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ABSTRACT

Breast cancer is a genetically heterogeneous disease, and multiple genes remain to be identified among non-BRCA1 and BRCA2 breast cancer-prone families. This statement is supported by the evidences that BRCA1 and BRCA2 mutations are associated with between 20 to 60% of hereditary breast cancer families, which is less than originally estimated, especially in population-based studies. There is mounting evidence which suggests that there may be additional, less prevalent breast cancer susceptibility genes, however the identification of these genes remain elusive. Several groups are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depend on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next BRCA genes will be those that encode for proteins whose functions are linked to important cell regulatory pathways. We have recently found one such candidate BRCA3 protein, referred to as BRCC36.

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INTRODUCTION

Breast cancer is a genetically heterogeneous disease, and multiple susceptibility genes are remaining to be identified among non-*BRCA1* and *BRCA2* breast cancer-prone families. This statement is supported by evidence that *BRCA1* and *BRCA2* mutations are associated with only 20 to 60% of hereditary breast cancer, which are less than originally estimated, especially in studies on population-based family materials (1, 2). There is mounting evidence to suggest that there may be additional, less prevalent high penetrance breast cancer susceptibility genes, however, the identification of these genes remains elusive (3). In addition, germline mutations of *PTEN*, *LKB1*, *ATM*, *TP53*, *MSH2/MLH1*, *CHK2*, and *BACH-1* are associated with breast cancer, but to a much more limited extent than *BRCA1* and *BRCA2*. Several cooperative groups including the Breast Cancer Linkage Consortium (BCLC) are currently collecting high-risk breast cancer families and evaluating them by standard linkage analysis approaches for evidence of new breast cancer susceptibility loci (4). These types of studies are often hampered by the lack of sufficient numbers of large families with multiple living affected individuals for which blood samples are available and the likelihood that familial breast cancer is heterogeneous. Therefore, other approaches must be used to identify new genes that predispose to breast cancer. A valid alternative approach is to examine candidate genes in families with multiple cases of breast cancer to determine whether such candidates carry mutations that might account for a proportion of these families with unknown genetic etiology. The most likely strategy is to evaluate genes that code for proteins with equivalent or complementary functions or function in the same pathway as BRCA1 and BRCA2. This strategy may also help to uncover important new insight into the role of BRCA1 and BRCA2 in sporadic breast and ovarian cancer. In comparison to the facts that *BRCA1* and *BRCA2* mutations contribute to hereditary breast/ovarian cancer predisposition, it is surprising that these genes are rarely found mutated in sporadic breast/ovarian cancers. Nevertheless, evidence is accumulating that dysfunction of other genes, coding for proteins in the same pathway as BRCA1 and BRCA2, might be important in the pathogenesis of a significant proportion of sporadic, non-familial cancers. This speculation comes from several line of evidence, including both phenotypic analyses of breast and ovarian tumors and mechanistic studies of BRCA pathways (5, 6).

One of major problems of this strategy is that the "true" function(s) of BRCA1 and BRCA2 have not been completely established, nor have all of the proteins been identified which interact with BRCA1 and BRCA2 (7). Wang *et al* have previously reported that a set of proteins associate with BRCA1 to form a large mega-Dalton protein complex, referred to as BASC (BRCA1-Associated Genome Surveillance Complex). This complex includes several tumor suppressors, the DNA damage repair proteins MSH2, MSH6, MLH1, ATM, BLM, the RAD50–MRE11–NBS1 protein complex, and is responsive to double stranded breaks (8). However, the entire BRCA1/2 associated protein complex has yet to be purified. In this aspect, our collaborators, Dr. Shiekhattar's research group have isolated a novel multiprotein complex, termed BRCC (BRCA Containing Complex), which contains seven polypeptides including BRCA1, BRCA2 and RAD51 (9). BRCC is an ubiquitin E3 ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53. Importantly, one of these proteins, BRCC36, appears to be a novel component of the complex with sequence homology with a subunit of the signalosome and proteasome complexes. Cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC (9).

BODY

Task 1: To expand the evaluation of the expression of BRCC36 in clinical breast tumor samples, and to determine if over-expression of BRCC36 is associated with gene amplification in breast cancer.

We have clearly shown that *BRCC36* is aberrantly expressed in the vast majority of sporadic breast tumors. Quantitative real-time PCR (qPCR) was performed to evaluate the expression of *BRCC36* mRNA levels in multiple independent normal breast organoids, primary epithelial cell cultures, nontumorgenic breast epithelial cell lines, breast cancer cell lines, and human breast tumor tissue specimens. In comparison with non-tumorigenic breast epithelial cell lines, the expression levels of *BRCC36* mRNA were dramatically increased in about 75% of breast cancer cell lines (3 of 4). Furthermore, the expression levels of *BRCC36* mRNA were elevated in 58% (11 of 19) of the clinical breast tumors evaluated when compared to normal breast organoids (**Figure 1A**). A subset of these tumors showed very high levels of expression relative to both the organoids and primary epithelial cultures. In comparison, only 16% (3/19) of these breast tumors showed c-*ERBB2* amplification/over-expression. To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser captured micro-dissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells. Two normal breast tissues and 9 invasive ductal carcinomas were micro-dissected and the RNA evaluated for expression of *BRCC36*. We found that 100% of these tumors (9 of 9) showed elevated levels of *BRCC36* mRNA relative to the normal mammary ductal epithelium (**Figure 1B**). Seventy-seven percent of these tumors expressed very high levels of *BRCC36* (>20-fold) as compared to normal epithelial cells.

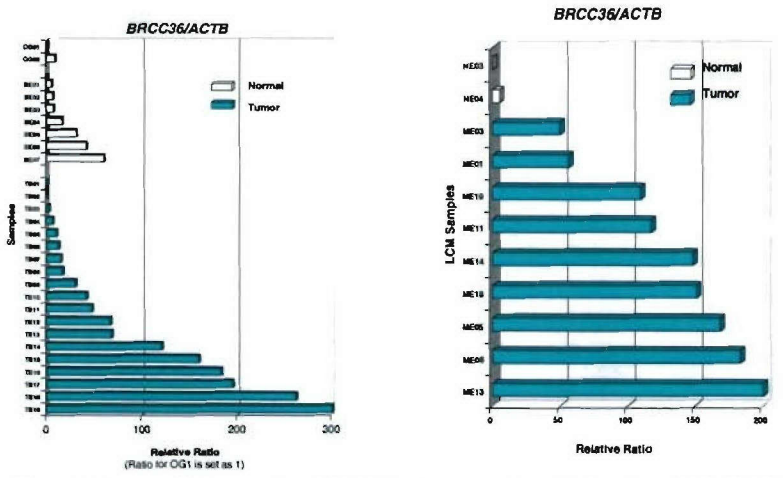

Figure 1. Aberrant mRNA expression of *BRCC36* in breast samples. (A) Quantitative PCR (QPCR) was performed to evaluate the *BRCC36* gene expression in the RNA samples isolated from breast mammary organoids (OG), primary breast epithelial cells (BE), and breast tumors (TB) *(right panel).* **(B)** QPCR was performed to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by laser micro-dissection (LCM). All levels of *BRCC36* gene expression were adjusted for equivalent *β-actin* gene expression *(left panel).*

Task 2: To determine the frequency of BRCC36 germline mutation in BRCA1 and BRCA2 mutation negative breast cancer prone kindreds.

We have made progress in regards to this task and have screened only a small number of breast cancer cell lines for mutations in BRCC36. A set of 4 oligonucleotide primer pairs were designed to amplify cDNA for the coding region of BRCC36, and a set of 11 oligonucleotide primer pairs were designed to amplify genomic DNA region for the BRCC36 coding exons.

Blood DNA from probands affected with breast cancer and reporting at least 2 first-degree and/or second-degree relatives with breast cancer were evaluated for germline mutations. In this initial screen, no germline BRCC36 mutations were identified in the 25 BRCA1/2 mutation negative breast cancer-prone kindreds examined. In addition, no somatic mutations were identified in 7 breast tumor cell lines, and an intron alteration (IVS2-29delT) was identified in one primary breast tumor. However, during the process of amplifying various regions of the cDNA, we identified one potential splicing variants of *BRCC36* that appears to be tumor specific (**Figure 2**). This alternative splicing leads to in-frame 25 amino acids insertion. The functional significance of this variant is under investigation.

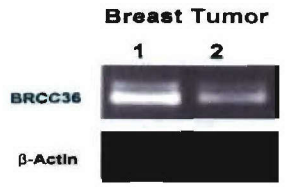

Figure 2. Splicing variants of *BRCC36* in breast tumor.

Task 3: To determine the role of BRCC36 in tumorigenesis by studying if its over-expression contributes to malignant transformation of mammary epithelial cells.

To further elucidate the functional consequence of *BRCC36* over-expression in breast cancer, we performed *in vitro* silencing studies using small interfering RNAs (siRNA) targeting *BRCC36* in MCF-7 breast cancer cell line which constitutively expresses high levels of *BRCC36*. Quantitative PCR analysis revealed a >80% decrease in mRNA levels in *BRCC36* siRNA transfected cells in comparison to those control siRNA transfected cells ($p<0.05$) (**Figure 3**). Since BRCC36 directly interacts with BRCA1, and previous studies indicated that the BRCA1 protein is activated via the ATM/CHK2 signaling pathway following the exposure of cells to DNA damaging agents such as ionizing radiation (IR) (7), we sought to determine the role, if any, of BRCC36 in this response. Therefore, siRNA transfected MCF-7 cells were exposed to IR and examined for DNA damage-induced cell apoptosis. There was no significant difference in the apoptotic fraction of untreated cells transfected with either *BRCC36* siRNA or control siRNA. However, when combined with BRCC36 abrogation, IR exposure led to an increase in apoptotic cells (45.9% ± 4.3% vs. 34.9% ± 1.9%) ($p<0.05$) and a lower fraction of viable cells (50.9% ± 5.8% vs. 58.4% ± 5.7%) when compared to the siRNA control group.

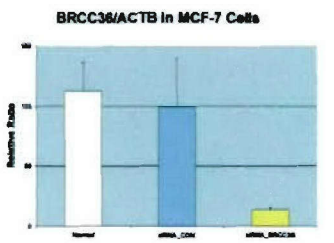

Figure 3. Abrogation of BRCC36 Expression by siRNA Treatment.

Previous studies have indicated that the BRCA1 protein is phosphorylated in response to DNA damaging agents. As part of the BRCC complex, we wanted to examine the possible interaction between BRCA1 and BRCC36 and how that might alter the DNA repair pathway. We examined the effect of abrogation of BRCC36 on the DNA damage pathway on targets both up and downstream of the BRCC complex, and in particular BRCA1. Cells were treated with siRNA targeting BRCC36 followed

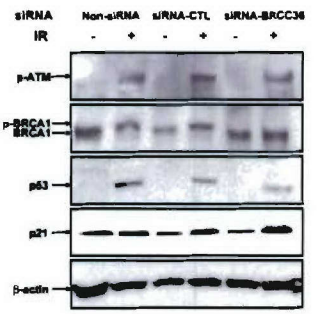

Figure. 4. Activation of BRCA1 in Response to IR treatment. MCF-7, MCF-7/siRNA control and MCF-7/siRNA-BRCC36 cells were un-treated or treated with IR (4Gy), and cells were lysed at 2h after radiation. The phosphorylated BRCA1 was evaluated by immunoblotting with anti-BRCA1 antibody. The protein levels of phosphorylated ATM, p53, and p21 were determined by immunoblotting with anti-p-ATM, anti-p53, and anti-p21 antibodies, respectively. The protein levels of were determined by immunoblotting with Protein loading levels were evaluated by immunoblotting with anti-beta-actin antibody.

by 4 Gy of ionizing radiation to induce DNA damage. Western blot analysis was then carried out, examining BRCA1, p21, p53, and ATM. Western analysis clearly showed that DNA damaging ionizing irradiation increased the expression of p21, p53 and phosphorylated ATM. But, there was no significant difference noted in expression of these proteins in those cells that were BRCC36 depleted, thus showing that BRCC36 had no direct effect on the expression of p21, p53, or ATM. In contrast, BRCC36 abrogation clearly disrupted BRCA1 phosphorylation in BRCC36 depleted cells (**Figure 4**).

Furthermore, it is known that BRCA1 localizes to discrete nuclear foci (dots) during S phase or in response to DNA damage induced by IR (7). In our previous report, we have domenstrated that BRCC36 enables to associate directly with BRCA1 at the region encompassing amino acids 502-1,054. This predicted sites where BRCC36 binds to BRCA1 matches with this BRCA1 DNA binding domain (aa452-1079). Since the DNA binding domain has been indicated to contribute the BRCA1 nuclear localization. Therefore, we decide to evaluate the role of BRCC36 in the formation of BRCA1 nuclear foci in response to DNA damage. MCF-7/non-siRNA, MCF-7/siRNA-control and MCF-7/siRNA-BRCC36 cells were exposed to 4 Gy IR. The cells were then recovered for 2 or 4 hours before immunostaining for BRCA1 and γH2AX. As shown in Figure 5A and 5B, many MCF-7/siRNA-BRCC36 cells showed clear decreased BRCA1 nuclear formation in comparison to the MCF-7/non-siRNA and MCF-7/siRNA-control (**Figure 5A** and **5B**). After quantification utilizing Metamorph, the nuclear foci formation of BRCA1 in MCF-7/siRNA-BRCC36 decreased about 62.7% and 52.1% comparing with MCF-7/siRNA-control at 2h and 4h post-IR, respectively ($p<0.05$) (**Figure 5C**).

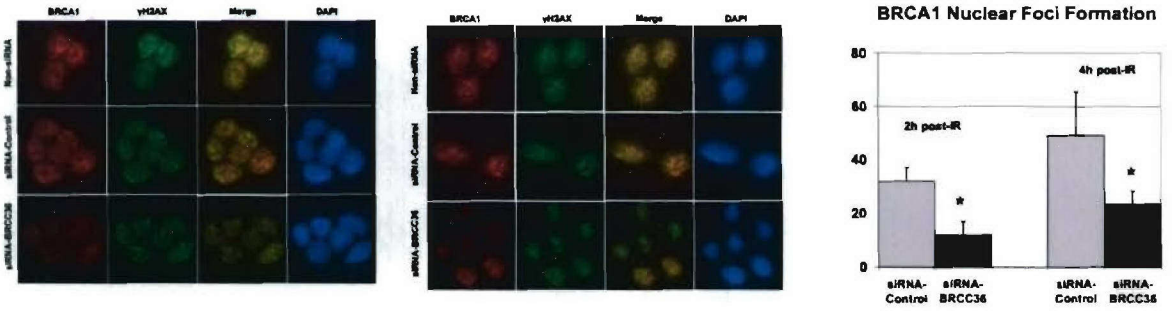

Figure 5. BRCA1 Nuclear Foci Formation in Breast Cancer Cells Following IR Exposure.
MCF-7 cells were transfected with either GFP (SiRNA control) or BRCC36-siRNAs. At 24 h post-transfection, cells were treated with 4 Gy IR were then incubated for 2 or 4 hours. After pre-extraction and fixation, transfection cells then were immunostained for BRCA1 and gH2AX. Microscopic analysis was carried out using the Nikon Eclipse TE2000 and a Cascade 650 monochrome camera. Quantification of BRCA1 nuclear foci formation was performed with Metamorph® software (v6.1.) was performed with Metamorph® software (v6.1.). (A). BRCA1 and γH2AX nuclear foci formation at 2h post-IR exposure. (B). BRCA1 and γH2AX nuclear foci formation at 4h post-IR exposure. (C). Quantification of BRCA1 nuclear foci formation at 2h and 4h post-IR exposure.

KEY RESEARCH ACCOMPLISHMENTS

1. Using cell lines expressing a stable Flag-BARD1, the BRCA1-associated RING domain protein, we isolated an E3 ubiquitin ligase complex termed BRCC containing seven polypeptides including BRCA1, BRCA2 and RAD51.
2. Demonstrated that cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC.
3. Reported that depletion of BRCC36 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation.
4. Reported that the abrogation of BRCC36 prevents the phosphorylation of BRCA1 and does not affect the levels of ATM, p53 and p21 in the breast cancer cells response to IR.
5. Reported that the abrogation of BRCC36 disrupts the BRCA1 nuclear foci formation in the breast cancer cell following IR other than disrupts the BRCC complex integrity.
6. Demonstrated that BRCC36 is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.
7. Evaluated *BRCA1* and *BRCA2* negative families for germline mutations in *BRCC36*, no deleterious mutations have been found as of yet.

REPORTABLE OUTCOMES

1. Abstracts

Chen X. W., Dong Y, Hakimi M-A, Shiekhattar R, Godwin AK. Aberrant expression of BRCC36, a novel subunit of a BRCA1 E3 ubiquitin ligase complex, in sporadic breast cancer. AACR Anual Meeting (Abstract Number: #3643); 2004.

Inhibition of BRCC36, a novel subunit of a BRCA1 E3 ubiquitin ligase complex, promotes ionizing radiation-induced apoptosis in breast cancer cells. Chen, X., Arciero, C.A., Wang, C., Broccoli, D., Shiekhattar, R., and Godwin, A.K. AACR Anual Meeting (Abstract Number: #5701); 2005.

2. Publications

Dong, Y., Hakimi, M-A., X. Chen, Kumaraswamy, E., Cooch, N.S., Godwin, A.K., and Shiekhattar, R. Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and its Role in DNA Repair. Molecular Cell, 12:1087-1099, 2003.

Chen, X., Arciero, C.A., Wang, C., Broccoli, D., Shiekhattar, R., and Godwin, A.K. Inhibition of BRCC36 enhances apoptosis by disrupting BRCA1 phosphorylation and nuclear foci formation in breast cancer cells exposed to ionizing radiation. Submitted, 2005.

CONCLUSIONS:

The biochemical pathways that are disrupted in the genesis of familial and sporadic breast cancers remain unclear. Moreover, the present prognosticating markers used to determine the prognosis of node-negative-patient leads to probabilistic results and the eventual clinical

course is far from certain. Here we identified the human BRCC36 complex, an E3 ubiquitin ligase complex containing eight polypeptides including BRCA1, BRCA2 and RAD51. We show by LCM and real-time PCR approaches that while BRCC36 is expressed at very low levels in normal breast epithelial cells, it is highly expressed in breast tumors. Reduction of BRCC36 levels in breast cancer cell lines by siRNA results in increased sensitivity to ionizing radiation. Abrogation of BRCC36 prevents the phosphorylation of BRCA1, and disrupts the BRCA1 nuclear foci formation in the breast cancer cell following IR. These results suggest that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR and appears to sensitize breast cancer cells to IR-induced apoptosis. Therefore, BRCC36 may be a therapeutic target for the management of radiation resistant breast tumors.

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APPENDICES

None