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Award Number: DAMD17-03-1-0707

TITLE: BRCC36, A Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex:  
Candidate Breast Cancer Susceptibility Gene

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REPORT DATE: September 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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**20060508036**

# REPORT DOCUMENTATION PAGE

*Form Approved*  
**OMB No. 0704-0188**

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<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-09-2005			<b>2. REPORT TYPE</b> Final		<b>3. DATES COVERED (From - To)</b> 1 Sep 2003 - 31 Aug 2005	
<b>4. TITLE AND SUBTITLE</b> BRCC36, A Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> DAMD17-03-1-0707	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Andrew K. Godwin, Ph.D.  E-mail: Andrew.godwin@fcc.edu					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Fox Chase Cancer Center Philadelphia, PA 19111-2412					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some years ago, when the breast-cancer susceptibility genes, BRCA1 and BRCA2 were identified. However, recent studies have suggested that mutations in these genes are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups including ours 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 the 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.						
<b>15. SUBJECT TERMS</b> Breast cancer susceptibility, BRCA1 interacting protein						
<b>16. SECURITY CLASSIFICATION OF:</b>				<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U	<b>19b. TELEPHONE NUMBER (include area code)</b>			
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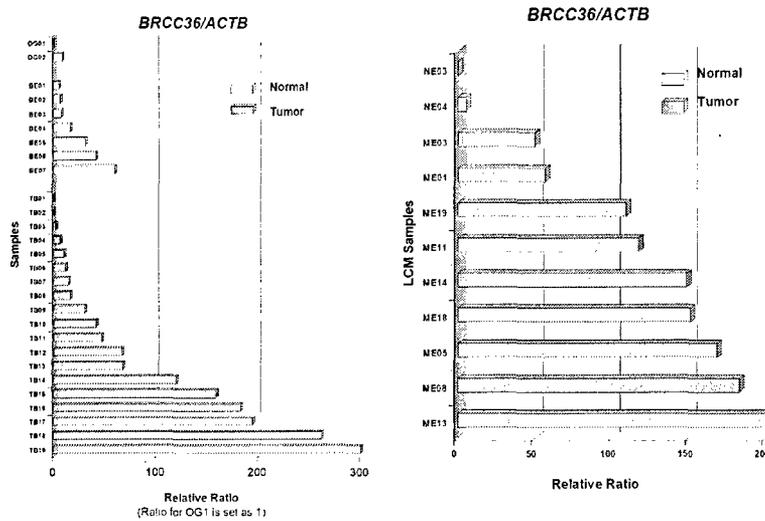
**INTRODUCTION:**

The strongest known epidemiological risk factor for breast cancer is a positive family history and studies of breast and ovarian cancer patients and their relatives consistently find statistical evidence for involvement of autosomal dominant genes. As a result of the cloning of two prominent breast-ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, clinical screening of women from high-risk families for germ-line mutations has been realized. Mutations in these two breast cancer susceptibility genes accounts for most multiple-case breast cancer families containing members with ovarian cancer or male breast cancer (1, 2). However, mutations in the *BRCA1* and *BRCA2* genes have been identified in only about 40% of families with four or five cases of female breast cancer but not cases of male breast cancer (1, 2). Several groups are searching for these genes by use of whole genome scanning, but the success of this approach depends on the underlying heterogeneity of the remaining cancer susceptibility loci. Furthermore, these suspected breast cancer families are too numerous to be statistical "accidents" of non-hereditary breast cancer. Therefore, 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 those of *BRCA1* and *BRCA2* (e.g., associate with *BRCA1* and/or *BRCA2* to form large functional protein complexes). We have recently found one such candidate *BRCA3* protein, referred to as *BRCC36*.

**OVERVIEW:**

We hypothesize that proteins whose functions are linked to those of *BRCA1* and/or *BRCA2* are likely to contribute to the remaining breast cancer susceptibility. Furthermore, we believe, based on lack of success of genome wide association studies to uncover tumor suppressor type susceptibility genes that mutations in a proto-oncogene may also predispose to breast cancer. A survey of the 30+ genes identified that have been associated with various hereditary cancer syndromes find that at least four are oncogenes (i.e., *RET*, *MET*, *KIT*, *CDK4*). Using cell lines expressing a stable Flag-BARD1, the *BRCA1*-associated RING domain protein, a novel E3 ubiquitin ligase complex termed BRCC was isolated containing eight polypeptides including *BRCA1*, *BRCA2* and *RAD51* (Dong et al., Molecular Cell, 2003). BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 *in vitro* (Dong et al., Molecular Cell, 2003). 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 (Dong et al., Molecular Cell, 2003). Moreover, *BRCC36* is aberrantly expressed in a number of breast cancer tumors (42%; 8/19) and cell lines (75%; 3/4%). In comparison, only 16% (3/19) of these breast tumors showed *c-ERBB2* amplification/overexpression. Furthermore, over-expression of *BRCC36* abolished *BRCA1* coactivation of p53 transcriptional responsiveness. These findings identify BRCC as an ubiquitin E3 ligase complex and suggest that aberrant expression of its components, e.g., *BRCC36*, may contribute to various forms of breast cancer.

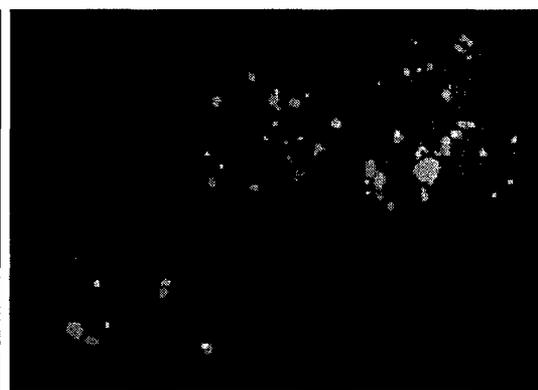
**BODY****Final Report****Task 1. Evaluate breast tumors that over-express *BRCC36* for gene amplification.**



**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) (left 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 with *beta-actin* gene expression (right panel).

We have clearly shown that *BRCC36* is aberrantly expressed in the vast majority of sporadic breast tumors.

Real time quantitative PCR (qPCR) was performed to evaluate the expression of *BRCC36* mRNA levels in multiple independent normal breast organoids (uncultured breast ducts composed of luminal and myoepithelial cells), primary epithelial cell cultures, non-tumorigenic breast epithelial cell lines, breast cancer cell lines, and human breast tissue specimens surgically obtained from patients with primary invasive carcinoma as described in our manuscript (3). The expression levels of *BRCC36* mRNA were elevated in 58% (11 of 19) of the breast tumors evaluated when compared to normal breast organoids (Figure 1A). A subset of these tumors (6 of 19) showed very high levels of expression relative to both the organoids and primary epithelial cultures (Figure 1A). To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser captured microdissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells. Two normal breast tissues and 9 invasive ductal carcinomas were microdissected and the *BRCC36* mRNA levels were evaluated by RT-PCR (Figure 1B). 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). We next obtained DNA from these tumors that highly expressed *BRCC36* and evaluate gene copy number by southern blotting. Gene amplification was not detected in these samples relative to normal blood DNA (data not shown), suggesting that gene amplification was not the primary means of increased expression. However, we also developed a Fluorescence In Situ Hybridization (FISH) assay to evaluate *BRCC36* gene amplification in additional clinical samples. The FISH probe to detect *BRCC36* was derived from a Homo sapiens BAC clone (RP11-14H17) containing the entire *BRCC36* gene and flanking sequences (GenBank accession no. BX293995). FISH probe DNA was labeled following standard protocols and hybridized to metaphase



**Figure 2.** Fluorescence images showing that the *BRCC36* gene is amplified in male breast cancer cases by FISH. Green signals represent *BRCC36* and red signals indicate chromosome 7.

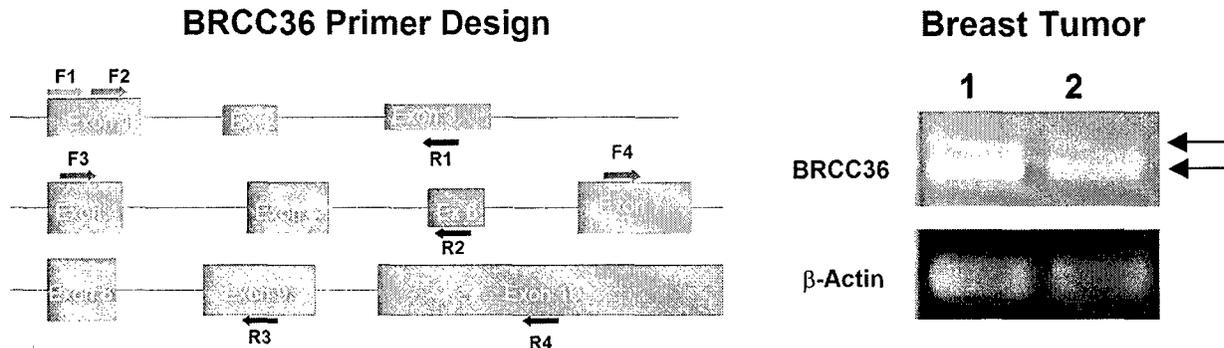
chromosome spreads and interphase cells. We first confirmed that the location of the BAC probe for *BRCC36* is on chromosome X (data not shown).

Since, *BRCC36* is located on chromosome X, we decided to evaluate a limited number of male breast cancers by FISH and found that multiple copies of the

*BRCC36* gene were present in some male breast cancer cases (Figure 2). However, we also notice that these tumors were aneuploid based on increased copies of chromosome 7. We have evaluated 9 male breast cancer cases and found that 2 of 9 showed increased *BRCC36* gene copies. We are developing a PCR-based assay to more rapidly evaluate *BRCC36* gene copy number in DNA from breast tumors.

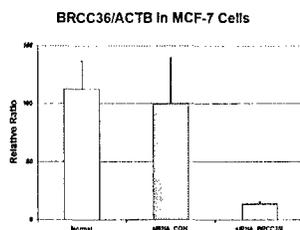
**Task 2. Evaluate *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *BRCC36* mutations.**

We screened a number of breast cancer cell lines and cancer prone individuals for mutations in



**Figure 3.** (A) RT-PCR primer design for *BRCC36*. A set of 4 oligonucleotide PCR primer pairs was designed to amplify the coding region of *BRCC36* (left panel). (B) RT-PCR analysis of breast tumor RNA detected a similar splice variant in *BRCC36* (right panel).

*BRCC36*. A set of 4 oligonucleotide primer pairs were designed to amplify the coding region of *BRCC36* (Figure 3A). We first evaluated RNA by RT-PCR and direct sequencing for evidence of sequence variants in 7 breast tumor cell lines (i.e., MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-486, SK-BR-3, ZR-25-1, T-47D). No alterations were found in any of the tumor cell lines when compared to the wild-type sequence that we had established previously. We next evaluated RNA isolated from lymphoblastoid cell lines derived from women affected with breast cancer who reported a significant family history of breast and/or ovarian cancer. All had tested negative for germline mutations in *BRCA1* and *BRCA2*. Our first evaluation found no evidence for deleterious mutations in *BRCC36* in any of the 25 individuals screened. However, during these evaluations, we identified one potential splicing variants of *BRCC36* using the primer set F3/R3 (Figure 3B). Studies are underway to evaluate the frequency of this splice variant and determine its origin. Furthermore, we have yet to identify a sequence variant (either germline or somatic) in *BRCC36*. This is somewhat unusual and may reflect an allele bias by evaluating only RNA. Studies are underway to evaluate DNA using high-throughput methods established in the lab (4-6).

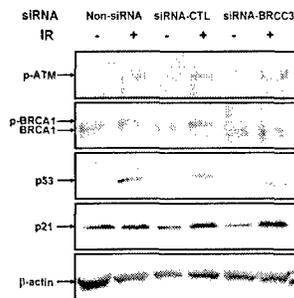


**Figure 4.** Abrogation of *BRCC36* expression by siRNA treatment.

We also have made progress in understanding the role of *BRCC36* in the pathogenesis of breast cancer (which was not part of the original objectives of this concept grant). To potentially elucidate the functional consequence of aberrant *BRCC36* 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 express 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 (Figure 4). In addition, we sought to determine the role, if any, of *BRCC36* in DNA repair and cell apoptosis pathway. 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 cells transfected with either

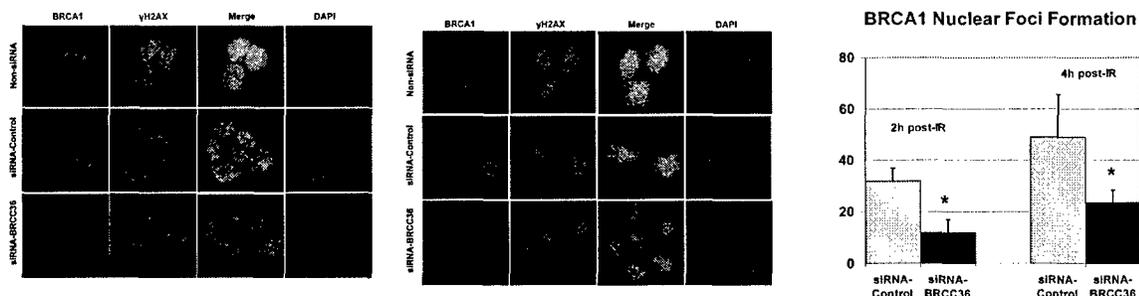
*BRCC36* siRNA or control siRNA. However, when combined with *BRCC36* abrogation, IR exposure led to an increase in apoptotic cells ( $45.9\% \pm 4.3\%$  vs.  $34.9\% \pm 1.9\%$ ) ( $p < 0.05$ ) and a lower fraction of viable cells ( $50.9\% \pm 5.8\%$  vs.  $58.4\% \pm 5.7\%$ ) when compared to the siRNA control group.

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 by 4 Gy of ionizing radiation to induce DNA damage. Western blot analysis was then carried out to examine *BRCA1*, p21, p53, and ATM expression and phosphorylation. Western analysis clearly showed that DNA damaging ionizing irradiation increased the expression of p21, p53 and phosphorylated ATM. There was no significant difference noted in expression of these proteins in those cells that were *BRCC36* depleted, thus suggesting that *BRCC36* had no direct effect on the expression of p21, p53, or ATM. In contrast, *BRCC36* abrogation disrupted *BRCA1* phosphorylation in response to IR (Figure 5).



**Figure 5. 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 loading were evaluated by immunoblotting with anti-beta-actin antibody.

Furthermore, it is known that *BRCA1* localizes to discrete nuclear foci (dots) during S phase or in response to DNA damage induced by IR. In our previous report, we have demonstrated that *BRCC36* is able to associate directly with *BRCA1*, at the region encompassing amino acids 502 to 1,054, which overlaps with the *BRCA1* DNA binding domain (a.a. 452 to 1,079). Since the DNA binding domain has been implicated in *BRCA1* nuclear localization we decided 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 allowed to recover for 2 or 4 hours before immunostaining for *BRCA1* and  $\gamma$ H2AX. As shown in Figure 6A & B, *BRCC36*



**Figure 6. *BRCA1* Nuclear Foci Formation in Breast Cancer Cells Following IR Exposure.** MCF-7 cells were transfected with either GFP (siRNA control) or *BRCC36*-siRNAs. Approximately 24 h post-transfection, cells were treated with 4 Gy IR and were then incubated for 2 or 4 hours. After pre-extraction and fixation, siRNA treated cells were immunostained for *BRCA1* and  $\gamma$ H2AX. 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.). (A) *BRCA1* and  $\gamma$ H2AX nuclear foci formation at 2h post-IR exposure. (B) *BRCA1* and  $\gamma$ H2AX nuclear foci formation at 4h post-IR exposure. (C) Quantification of *BRCA1* nuclear foci formation at 2h and 4h post-IR exposure.

depleted MCF-7 cells showed decreased *BRCA1* nuclear formation in comparison to controls. Quantification of these *BRCA1* containing nuclear foci demonstrated a decreased of ~62% and ~52% compared with MCF-7/siRNA-control at 2h and 4h post-IR, respectively ( $p < 0.05$ ) (Figure 6C).

Overall, our studies have shown that *BRCC36* is aberrantly expression in vast majority of breast tumors and abrogating *BRCC36* significantly increases the sensitivity of breast cancer cells to ionizing radiation-induced

apoptosis. These results suggest a potential role for BRCC36 in the DNA damage response pathway and BRCC36 may be a therapeutic target for the management of radiation resistant breast tumors.

## C- KEY RESEARCH ACCOMPLISHMENTS:

### C.1. "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"

- 1.a. Using cell lines expressing a stable Flag-BARD1, the BRCA1-associated RING domain protein, we isolated an E3 ubiquitin ligase complex termed BRCC containing eight polypeptides including BRCA1, BRCA2 and RAD51.
- 1.b. Reported that BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 *in vitro*.
- 1.c. Demonstrated that cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC.
- 1.d. Reported that depletion of BRCC36 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation, loss of G2/M checkpoint, decreased homology-directed DNA repair and deregulation of BRCC ubiquitin E3 ligase activity.
- 1.e. Demonstrated that *BRCC36* is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.
- 1.f. Found that expression of *BRCC36* was not apparently associated with gene amplification in female breast cancer.
- 1.g. Evaluated *BRCA1* and *BRCA2* negative families for germline mutations in *BRCC36*, no deleterious mutations were identified, however, a tumor associated splicing variant of *BRCC36* was detected in breast tumors.
- 1.h. Isolated a BAC clone for *BRCC36*, mapped the gene, and developed a FISH assay to assess gene copy number.
- 1.i. Reported that depletion of *BRCC36* by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation.
- 1.j. Reported that the abrogation of *BRCC36* prevents the IR-induced phosphorylation of *BRCA1*, while the levels of ATM, p53 and p21 do not appear to be altered
- 1.k. Reported that the abrogation of *BRCC36* disrupts the *BRCA1* nuclear foci formation in the breast cancer cell following IR, without disrupting integrity of the BRCC complex.

**D-REPORTABLE OUTCOMES:**

**D.1. "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"**

**1.a. 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. The 95<sup>th</sup> Proceedings of the American Association for Cancer Research, abstract #3643, 2004.

Chen, X., Arciero, C.A., Wang, C., Broccoli, D., Shiekhattar, R., and Godwin, A.K. Inhibition of BRCC36, a novel subunit of a BRCA1 E3 ubiquitin ligase complex, promotes ionizing radiation-induced apoptosis in breast cancer cells. The 96<sup>th</sup> Proceedings of the American Association for Cancer Research, abstract #5701, 2005.

**1.b. Publications**

Dong, Y., Hakimi, M-A., X. Chen, Kumaraswamy, E., Cooch, N.S., Godwin, A.K., 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, 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.

**Book chapters and review articles:**

Pan, Z-Z., Godwin, A.K. Oncogenes. *Encyclopedia of Molecular Cell Biology and Molecular Medicine*. Edited by R.A. Meyers, Second Edition, Volume 9. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp., 435-495, 2005.

C.T. Bui, E. Nicolas, G. Sallmann, M. Chiotis, A. Lambrinakos, K. Rees, I. Trounce, R.G.H. Cotton, L. Hancock, A.K. Godwin, And Anthony T. Yeung. Enzymatic and Chemical Cleavage Methods to Identify Genetic Variation . In *Molecular Diagnostics* (Ed. G. Patrinos and W Ansorge) in press 2005.

**E-CONCLUSIONS:**

***E.1. "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"***

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 result in increased sensitivity to ionizing radiation, loss of G2/M checkpoint, decreased homology-directed DNA repair and deregulation of BRCC ubiquitin E3 ligase activity. These findings identify BRCC as an ubiquitin E3 ligase complex that enhances cellular survival following DNA damage. Although BRCC36 does not appear to be mutated in a limited series of clinical samples, it is abnormally expressed in the majority of breast tumors and may be amplified in some male breast cancer cases.

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