

AD _____

Award Number: DAMD17-00-1-0328

TITLE: Characterization of BRCA2 Mutations in a Series of
Functional Assays

PRINCIPAL INVESTIGATOR: Fergus J. Couch, Ph.D.

CONTRACTING ORGANIZATION: Mayo Clinic Foundation
Rochester, Minnesota 55905

REPORT DATE: May 2001

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010925 173

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2001	3. REPORT TYPE AND DATES COVERED Annual (1 May 00 - 30 Apr 01)	
4. TITLE AND SUBTITLE Characterization of BRCA2 Mutations in a Series of Functional Assays			5. FUNDING NUMBERS DAMD17-00-1-0328	
6. AUTHOR(S) Fergus J. Couch, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mayo Clinic Foundation Rochester, Minnesota 55905 E-Mail: couch.fergus@mayo.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The BRCA2 breast and ovarian cancer predisposition gene was identified in 1995. Mutations in the gene account for approximately 20% of all hereditary breast cancer and perhaps 2% of all breast cancer cases. Many individuals undergo expensive clinical testing for mutations in the BRCA2 gene in order to provide information to their family members about risk of breast cancer. The majority of mutations identified during clinical testing result in truncation of the protein, while approximately 20% are missense mutations. The affect of these missense mutations on BRCA2 function is not known. Thus, these mutations are termed unclassified variants and women carrying these mutations are informed that their results are inconclusive. To improve upon this situation we have proposed to develop a series of functional assays that can be used to determine if particular missense mutations disrupt BRCA2 function and are disease associated or not.				
14. SUBJECT TERMS Breast Cancer, BRCA2, missense mutation, functional assay			15. NUMBER OF PAGES 8	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover	1
SF 298	2
Introduction	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusions	6
References	6
Appendices	7

Introduction

The BRCA2 breast and ovarian cancer predisposition gene was identified in 1995. Mutations in the gene account for approximately 20% of all hereditary breast cancer and perhaps 2% of all breast cancer cases. Many individuals undergo expensive clinical testing for mutations in the BRCA2 gene in order to provide information to their family members about risk of breast cancer. The majority of mutations identified during clinical testing result in truncation of the protein, while approximately 20% are missense mutations. The affect of these missense mutations on BRCA2 function is not known. Thus, these mutations are termed unclassified variants and women carrying these mutations are informed that their results are inconclusive. To improve upon this situation we have proposed to develop a series of functional assays that can be used to determine if particular missense mutations disrupt BRCA2 function and are disease associated or not.

Body

Aim #1: To assess the role of BRCA2 in cell growth.

In the first aim of this study we proposed to establish Capan-1 BRCA2 mutant cells that stably express wildtype BRCA2. Capan-1 cells were chosen because of the absence of endogenous wildtype BRCA2. This will allow observation of the effect of wildtype and mutant forms of BRCA2 on the cell. However, we have encountered significant technical problems with this aim. Specifically, using multiple different transfection techniques we have been unable to achieve greater than 0.01% transfection efficiency, which is insufficient for the outlined experiments. The only possible alternative is the use of recombinant retrovirus nor adenovirus vectors. However, the BRCA2 gene is too large to be cloned into these vectors.

To overcome this problem we propose to overexpress the BRCA2 constructs in MCF7 breast cancer cells. While these cells express wildtype BRCA2 we believe that the overexpression of mutant forms will competitively inhibit wildtype BRCA2 function. Evidence for this claim is derived from a series of transient expression studies in MCF7 cells. MCF7 cells transiently transfected with BRCA2 constructs were serum starved and the apoptotic response was measured by flow cytometry. Overexpression of the 6174delT mutant form of BRCA2 inhibited the proapoptotic activity of wildtype BRCA2 indicating that mutant BRCA2 can function in a dominant negative manner and that this method can be used to evaluate the functional significance of missense mutations.

A second alternative involves using BRCA2 mutant mouse embryo fibroblast (MEF) cells that have been provided by Dr. Roger Wiseman (NIEHS). These cells contain a deletion of exon 27 of BRCA2 and are missing only the last exon of the gene. Previous studies of BRCA2 revealed that exon 27 encodes two functional nuclear localization sites. Thus, the exon 27 mutant is localized in the cytoplasm while the wildtype BRCA2 is localized to the nucleus. This suggests that we can use these cells to discriminate between mutant and wildtype forms of BRCA2. However, because these are primary cell lines that are not immortalized, we cannot make stable cell lines and are forced to use these lines only for transient assays.

We have focused our efforts on the use of MCF7 cells stably expressing wtBRCA2, vector alone, and 6174delT-BRCA2. These cells were assessed for alterations in growth rate (MTT assays),

anchorage independence (soft agar assays), colony formation, and cell cycle (FACS) as proposed in Task 3 (Appendix 1). In MTT assays two independent wtBRCA2 expressing cell lines grew significantly slower than vector transfected cells, while cells expressing mutant BRCA2 grew the most rapidly. In soft agar and colony formation assays more colonies formed from mutant or vector expressing cells. Cell cycle analysis revealed no change in the cell cycle over time. Overall, the data shows that BRCA2 regulates cell growth and transformation in a cell cycle independent manner, and that mutants of BRCA2 can be identified using these assays. We have not yet performed a tumorigenesis assay in nude mice, but this experiment will soon be carried out.

A total of 8 BRCA2 mutant constructs containing 7 missense mutations and 1 truncating mutation in different domains of BRCA2 have been generated (Appendix 2). This was accomplished using a shuttle vector system. All full length constructs were completely sequenced to ensure that only a single mutations was present in each construct. The Y42C mutation is located in a putative transactivation domain, while E462G, P655R, 4812C>T, and K1690N are all located in the BRC repeat region that has been associated with DNA repair activity. The remaining mutations are located in previously uncharacterized regions.

We have not, as yet, completed Task 6 and 7 of Aim 1. The reason for the delay is that the postdoctoral fellow carrying out the research left the laboratory unexpectedly in early January. We have been actively recruiting a replacement, but as a result of the lack of personnel, we have been unable complete all of our aims and tasks. However, once another fellow is in place we will make every effort to complete the outlined studies within the lifetime of the grant.

Aim #2: To test the effect of missense mutations and polymorphisms on BRCA2 function in a series of functional assays.

We have expanded upon the series of assays that we propose to use in the functional studies. Originally we aimed to look at DNA repair, apoptosis, hmdm2 transcriptional regulation, and cell growth. However, we have recently shown that mutant forms of BRCA2 induces centrosome amplification and aneuploidy in cells. Specifically, centrosomes in Hela cells overexpressing 6174delT-BRCA2 were identified using anti-centrin antibodies and counted by confocal microscopy. After 2 days the mutant BRCA2 construct had induced a significant increase in the number of centrosomes while wildtype BRCA2 resulted in retention of two centrosomes only. This relatively straightforward assay will now be used to study the other BRCA2 mutants.

In the coming year, we will use this and other assays to assess the affect of a variety of BRCA2 missense mutations.

Key Research Accomplishments

- 1) Established wildtype BRCA2, 6174delT-BRCA2, and pcDNA3.1 stable cell lines in MCF7 cells.
- 2) Demonstrated that wt BRCA2 regulates cell growth rate, anchorage independence, and colony formation.

- 3) Generated 8 different BRCA2 mutant constructs, 7 containing previously reported missense mutations.
- 4) Established that BRCA2 mutants result in centrosome amplification

Reportable Outcomes

No manuscripts have yet resulted from this work.

No other grant applications based on this work have been submitted.

Conclusions

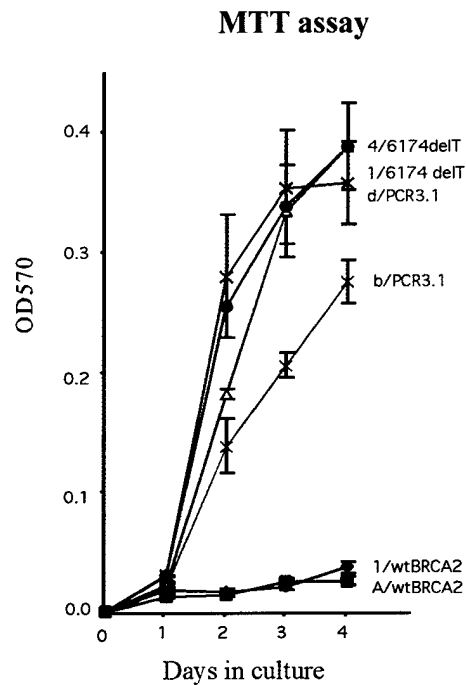
Our conclusions at this point are that it is possible to identify biologically relevant missense mutations in the BRCA2 gene using a series of functional assays. In previous studies we have shown that we can measure DNA repair, apoptosis in response to BRCA2 expression. In this work, we have shown for the first time that BRCA2 regulates centrosome amplification, and that BRCA2 has a direct suppression effect on cell growth and transformation. The combination of these measurable outcomes should facilitate identification of the majority of disease associated BRCA2 missense mutations, although some may still be overlooked. Having generated several mutant forms of BRCA2 we are now poised to begin to differentiate between benign polymorphisms and functional missense mutations.

References

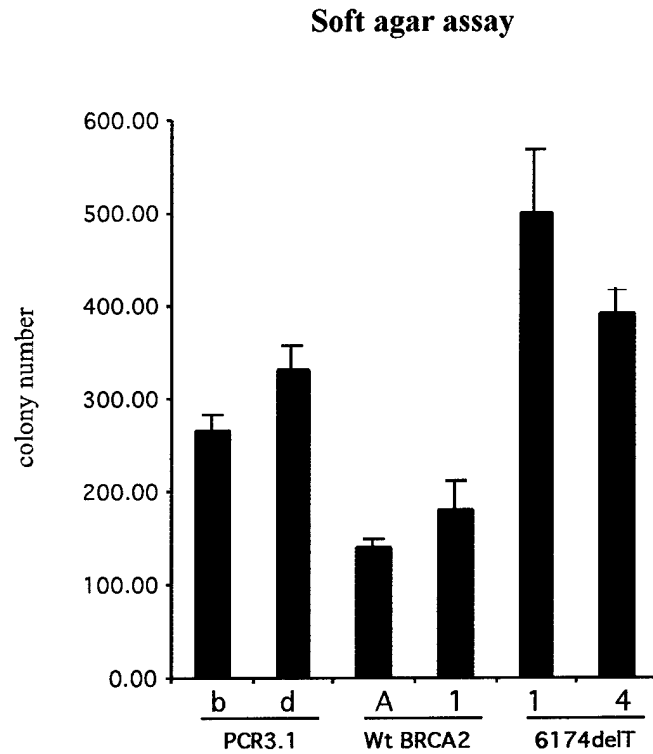
None.

Appendix 1.

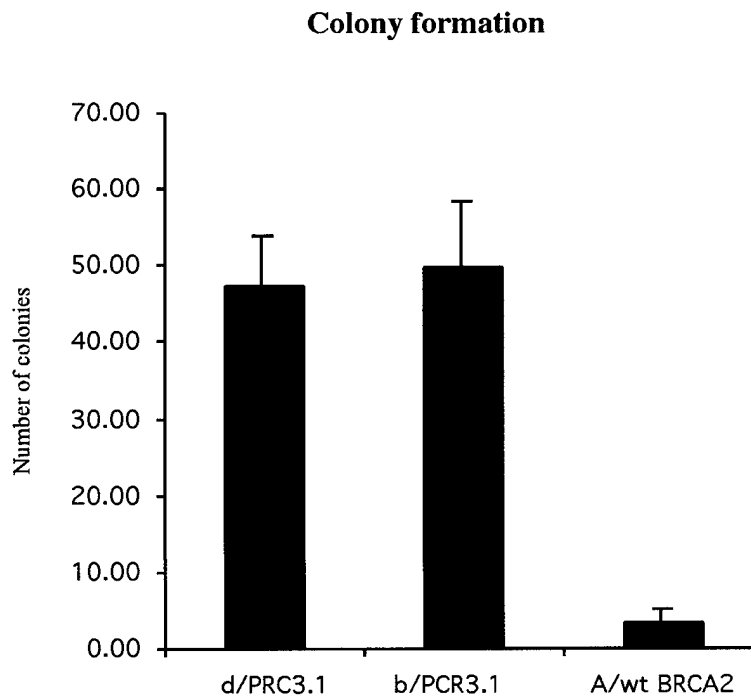
A



B



C



Growth retardation by BRCA2: MCF-7 cells stably expressing vector controls (b/PCR3.1, d/PCR3.1), wild type BRCA2 (1/wtBRCA2, A/wtBRCA2) and 6174delT mutant BRCA2 (1/6174delT, 4/6174delT) were plated at equal density and used for A) MTT, B) soft agar, and C) colony formation assays.

Appendix 2.

Mutant Name	Exon	NT	Codon	Base	AA Change	Designation	Mutation Type	BRCA2 Fragment
353	3	353	42	A to G	Tyr to Cys	Y42C	M	1
1613-1614	10	1613	462	A to G	Glu to Gly	E462G	M	1
2192-2193	11a	2192	655	C to G	Pro to Arg	P655R	M	1
4812	11d	4812	1528	C to T	Ser to Ser	4812C/T	P	3
5298	11d	5298	1690	A to C	Lys to Asn	K1690N	M	3
6174	11e	6174	1982	del T	Stop 2003	6174delT	F	4
6995	11f	6995	2256	G to A	Cys to Tyr	C2256Y	M	4
7625	14	7625	2466	C to A	Ala to Val	A2466V	M	4