysfunctional in greater than 50% of solid malignancies (10-12). Dysfunctional p53 correlates with poor prognosis in breast and many other types of cancer (12-14). Given that BRCA1 subcellular localization controls its function in DNA repair and apoptosis (6, 9), and p53 regulates BRCA1 NE (8), it is possibility that genetically wt BRCA1 is rendered functionally abnormal through compromised nuclear-cytoplasmic shuttling in sporadic breast cancer with dysfunctional p53. Specifically, this altered shuttling may affect tumor response to DNA damage-based therapies (6, 9).

In this study, we sought to determine the mechanism of p53-dependent BRCA1 subcellular distribution and DNA damage-induced NE and the impact on cytotoxic response to
therapy in breast cancer. Interestingly, our findings suggest that p53-dependent BRCA1 NE is independent of its transcriptional activity, but instead depends on the binding of the two proteins. Furthermore, we find that dysfunctional p53 strongly correlates with nuclear retention of sequence-verified wild type BRCA1 in sporadic breast cancer specimens. Furthermore, interrupting BRCA1-BARD1 binding may be one of the mechanisms of p53-mediated BRCA1 NE. Re-expression of wt p53, but not the mutant deficient in binding to BRCA1, restored BRCA1 cytoplasmic shuttling and enhanced IR-induced cytotoxicity. Lastly, augmenting cytosolic BRCA1 significantly enhances p53-deficient cancer cell susceptibility to ionizing radiation (IR). These results suggest that regulation of subcellular localization and NE of genetically wt BRCA1 in sporadic breast cancer by other regulatory proteins, such as p53, may be an important determinant of tumor response to DNA-damaging agents.
Materials and methods

Cell cultures and treatment

HCC1937 cells were purchased from American Type Culture Collection. The genetic background, including expression and function of BRCA1, BARD1, p53, p21, and caspase 9, as well as the growth characteristics and their response to genotoxic agents, was tested most recently on May 2010 using Western blot analysis, immunohistochemistry, and colony formation assays. HCC1937 cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal bovine serum (FBS), 100 U of penicillin (pen), and 100 µg/mL of streptomycin (strep). MCF7/E6 cells were previously described (8) and were maintained in DMEM supplemented with 10% FBS, 100 U pen, and 100 µg/mL strep. EJ-p53 cells (a gift from Dr. Stuart A. Aaronson, Mount Sinai School of Medicine, New York). Cells were cultured in complete DMEM medium, containing 10% FBS, 100 U pen, 100 µg/mL strep, 1 µg/ml tetracycline (Tet), 75 µg/ml Hygromycin B, (Sigma-Aldrich), and 500 µg/ml G418 (Cellgro). Removal of Tet from the medium results in p53 induction (15). For irradiation of cells, the Mark I 137Cs irradiator (J.L. Shepherd and Associates) was used, delivering 1.84 Gy/min. A turntable ensured that the radiation was equally distributed. As a control, mock irradiation consisted of placing plates containing cells in the irradiator for the designated times without turning on the machine.

Plasmids and adenovirus (Supplementary data)

Immunofluorescence (Supplementary data)

Subcellular fractionation, immunoprecipitation, and Western blot analysis (Supplementary data)

Colony formation assays (Supplementary data)

Analysis of apoptosis (Supplementary data)
Flow cytometry (Supplementary data)

Statistical Analysis (Supplementary data)

Results

Regulation of BRCA1 shuttling is dissociated from p53 transcriptional activity

Transcriptional regulation is one of the commonly employed mechanisms of p53 function in controlling genotoxic stress-induced cell cycle arrest and apoptosis. We hypothesize that p53 controls BRCA1 NE through transcriptional regulation. The effect of p53 transcriptional activity on the subcellular distribution of BRCA1 was determined in HCC1937 human breast cancer cells that are functionally null in both p53 and BRCA1 (16, 17) following co-expression of HA-tagged wt-BRCA1 with p53-wt or two tumor-associated p53 mutants (p53-179Q and p53-273H). The transcriptional activation deficiency of both p53 mutants (p53-179Q and p53-273H) was confirmed in HCC1937 cells (Supplemental Figure 1a). The location of BRCA1 in nuclear or/and cytoplasmic compartments was assessed by subcellular fractionation. As shown in Fig. 1a, the presence of p53-wt resulted in BRCA1 cytoplasmic translocation following IR. Nuclear BRCA1 levels decreased by 2 fold (p<0.05) while cytoplasmic levels increased by more than 2 fold (p<0.05). Interestingly, expression of the p53-179Q mutant resulted in a similar level of IR-induced BRCA1 NE as in cells with p53-wt expression. This effect, however, was significantly reduced in cells expressing the p53-273H mutant, suggesting that the p53-273H mutant, but not p53-179Q, is deficient in regulating BRCA1 shuttling. To our surprise, these results did not support our initial hypothesis and indicate that p53 transcriptional activity is not required to regulate BRCA1 nuclear export following IR. To confirm the results obtained by cell fractionation, immunohistochemistry was performed on these cells to assess BRCA1 localization.
as a function of p53 by co-immunostaining of exogenously expressed BRCA1 and p53 using anti-HA and anti-p53 antibodies (Fig. 1b). BRCA1 subcellular distribution was scored as nuclear (N), nuclear/cytoplasmic (N/C), or cytoplasmic (C). Consistent with our nuclear-cytoplasmic fractionation results, an increased percentage of cells with cytoplasmic BRCA1 was seen in cells expressing p53-wt or p53-179Q following DNA damage, but not in cells expressing p53-273H. Together, these data imply that p53-mediated regulation of BRCA1 NE is not dependent on the transcriptional activation of p53.

**p53-BRCA1 interaction is critical for p53-dependent BRCA1 nuclear export**

It has been reported that BRCA1 and p53 physically interact (18-20). Because our results revealed that regulation of BRCA1 NE is not dependent on p53 transcriptional activity, we proposed the alternative hypothesis that a protein–protein interaction may be the mechanism by which p53 regulates DNA damage-induced BRCA1 NE.

To determine the association of p53 and BRCA1, we performed co-immunoprecipitation experiments in HCC1937 cells described above. As shown in Fig. 2a, similar amounts of wt BRCA1 were present in the p53-immunocomplexes pulled down in cells expressing either p53-wt or p53-179Q. In contrast, a significantly decreased amount of BRCA1 was present in the p53-immunocomplex in cells expressing the p53-273H mutant. Quantitative analysis revealed a 2-fold decrease in BRCA1 co-precipitated with p53-273H compared to that with p53-wt (p<0.05, Fig. 2a lower panel). Reciprocal immunoprecipitation experiments using HA antibody yielded similar results (p<0.05, Fig. 2b). These findings suggest that a protein–protein interaction, and not transcriptional activity, may be important for p53 to regulate BRCA1 NE, and the deficiency
of the p53-273H mutant in promoting DNA damage-induced BRCA1 NE may be due, at least in part, to its compromised ability to interact with BRCA1.

In contrast to p53, BARD1 inhibits BRCA1 export by binding and masking the BRCA1 nuclear export sequence (NES) (7). To further dissect the mechanisms by which p53 promotes BRCA1 cytosol translocation, we examined whether binding of p53 to BRCA1 affects the interaction between BRCA1 and BARD1. As shown in Fig 2b, when BRCA1 was immunoprecipitated using HA antibody, similar amounts of BARD1 were present in the BRCA1-immunocomplexes pulled down in cells expressing either p53-wt or p53-179Q. In contrast, a significantly 1.9-fold increased amount of BARD1 was present in the BRCA1-immunocomplex pulled down in cells expressing the p53-273H mutant compared to that in cells expressing BRCA1-binding proficient p53-wt or p53-179Q (p<0.05, Fig. 2b lower panel). These findings support the model that binding of p53 to BRCA1 disrupts the interaction of BRCA1 with BARD1. The dissociation of BRCA1 from BARD1 uncovers the BRCA1 NES and thereby promotes BRCA1 NE.

**p53–BRCA1 binding confers cellular sensitivity to radiation-induced cytotoxicity**

We next determined whether the p53–BRCA1 interaction which promotes BRCA1 NE would also enhance DNA damage-induced cell killing. To address this, we assessed the cytotoxic response of HCC1937 cells co-transfected with wt BRCA1 and wt p53 or p53 mutants. IR-induced cytotoxicity was determined by colony formation capacity. As shown in Fig 3a, increased radiosensitivity was observed in cells expressing p53-wt or p53-179Q mutant compared to that in cells expressing BRCA1-binding deficient p53-273H mutant (Fig. 3a, p<0.05).
Consistent with the colony formation assay results, we observed a significant 2-fold decrease in the levels of cleaved caspase 9 following the induction of DNA damage for the p53-273H mutant compared to that for p53-wt and p53-179Q (Fig. 3b, p<0.05). This reduction in IR-induced apoptosis was verified using annexin V staining. As shown in Fig. 3c, apoptosis was decreased by at least 2 fold in cells expressing p53-273H compared to p53-wt or p53-179Q (p<0.05). Thus, our results support the hypothesis that loss of p53–BRCA1 binding, which fails to promote BRCA1 export, leads to a reduced cytotoxic response to DNA damage.

The capacities of BRCA1 mutants to bind p53 affect their nuclear export and the subsequent cytotoxic response following DNA damage.

Our data thus far support the importance of p53–BRCA1 interaction in BRCA1 shuttling and subsequent IR-induced cytotoxicity. We reasoned that BRCA1 mutants that cannot bind to p53 should also be aberrant in their location and cytotoxic response to DNA damage. To test this, HA-epitope-tagged BRCA1-wt or the BRCA1-5382insC BRCT-truncated mutant, or BRCA1-S1423/1524A mutant which resists to ATM protein kinase-mediated phosphorylation and is deficient in G2/M-phase checkpoint (21), was co-expressed with wt p53 or vector control in HCC-1937 cells. Wild type p53 function was verified by assessing IR-induced p21 induction (Supplemental Figure 1b). Reciprocal immunoprecipitation experiments were performed to assess the BRCA1–p53 interaction. As shown in Figure 4a, p53 and BRCA1 were detected in the same immunocomplexes in cells expressing wt p53 with wt or S1423/1524A BRCA1. In contrast, in cells expressing wt p53 and 5382insC BRCA1, at least a 4-fold reduction in interaction was observed (p<0.05). Similar results were obtained with reciprocal co-immunoprecipitation (Fig...
4b, p<0.05). These results confirm the observations from a previous study (19) that the BRCT domain of BRCA1 is important for interaction with p53 (22).

To further test the model that BRCA1-p53 interaction and the subsequent BRCA1 NE are critical in DNA damage-induced cytotoxicity, we next determined whether p53-binding capacity of BRCA1 mutants affects its response to p53-mediated and DNA damage-induced NE. The subcellular distribution of exogenous BRCA1 in HCC 1937 cells was analyzed in relation to p53 status by subcellular fractionation (Fig. 4c, 4d, 4e) and immunostaining (Supplement Fig. 2) using anti-HA antibody following either mock or 5 Gy IR. Consistent with our previous findings (8), wt BRCA1 was predominantly retained in the nucleus when p53 was dysfunctional, and this nuclear retention was attenuated by wt p53 co-expression (Fig. 4C p<0.01, and Supplement Fig. 2a, p<0.05). Interestingly, the subcellular distribution of p53-binding proficient S1423/1524A mutant was similar to that of wt BRCA1 when co-expressed with wt p53 or control vector (Fig. 4d, p<0.01, and Supplement Fig. 2b, p<0.05). In contrast, co-expression of p53-binding deficient 5382insC mutant exhibited an extensive cytoplasmic distribution irrespective of p53 status and IR treatment (Fig. 4e and Supplement Fig. 2c), which is consistent with previous reports (23). These data suggested that p53-dependent BRCA1 NE following DNA damage is intact in the p53-binding-proficient BRCA1 S1423/1524A mutant, but impaired in the p53-binding-deficient BRCA1 5382insC mutant.

However, the cytosolic accumulation of the 5382insC mutant seems to conflict with the results shown in Figures 1 and 2 and our overall model: we would have predicted that the defect in binding to p53 would lead to retention of the 5382insC mutant in the nucleus. To further investigate this, we examined the interaction of the 5382insC mutant with BARD1, which enhances BRCA1 nuclear import and prohibits BRCA1 NE. Interestingly, a 4-fold reduction in
the association of 5382insC BRCA1 with BARD1 was observed (Figure 4b, \( p<0.05 \)), compared to that with wt or S1423/1524A BRCA1. The lack of interaction between BRCA1 and p53, as well as BRCA1 and BARD1, may be due to restriction of BRCA1 to the cytoplasmic compartment where it is inaccessible to its nuclear interacting partners p53 and BARD1. To rule out this possibility, we expressed 3XNLS-5382insC BRCA1 fusion protein, in which 3X-nuclear localization sequence (NLS) was inserted in-frame at the N-terminus of 5382insC-BRCA1, in HCC1937 cells. As shown in Supplement Fig. 3, increased nuclear NLS-5383insC BRCA1 remains incapable of interacting with either p53 or BARD1 and is unable to translocate to the cytoplasm following IR regardless of p53 status.

These results suggest that the BRCT domain of BRCA1 is critical not only for its interaction with p53 but also for its association with other proteins, including BARD1. In support of this interpretation, it has been previously reported that cancer-associated mutations within the BRCT domain cause significant relocalization of BRCA1 from the nucleus to the cytoplasm (23). Since BARD1 binds to the N-terminal of BRCA1 to facilitate BRCA1 nuclear import and preventing its export to the cytosol, we thus propose that the effect of BRCA1-5382insC dissociation from BARD1 on BRCA1 shuttling overrides p53’s regulation and results in its exclusively cytosolic location.

To investigate whether the capacity of BRCA1 mutant to interact with p53 also determines its effect on DNA damage induced cytotoxicity, IR-induced cytotoxicity was assessed by colony formation assays, caspase-9 cleavage, and annexin V staining. Consistent with previous reports (9, 17, 21), HCC1937 cells expressing wt BRCA1 alone, in which BRCA1 accumulated in the nucleus (Fig. 4c and Supplement Fig. 2a), increased cellular radioresistance was observed compared to cells co-expressing wt BRCA1 and wt p53, in which BRCA1 export
to cytoplasm following IR (Fig. 5a, p<0.01). In contrast, cells expressing the 5382insC mutant which is predominantly located in the cytoplasm were exquisitely sensitive to IR-induced toxicity regardless whether p53 was co-expressed (Fig. 5b). Interestingly, the BRCA1 S1423/1524A mutant, which is retained in the nucleus following DNA damage without p53, rendered HCC1937 cells more radioresistant. Co-expression of wt p53, however, restored nuclear export and re-sensitized cells to IR (Fig. 5c, p<0.01). These results again support the notion that p53-mediated and DNA-damage-induced BRCA1 NE is critical for the cytotoxic response induced by DNA damage.

To confirm our cytotoxicity results, we next analyzed the effect of the various BRCA1 mutants on IR-induced apoptosis by assessing cleaved caspase 9 and annexin V. Increased caspase cleavage was observed following IR in cells expressing wt BRCA1 and p53 (Fig. 5d, p<0.05). Consistent with colony formation assays, no significant difference was observed in cells expressing the 5382insC BRCA1 mutant (Fig. 5e). However, the BRCA1 S1423/1524A mutant, which binds and response to p53-mediated shuttling, behaves similarly to wt BRCA1 (Fig. 5f, p<0.05). As shown in Figure 5g, restoration of p53-mediated BRCA1 shuttling similarly increased the annexin V-positive cells, indicative of enhanced apoptosis (p<0.01). Thus, Our data supports our hypothesis that p53-BRCA1 interaction and p53-mediated BRCA1 NE is an important determinant of cancer cell sensitivity to DNA damage-based therapy.

The subcellular distribution of genetically wt BRCA1 strongly correlates with p53 status in human breast cancer specimens

Although BRCA1 mutations are rarely found in sporadic breast cancers, aberrant localization of BRCA1 in patient specimens has been reported (24). We next determined whether
p53 functional status affects the subcellular distribution of wt BRCA1 in sporadic breast cancer patients.

To address this question, we assessed BRCA1 subcellular distribution in 31 specimens from breast cancer patients confirmed to have wt BRCA1 by direct sequencing under an IRB-approved protocol. p53 functional status and BRCA1 subcellular distribution were evaluated by co-immunofluorescence staining using p53 and BRCA1 antibodies. Increased protein stability and nuclear accumulation of p53 have been well documented and are routinely used to represent dysfunctional p53 (12). The intensity of nuclear p53 immunostaining was graded as: 0 for negative, 1 for weak, 2 for moderate, and 3 for strongly positive staining. Samples with intensity of grades 1–3 for positive nuclear p53 staining were scored as having dysfunctional p53, while samples with negative nuclear staining of grade zero intensity were scored as having functional p53. The BRCA1 subcellular distribution was scored as nuclear (N), nuclear/cytoplasmic (N/C), or cytoplasmic (C). Examples of each of these BRCA1 distribution patterns in specimens with dysfunctional or functional p53 are shown in Fig. 6a, 6b, and supplemental Fig. 4. As expected (10, 12), p53 functional status was heterogeneous within each patient specimen. Cells were divided into functional and dysfunctional p53 groups, and BRCA1 subcellular distribution was scored with respect to p53 status. Consistent with our previous findings, the combined data from the 31 patients showed that the median distribution of BRCA1 with functional p53 was 36% N, 41% N/C, and 23% C (Fig. 6c). However, when p53 was dysfunctional, increased nuclear BRCA1 was observed, and the fraction of cells exhibiting nuclear BRCA1 was increased (67% N, 25% N/C, and 8% C) (Fig. 6c).

A ternary diagram (Fig. 6d) depicts BRCA1 subcellular distribution with respect to p53 status for each individual patient. Strikingly, in every patient dysfunctional p53 was associated
with a dramatic shift of wt BRCA1 to the nucleus (p<0.0001). The substantial increase in nuclear localization of BRCA1 when p53 is dysfunctional was consistent across all 31 patients, which demonstrates that loss of functional p53 strongly correlates with the nuclear accumulation of wt BRCA1 in breast cancer and suggests that localization of wt BRCA1 plays a pivotal role in sporadic breast cancer.

**Targeted cytosolic localization of BRCA1 enhances the cytotoxic response to DNA damage**

Loss of p53 is associated with a worse prognosis and, as we have shown here, results in nuclear retention of wt BRCA1 in sporadic breast cancer patients and increased resistance to IR-induced cytotoxicity in breast cancer cells. We further hypothesized that enhancing cytosolic accumulation of wt BRCA1 can be used as a means of sensitizing cancer cells to DNA-damaging agents in a p53-dysfunction background.

BARD1 prevents BRCA1 nuclear export by interacting with RING domain and thereby masks the BRCA1 NES. Expression of a truncated BRCA1 RING domain has been shown to enhance wt BRCA1 NE by removing this competing interaction (6, 7). We created a BRCA1 RING domain-yellow fluorescent fusion protein (YFP-RING) (Supplement fig 5a ) to increase cytosolic BRCA1 in wt BRCA1-expressing MCF7/E6 cells, in which p53 function is inactivated by expression of the HPV16-E6 protein (8). Cells were sorted by flow cytometry for YFP expression, 24 hours following YFP-RING or control vector transfection. The BRCA1 distribution was determined by immunocytochemistry using the BRCA1 antibody Ab-1, which recognizes the N-terminus of BRCA1. As shown in Fig. 7a, expression of the YFP-RING fusion protein efficiently augmented cytosolic wt BRCA1, resulting in an approximately 40% decrease in nuclear BRCA1 (p<0.001) and a concomitant 22% increase in nuclear/cytoplasmic BRCA1
(p<0.001) and 18% increase in cytoplasmic BRCA1 (p<0.01) compared to vector control. Similar results were obtained using the BRCA1 antibody Ab-4 (Supplemental Fig. 5b), which recognizes exon 11 of BRCA1 and does not recognize YFP-RING.

We next determined whether YFP-RING-induced increasing cytosolic enhances the cytotoxic response to IR. The expression of YFP-RING resulted in radiosensitization of MCF7/E6 cells measured by colony formation ability (Fig. 7b, p<0.05), and significant enhancement of IR-induced apoptosis measured by Annexin V (Fig. 7c, p<0.05) and caspase 9 cleavage (Fig. 7d, p<0.05). These findings confirmed that BRCA1 subcellular location is an important determinant of the cytotoxic response to DNA damage.
Discussion

While the importance of BRCA1 mutations in the development of familial breast cancers is well established, sporadic BRCA1 mutations are rare. Because of this, the significance of BRCA1 function in sporadic breast cancers has been unclear. We have previously reported p53-mediated BRCA1 cytoplasmic translocation, which may play a novel role not only in inhibiting DNA repair functions but also in activating cell death processes following DNA damage (9). In this study, we provide evidence that the p53–BRCA1 interaction—and not p53 transcriptional activity—is one of the mechanisms for p53-mediated regulation of BRCA1 NE following DNA damage. Our results were validated in breast cancer patient specimens with dysfunctional p53 that showed increased nuclear accumulation of wt BRCA1. We further determined that p53 regulates BRCA1 subcellular localization through interaction with the BRCA1 C-terminal region, disrupting BRCA1 association with BARD1, and this regulation of BRCA1 export determines DNA damage-induced cytotoxicity. Finally, augmenting cytosolic wt BRCA1 by expressing a truncated BRCA1 RING domain enhanced cancer cell sensitivity to IR in p53-deficient cancer cells. Our findings therefore indicate that the location of BRCA1 can serve as a therapeutic target for p53-mutated tumors.

Complex signaling networks contribute to cell sensitivity to DNA damage, making it difficult to pinpoint the key determinants of sensitivity. It has been shown that BRCA1 functions in multiple DNA damage response and repair pathways, and BRCA1-deficient cells are sensitive to various types of DNA damage, including oxidative stress-induced lesions (17, 21, 25, 26), but it is not clear which function of BRCA1 is responsible for this sensitivity. Work by others has shown that loss of the G2/M checkpoint function of BRCA1 does not render cells more sensitive to DNA damage(21). Results from this study with the BRCA1 S1423/1524A mutant are in
agreement with these findings. Interestingly, we have recently reported that subcellular localization of BRCA1 is the predominant determinant of its ability to regulate cellular response to different types of DNA damage, in particular the increased persistent γ-H2AX foci following IR due to decreasing overall DSB repair efficiency and the increasing apoptotic response when BRCA1 is cytosolic (9). The complex role of cytosolic BRCA1 in conferring cytotoxicity is further supported by the enhanced cellular sensitivity not only to IR but also to UV exposure (9).

Our results indicate that cytosolic BRCA1 may compromise its nuclear functions in processing oxidative DNA lesions and in various DNA repair pathways including HR, NHEJ, and nucleotide excision repair. As BRCA1 has been shown to be required for DNA damage-induced apoptosis (27), it is intriguing to speculate that, besides inhibition of overall DNA repair, the effect of BRCA1 cytosolic localization on sensitivity to IR can also be partially attributed to activation of BRCA1 cell death functions. Alternatively, sequestration of BRCA1 away from critical binding partners may block multiple nuclear processes, including DNA repair, required for cell survival after DNA damage.

The results from this study support a model (Fig. 7e) in which physical interaction of p53 with BRCA1 at the BRCT domain may alter BRCA1 conformation and interrupt BRCA1 to interact with BARD1. In this case, the exposure of the BRCA1 NES results in BRCA1 cytoplasmic translocation. We previously have shown that p53-mediated BRCA1 nuclear export takes places in all phases of the cell cycle, it is less likely that an aberrant G1 checkpoint in p53-deficient tumor cells plays an important role in regulation of BRCA1 subcellular distribution. Further investigation is warranted to shed light on the molecular basis of these interactions and the resulting effect on their tumor suppressor roles.
On the other hand, mutations in BRCA1 can affect its interaction and response to p53-mediated nuclear export. ATM phosphorylation of BRCA1 at serine 1423 and 1524 was not required for p53-mediated BRCA1 shuttling. However, The BRCT domain has been shown to play a crucial role for BRCA1 protein folding, conformation (22), nuclear foci formation, and association with numerous other proteins (18, 23, 28-30). mutations that target the BRCT region of BRCA1 have been shown to located in the cytoplasm (31). Our data revealed that the 5382insC mutation at the BRCT domain compromises its interaction with not only p53 but also BARD1, suggesting that the dissociation of 5382insC BRCA1 from BARD1 may compromise BRCA1 nuclear import and enhance BRCA1 NE (18, 28, 29), and override p53’s regulation on BRCA1 shuttling and lead to exclusive cytoplasmic accumulation of 5382insC BRCA1.

While the majority of known p53 mutations have been shown to lead to increased protein stability (12, 32), we acknowledge that we cannot detect mutations that do not lead to nuclear accumulation in our study. However, the nuclear accumulation of BRCA1 when p53 was determined to be dysfunctional was consistent across all 31 patients. Furthermore, the clinical data is supported by our previous (8) and current biochemical and genetic studies. There is abundant evidence showing that p53-mutated tumors have a poor prognosis and increased resistance to therapies, including DNA-damaging agents. Because tumors with p53 mutation retain BRCA1 in the nucleus and are subsequently more resistant to treatment, The worse prognosis seen in patients with dysfunctional p53 may also be attributed, at least in part, to nuclear accumulation of wt BRCA1. Our findings also provide a new target in the RING domain. We have shown that the RING domain disrupts the BRCA1–BARD1 interaction, which results in BRCA1 nuclear export and a significantly enhanced sensitivity to DNA damage. It is thus a
promising target and warrants further investigation of this mechanism as a potential therapeutic strategy in p53-mutated tumors (Fig. 7e).

The findings in this study have these important implications: (1) genotyping of BRCA1 and protein expression level may not be sufficient to determine BRCA1 function in human cancer specimens; (2) BRCA1 localization may be an important prognostic factor and predictor of cancer cell response to therapies; and finally, (3) BRCA1 shuttling is a potential molecular target for improving treatment using DNA-damaging agents in sporadic breast cancer, especially when p53 is dysfunctional.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was supported by grants from the NIH (R01 CA118158-02, to F.X.), the Vanderbilt CTSA (UL1 RR024975, to J.J.), and the University of Alabama-Birmingham School of Medicine IMPACT Award (to E.S.Y.).
References


Figure legends

Figure 1. BRCA1 nuclear export following DNA damage does not require p53 transcriptional activity. (A) (Top panels) BRCA1 subcellular localization at 24 hours following mock or 5 Gy IR was determined as a function of p53 status by subcellular fractionation (8, 9). The fold change relative to BRCA1 expression without treatment in each respective fraction is shown (*p<0.05, Bottom panels). (B) BRCA1 subcellular localization was confirmed by immunofluorescence using anti-HA antibody (9). (Top panel) Representative images of p53 (green, thin arrows), BRCA1 (red, thick arrows), DAPI (blue), and merged staining. (Bottom panel) BRCA1 staining was scored as nuclear only (N), cytoplasmic only (C), or mixed nuclear and cytoplasmic (N/C). Shown is the average from at least 3 independent experiments. Error bars represent the SEM (*p<0.001, **p<0.01).

Figure 2. BRCA1 interaction with p53 is attenuated in the p53-273H mutant. Reciprocal immunoprecipitation experiments with (A) p53 or (B) HA were performed in HCC1937 cells co-transfected with HA-tagged BRCA1 and WT p53, p53-179Q, or p53-273H. The levels of p53, HA-BRCA1, or BARD1 in immunocomplexes were normalized to the amount of the reciprocal protein that was pulled down. The fold change relative to cells expressing WT p53 is shown (*p<0.05).
Figure 3. p53-dependent BRCA1 nuclear export enhances IR-induced cytotoxicity. (A) Cytotoxicity following various doses of IR was assessed by colony formation assays (p<0.05 for p53-273H versus wild type p53 or versus p53-179Q). Apoptosis was measured at 48 hours following mock or 5 Gy IR by (B) the levels of cleaved caspase 9 (the levels of cleaved caspase 9 were normalized to the levels of actin and the fold change relative to cells expressing WT p53 is shown), and (C) annexin V staining (*p<0.05, **p<0.01).

Figure 4. C-terminal BRCT domain is critical for BRCA1 to interact with p53 and response to p53-dependent nuclear export following DNA damage. Reciprocal immunoprecipitation experiments with (A) p53 or (B) HA were performed in HCC1937 cells co-transfected with wt p53 or vector control and HA-tagged WT or mutant (5382insC or S1423/1523A) BRCA1. The fold change relative to cells expressing WT BRCA1 is shown (*p<0.05). Subcellular localization of (C) WT BRCA1, (D) S1423/1524A BRCA1, or (E) 5382insC BRCA1 following to mock or 5 Gy IR was determined as a function of p53 by subcellular fractionation. The fold change relative to BRCA1 expression without treatment in each respective fraction is shown (bottom panels of B, D, F; *p<0.05, **p<0.01).

Figure 5. p53-mediated increase in IR-induced cytotoxicity and apoptosis requires the BRCA1 C-terminal region. Wild type, 5382insC, or S1432/1524A BRCA1 was co-expressed in HCC1937 cells together with wild type p53 or vector control. Cytotoxicity following various doses of IR was assessed by (A–C) colony formation assays. (D–F) Apoptosis was measured at 48 hours following mock or 5 Gy IR by the levels of cleaved caspase 9, and (C) annexin V staining (*p<0.05, **p<0.01).
Figure 6. **Loss of p53 function increases nuclear BRCA1 in breast cancer patients.** Tissue sections from breast cancer patients genotyped as wild type for BRCA1 were co-stained for BRCA1 and p53. (A) Representative staining of N, N/C, and C subcellular distributions of BRCA1 in cells with dysfunctional p53. (B) Representative staining of N, N/C, and C distributions of BRCA1 in cells with functional p53. (C) Box-whisker plots of BRCA1 subcellular distribution with respect to p53 functional status in all 31 patients. (D) Ternary diagram of BRCA1 subcellular distribution with respect to p53 functional status for each individual patient (p<0.0001) (detailed explanation of the Ternary diagram is in supplement data, material and methods). The filled circles indicate localization of BRCA1 in cells that retain p53 function. The open circles indicate BRCA1 localization when p53 function has been lost. The gray lines join data points for each of the individual 31 patients.

Figure 7. **BRCA1 RING domain expression increases cytosolic BRCA1 and sensitizes breast cancer cells to IR.** (A) BRCA1 subcellular distribution (N, N/C, and C) was determined by immunofluorescence using BRCA1 Ab-1 antibody (*p<0.001, **p<0.01). (B) Colony formation ability, (C) Annexin V staining, and (D) levels of cleaved caspase-9 were assayed on the collected YFP-positive cells (*p<0.05). (E) Model depicting potential targeting of BRCA1 to the cytoplasm to inhibit DNA repair, promote apoptosis, and sensitize cells to DNA-damaging agents. Following DNA damage, BRCA1 facilitates the repair of DNA in the nucleus, which is dependent on association of BRCA1 with BARD1. When unrepaired DNA damage is persistent, p53 binds to BRCA1 at the BRCT domain, which may dissociate BRCA1 from BARD1,
BRCA1 cytoplasmic translocation, and activate apoptosis. By targeting BRCA1 to cytoplasm, tumor cells are sensitized to DNA-damaging agents.
Jiang et al., figure 1

A

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Relative fold change

IR

- + - +
N C N C

p53-WT

p53-179Q

p53-273H

B

WT BRCA1 Vector

WT BRCA1

WT p53

% of cells

IR

- + - +
N C N C

p53-WT

p53-179Q

p53-273H
Figure 3

A. Survival Fraction vs. Dose of IR (Gy)

- p53-WT
- P53-179Q
- P53-273H

B. Cleaved Caspase-9 and β-actin

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C. Relative IR-induced fold change

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D. IR-induced apoptosis(%)

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*Significant difference
A, B, C, and D: Western blot images showing the expression levels of BRCA1, p53, HSP70, and Histone H1 under different conditions.

**A:**
- BRCA1: WT, S1423/1524A, and 5382 insC
- WT p53
- IP: IgG
- WB: BRCA1

**B:**
- p53
- IP: HA
- WB: BARD1
- WB: BRCA1

**C:**
- WT BRCA1
- WT BRCA1+P53
- IR: N, C
- Relative IR-induced fold change

**D:**
- S1423/S1524A BRCA1
- S1423/1524A BRCA1+P53
- IR: N, C

**E:**
- 5382 insC BRCA1
- 5382 insC BRCA1+P53
- IR: N, C
Jiang et al., figure 5

A

Survival Fraction

WT

Dose of IR (Gy)

Vector

WT p53

B

Survival Fraction

5382 insC

Dose of IR (Gy)

Vector

p53

C

Survival Fraction

S 1423/1524A

Dose of IR (Gy)

Vector

p53

D

IR-induced fold change

WT

Vector

p53

Cleaved Caspase-9

β-actin

E

IR-induced fold change

5382 insC

Vector

p53

Cleaved Caspase-9

β-actin

F

IR-induced fold change

S1423/S1524A

Vector

p53

Cleaved Caspase-9

β-actin

G

IR-induced apoptosis (%)

Vector

p53

BRCA1 WT S1423/1524A 5382 insC
p53-dependent BRCA1 nuclear export controls cellular susceptibility to DNA damage

Juhong Jiang, Eddy S. Yang, Guochun Jiang, et al.

Cancer Res  Published OnlineFirst July 8, 2011.

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