

AD _____

Award Number: DAMD17-02-1-0503

TITLE: ATM Mutations and the Development of Severe Radiation-Induced Morbidity Following Radiotherapy for Breast Cancer

PRINCIPAL INVESTIGATOR: Barry S. Rosenstein, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029-6574

REPORT DATE: July 2004

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.

20041214 090

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 July 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 2003 - 30 Jun 2004)	
4. TITLE AND SUBTITLE ATM Mutations and the Development of Severe Radiation-Induced Morbidity Following Radiotherapy for Breast Cancer			5. FUNDING NUMBERS DAMD17-02-1-0503	
6. AUTHOR(S) Barry S. Rosenstein, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mount Sinai School of Medicine New York, New York 10029-6574 <i>E-Mail:</i> Barry.rosenstein@mssm.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				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The hypothesis being tested in this project is that a greater proportion of patients who develop radiation-induced subcutaneous late tissue morbidity possess a variant allele in the ATM gene compared with patients who do not suffer these complications. An additional objective is to determine the functional impact upon the protein encoded by the ATM gene for each genetic alteration identified and subsequent cellular radiosensitivity. The specific aims of this project are to (1) screen 50 breast cancer patients for ATM genetic alterations who developed radiation induced late subcutaneous tissue morbidity, (2) establish a control group and screen 100 patients without evidence of this late radiation reaction, and (3) perform functional studies using cells from patients identified as ATM carriers to determine to what extent each ATM variant identified affects radiosensitivity and normal activity of the protein produced by the ATM gene. The main accomplishment during the second year of this study was to accrue a total of 104 patients into this study and complete a full DHPLC screening of the ATM gene in each of these subjects. In addition, functional studies were performed with a series of wild type, AT and ATM heterozygote cell lines to measure the ATM kinase activity in these cells.				
14. SUBJECT TERMS No subject terms provided.			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.....	3
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendices.....	8

INTRODUCTION

The majority of female breast cancer patients treated with breast conservation protocols consisting of limited surgery followed by adjuvant radiation therapy to the breast and surgical bed can develop tissue changes within the irradiated volume. These changes are both expected and temporary, and in most instances will resolve with conservative medical management. In contrast, there is a small subset of patients who manifest persistent or late subcutaneous tissue changes that can result in poor cosmesis and often painful sequelae. In some cases there are plausible explanations for such reactions that may include large breast size, excessive radiation dose-fractionation schedules, use of concurrent chemotherapy, and medical comorbidities such as collagen vascular diseases and diabetes. However, there exists an important subset of patients with no clear explanation for excessive post-treatment morbidity and the potential for a genetic basis must be considered. The purpose of this study is to investigate whether the *ATM* gene plays a role in enhanced radiation sensitivity in this population. This gene was selected because the protein it encodes plays a critical role

in the response of cells to radiation and the repair of radiation-induced damage. Furthermore, cells possessing a mutated copy of this gene are more radiosensitive than cells from individuals with a normal genotype. In addition, the results of a pilot study screening breast cancer patients are supportive of the hypothesis that patients who are carriers of an *ATM* mutation are more likely to develop late radiation-induced subcutaneous tissue complications.

The principal goal of this project is to determine whether women who inherit an altered copy of the *ATM* gene are more prone to the development of late radiation-induced morbidity. This will be accomplished through comprehensive screening of the *ATM* gene for germline variants. If a correlation is found between radiosensitivity and *ATM* genetic status, this would indicate that possession of an altered *ATM* gene results in susceptibility to subcutaneous tissue complications for breast cancer radiotherapy patients. In addition, a determination will be made as to the pathogenic consequences of each *ATM* variant through the use of functional studies that will examine the *ATM* protein in cells from patients who are carriers of an alteration in this gene. This project is innovative as it represents the first study to use the powerful DHPLC mutation screening technique to investigate the association between *ATM* heterozygosity and radiation-induced morbidity in the female breast cancer population. It is also the first study to examine whether there is a correlation between the presence of specific *ATM* genetic alterations, development of radiation-induced complications, and impairment of *ATM* protein function based upon cellular and molecular analyses.

Confirmation of this hypothesis will have important and direct implications upon patient care. It may suggest that all newly diagnosed female breast cancer patients considering breast conservation management should be tested for *ATM* heterozygosity using the relatively rapid and efficient mutation screening approach outlined in this proposal. Those women found to harbor an *ATM* variant may not be ideal candidates for standard breast conservation protocols and could possibly be better served by alternate treatment approaches such as modified radical mastectomy and breast reconstruction. Alternatively, these women may be ideal candidates for a dose reduction trial. A reduced total dose to the breast may result in equivalent local control rates as germline *ATM* gene alterations should be present in both tumor and normal cells and cause enhanced radiation sensitivity for both cell types. However, this remains to be tested. In either case, *ATM* mutation detection may help to prevent many women from experiencing the poor cosmetic and potentially painful side effects that can result from conventional breast radiotherapy in *ATM* carriers.

Body

Due to the substantial delay until April 30, 2003 from the HSRRB (Human Subjects Review Board) of the DOD for approval of the human subjects protocol and consent forms for this project, which was followed by an additional delay to obtain approval from both the Mount Sinai and NYU IRBs, subject accrual into this study could not be initiated until the beginning of the second year of this project. However, once

these approvals were obtained, all of the study investigators made a concerted effort to rapidly accrue patients and screen DNA samples for *ATM* variants. Hence substantial progress was made in the past year of this project with complete screening of the *ATM* gene accomplished for 104 breast cancer patients who received standard radiotherapy for treatment of their cancer, 46 of whom exhibited late subcutaneous responses and 58 who did not display these reactions. A full listing of the *ATM* variants detected in these patients is provided below. Of the total number of patients, 36% (37/104) were found to possess an *ATM* variant.

A full analysis of these data has not been performed, but the main finding of interest is that 73% of the patients found to possess an *ATM* alteration (27/37), displayed an adverse reaction. In contrast, only 28% (19/67) of the patients who did not harbor an *ATM* variant allele, developed a late subcutaneous response. This finding supports the hypothesis that alternations in the *ATM* gene render breast cancer patients susceptible to adverse effects of radiotherapy.

The following tables report the specific results of the *ATM* variants detected for subjects who either did, or did not, display a late subcutaneous tissue response. In these tables, N represents no reaction, F indicates fibrosis, T telangiectasia and the number preceding F or T indicates the level of response;

RT Treated Breast Cancer Patients with *ATM* Variant Displaying an Adverse Response

RT Response	<i>ATM</i> Variant
1F	1176C>G
1F	2119T>C
3F	2362A>C, 6088A>G
1F,1T	2442C>A
1F	2572T>C, 5557G>A
2F	334G>A
1F	378T>A
1F	378T>A, 5557G>A
1T	378T>A, 1176C>G, 4138C>T
1F	378T>A, 6176C>T
3F	4138C>T, 4400A>G
1F	4258C>T
1F	4578C>T
1F,1T	5071A>C
1F	5557G>A
1F	5557G>A
1T	5557G>A
2F	5557G>A
2F	5557G>A
3F	5557G>A
1F	5557G>A
2F,1T	5557G>A
1F	5557G>A, 5558A>T

1F,1T	5557G>A, 5558A>T, IVS62+8A>C
4F	735C>T, 5557G>A, 7397C>T
1F	IVS5-7C>T, 378T>A
1F	IVS62+8A>C

RT Treated Breast Cancer Patients with ATM Variant not Displaying an Adverse Response

RT Response	ATM Variant
N	1254A>G
N	2614C>T, 2685A>C, 4473C>T, IVS56+3INST
N	378T>A, 1176C>G
N	4138C>T
N	5557G>A
N	5557G>A
N	5557G>A
N	5557G>A, 5558A>T
N	5793T>C
N	IVS5-7C>T, 378T>A, 4578C>T

Additional work has been accomplished performing functional assays. Since we have only recently begun to EBV transform lymphocytes to lymphoblastoid cell lines from subjects shown to be positive for possession of an *ATM* alteration, most of the work done during this past year involved the use of cell lines derived from either cells not exhibiting an *ATM* variant (wild type), as well as cell lines derived from ataxia telangiectasia (AT) patients who possess two mutated copies of the *ATM* gene or from the parents of these AT patients who are obligate heterozygotes for a single mutated copy of the *ATM*.

Work was also performed to examine the functional status of the ATM protein in wild type and *ATM* mutant lymphoblastoid cell lines involving immunodetection of p53 which has been phosphorylated. Since it is known that ATM phosphorylates p53 at ser-15, and given that there are commercially available antibodies that specifically recognize p53 protein that has been phosphorylated at ser-15, it is possible to determine the kinase activity of ATM in various cell types indirectly by determining the amount of p53 in a cell that has been phosphorylated by ATM at ser-15.

Briefly, we sediment the cells and then resuspend the cells in lysis buffer (50mM Tris/HCl, 5mM Na₂EDTA, 150 mM NaCl, 0.5% NP40, 1mM DTT, 1mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM sodium orthovanadate, 1mM sodium fluoride) for 20 minutes on ice. The protein concentration is determined using a modified Bradford assay kit (RC-DC, BioRad). 1mg of cellular protein lysate is incubated with 1 µg of monoclonal anti-p53 (5 µl of a 200 µg/ml stock of 1C12 Mouse Monoclonal #2524, Cell Signaling technology) for 1 hour at 4°C with agitation. Protein-A conjugated to Sepharose beads (Sigma) is added to the mixture and incubated for 1 hr. at 4°C with agitation. The immunocomplex is precipitated by centrifugation, washed with 5 volumes of lysis buffer five times, and boiled in 20 µl Laemmli SDS-PAGE

loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) (BioRad). The beads are pelleted again using centrifugation.

The supernatant (containing the protein) is loaded onto a 7.5% precast gel (BioRad) and electrophoresed until the bromophenol blue dye front reaches the end of the gel. The proteins on the gel are transferred to a PVDF membrane (Immuno-Blot, BioRad) in Towbin buffer (25mM Tris/HCl, 192 mM glycine, 20% methanol, BioRad) for 2 hr at 4°C. The membrane is allowed to dry and washed in 25 ml phosphate buffered saline with 0.1% Tween-20 (PBST) twice for 5 min at room temperature (RT). The membrane is blocked with 25 ml PBST buffer with added protein blocker for 1 hr at RT and washed twice with 25 ml PBST for 5 min at RT. To the blocked membrane is added 10 ml of antibody dilution buffer (ADB) containing polyclonal rabbit Phospho-p53 (Ser15) antibody (Cell Signaling technology #9284, diluted 1:2000 [5 µl to 10 ml ADB]) and incubated for 1 hr at 4°C with rocking. The blot is washed twice in 25 ml PBST at RT and incubated for 1 hr at 4°C with rocking in secondary antibody (goat-anti-rabbit conjugated with horseradish peroxidase [GAR-HRP]) diluted to 1:5000 in ADB (2 µl antibody added to 10 ml ADB).

The blot is washed twice in 25 ml PBST at RT. The membrane is incubated in diluted BioRad Amplification Reagent (BAR) for 10 min at RT with rocking, washed four times in PBST with 20% DMSO 5 min at RT, and twice in PBST 5 min at RT. The membrane is incubated in diluted streptavidin-HRP for 30 min, followed by two washes in PBST 5 min RT. Using the Opti-4CN colorimetric detection from the BioRad kit, 15 ml of the colorimetric solution is prepared and the blot incubated in this solution for about 30 min or until the desired level of sensitivity is attained. The blot is washed in ddH₂O for 15 min. The colorimetrically labeled blot is scanned using a table scanner and each band in the scan quantitated using ImageJ (public domain software downloaded from <http://rsb.info.nih.gov/ij/>).

A total of 12 separate experiments were performed with wild type cells, while cells obtained from 6 individuals diagnosed with AT in addition to 9 *ATM* heterozygotes, and the level of phosphorylated p53 measured using densitometry. Cells were irradiated with either 0 or 4 Gy of x-rays and incubated either 0.5 or 2 hr. The densitometric results for each time point were divided by the value in each experiment for unirradiated cells to normalize these results. The mean values (with standard deviations) for wild type cells incubated either 0.5 or 2.0 hr were 3.9 ± 1.9 and 8.3 ± 4.4 . In contrast, the mean values for cells obtained from AT patients were at 0.5 and 2.0 hr, 1.2 ± 0.5 and 3.8 ± 2.2 . Hence, as expected, the AT cells displayed significantly less p53 phosphorylation compared with wild type cells following a 0.5 hr incubation. Although the value obtained after a 2 hr incubation was lower for the AT cells compared with wild type cells, there was not a statistically significant difference. In contrast, the mean ratios at 0.5 and 2 hr for obligate heterozygotes were 4.1 ± 1.9 and 7.1 ± 4.9 . Hence, we found similar values in heterozygotes compared with wild type cells. These experiments form a firm foundation for comparison of the *ATM* variant cell lines that are being generated in this project

Key Research Accomplishments

- Accrual and complete DHPLC screening of the *ATM* gene in 104 breast cancer patients who received standard radiotherapy, 46 exhibiting late subcutaneous responses and 58 who did not display these adverse reactions.
- Identification, through DNA sequencing, of the specific *ATM* genetic variants in 37 subjects
- Measurement of *ATM* functional activity through performance of western blots to measure p53 ser-15 phosphorylation with a series of wild type, AT and *ATM* heterozygote cell lines to establish a basis for comparison with the cell lines to be generated in this study.

Reportable Outcomes

None

Conclusions

Although it is still necessary to screen an additional 46 subjects, the preliminary results of this work are that 73% of the patients found to possess an *ATM* alteration (27/37), displayed an adverse reaction. In contrast, only 28% (19/67) of the patients who did not harbor an *ATM* variant allele, developed a late subcutaneous tissue response. This finding supports the hypothesis that alternations in the *ATM* gene render breast cancer patients susceptible to adverse effects of radiotherapy. During the remainder of this project, genetic screening of the full 150 patients will be accomplished, in addition to performance of the functional assays with lymphoblastoid cell lines generated from lymphocytes derived from subjects in this study to establish which of the *ATM* variants identified affect the activity of the protein encoded by this gene.

References

None

Appendices

None