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14. ABSTRACT 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 accomplishments during the past year were accrual and complete DHPLC screening of the *ATM* gene for additional subjects as well as the performance of functional studies with a series of patient derived cell lines to measure radiosensitivity, ATM protein levels and p53 phosphorylation by ATM.

15. SUBJECT TERMS
No abstract provided.

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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. Because of this delay, the U.S. Army Medical Research Acquisition Activity was notified on June 1,

2005 that we will exercise the option for a 12-month no-cost extension of this grant to enable completion of the work in this project. This will result in a revised termination date for this project of June 30, 2006. This was done per the Assistance Agreement, section 4.C. - "The recipient may make a one-time "no-cost" extension to the expiration date of the award for a period up to 12 months. The recipient shall notify the grants officer, in writing, at least 10 days prior to the expiration date of the award."

During the past year, the screening of 125 subjects was completed which began during the first year of the project. The first result of note, as indicated in the Table 1, was the substantial difference, and virtually no overlap, between the *ATM* variants detected in African-Americans women compared with the putative genetic alterations that play an important role for breast cancer patients who are not African-American.

Table 1. COMPARISON OF AFRICAN-AMERICAN AND NON-AFRICAN-AMERICAN PATIENTS

ATM Variant	Non-African-American Number (%)	African-American Number (%)
334G>A	0	1 (3)
378T>A	0	4 (10)
735C>T	1 (1)	0
1176C>G	0	2 (5)
2119T>C	1 (1)	0
2362A>C	1 (1)	0
2442C-A	1 (1)	0
2572T>C	1 (1)	0
4138C>T	0	2 (5)
4258C>T	1 (1)	0
4400A>G	0	1 (3)
4578C-T	0	1 (3)
5071A-C	1 (1)	0
5557G>A	14 (16)	3 (8)
5558A>T	2 (2)	0
6088A>G	1 (1)	0
6176C-T	1 (1)	0
7397C>T	1 (1)	0
IVS62+8A>C	3 (3)	0
IVS5-7C>T	0	1 (3)
IVS16-1G>A	1 (1)	0

Separating the patients between difference grades of fibrosis, the following results were obtained for the 125 subjects screened;

58 patients –grade 0 fibrosis
67 patients – grade 1-4 fibrosis
77% (34/44) of patients found to possess an *ATM* genetic alteration displayed at least grade 1 fibrosis.
41% (33/81) of patients who did not harbor an *ATM* variant displayed at least grade 1 fibrosis.
$p<0.001$ (chi-square)

100 patients –grade 0-1 fibrosis
25 patients – grade 2-4 fibrosis
32% (14/44) of patients found to possess an *ATM* genetic alteration displayed grade 2-4 fibrosis.
14% (11/81) of patients who did not harbor an *ATM* variant displayed grade 2-4 fibrosis.
$p = 0.02$ (chi-square)

115 patients –grade 0-2 fibrosis
10 patients – grade 3-4 fibrosis
14% (6/44) of patients found to possess an *ATM* genetic alteration displayed grade 3-4 fibrosis.
5% (4/81) of patients who did not harbor an *ATM* variant displayed grade 3-4 fibrosis.
$p = 0.172$ (chi-square)

In addition, a substantial amount of the work in the past year focused upon performance of functional assays to determine the effect of *ATM* sequence variants on the function of the ATM protein. This research was accomplished using lymphoblastoid cell lines derived from EBV transformed lymphocytes obtained from subjects, all of whom were never diagnosed with breast cancer. This work was performed using five cell lines from subjects who were not found to possess an *ATM* variant and eight subjects who were identified with *ATM* variants. Of these eight patients, four developed adverse responses from the radiotherapy while the remainder did not. Cellular radiosensitivity was measured in each cell line. However, the experiments are not complete to measure ATM and p53 phosphorylation in all cell lines and an NC (not complete) is indicated in these cases.

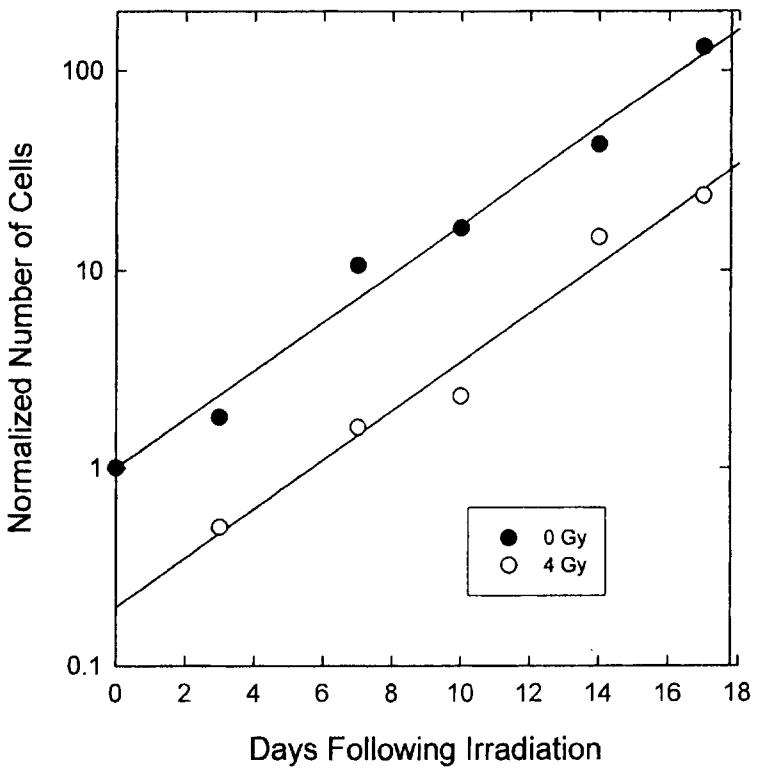
For experiments in which p53 phosphorylation was measured, cells were irradiated with either 0 or 4 Gy of x-rays and incubated either 0.5 or 2 hr. The densitometric results for each time point were divided by the value in each experiment for unirradiated cells to normalize these results. Each irradiation was performed a total of three times. The mean values (with standard deviations) for wild type cells incubated either 0.5 or 2.0 hr were 1.8+2.0 or 3.1+2.5, respectively. The results for the cell lines possessing variants are shown in Table 2. In addition, ATM protein levels were measured in each cell line in three separate experiments and divided by the average value obtained for the five wild type *ATM* cell lines.

Table 2. Functional Assays of Lymphoblastoid Cells Derived from Subjects Possessing *ATM* Variants

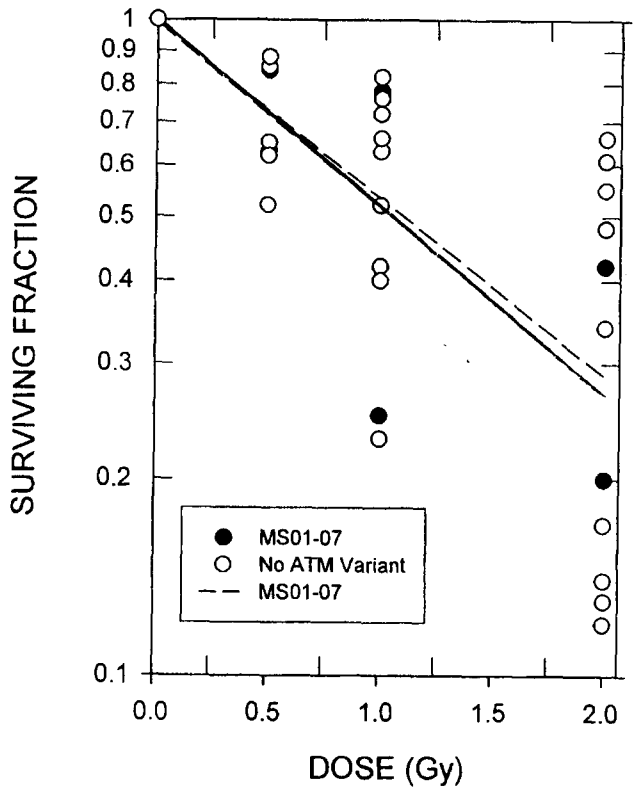
Cell Line	Radio-sensitive Yes/No	Nucleo-tide	Change	Amino Acid Position	Sub-stitution	ATM level	p53p 0.5 h	p53p 2 hr
MS01-07	No	4917	G>A	1639	P>P	NC	NC	NC
		5557	G>A	1853	D>N			
		5558	A>T	1853	D>V			
MS01-30	Yes	IVS5-7	C>T	n/a	n/a	NC	NC	NC
		378	T>A	126	D>E			
		4578	C>T	1526	P>P			
MS01-33	No	4138	C>T	1380	H>Y	1.11+0.55	5.06+4.37	5.37+2.99
MS01-37	Yes	378	T>A	126	D>E	1.59+0.16	2.06+1.21	1.99+1.20
		1176	C>G	392	G>G			
		4138	C>T	1380	H>Y			
MS01-39	Yes	5557	G>A	1853	D>N	1.29+0.89	1.35+0.98	0.68+0.38
		5558	A>T	1853	D>V			
MS01-45	No	5557	G>A	1853	D>N	0.39+0.04	NC	NC
MS01-51	Yes	IVS5-7	C>T	n/a	n/a	0.73+0.46	NC	NC
		378	T>A	126	D>E			
MS02-06	No	1254	A>G	418	Q>Q	1.06+0.18	0.87+0.24	0.90+0.38

The only instance of a statistically significant result was obtained was for MS01-45 which exhibited a significantly lower level of ATM protein. In addition, MS01-39 and MS02-06 displayed less p53 phosphorylation at 2 hours following irradiation compared with wild type cells.

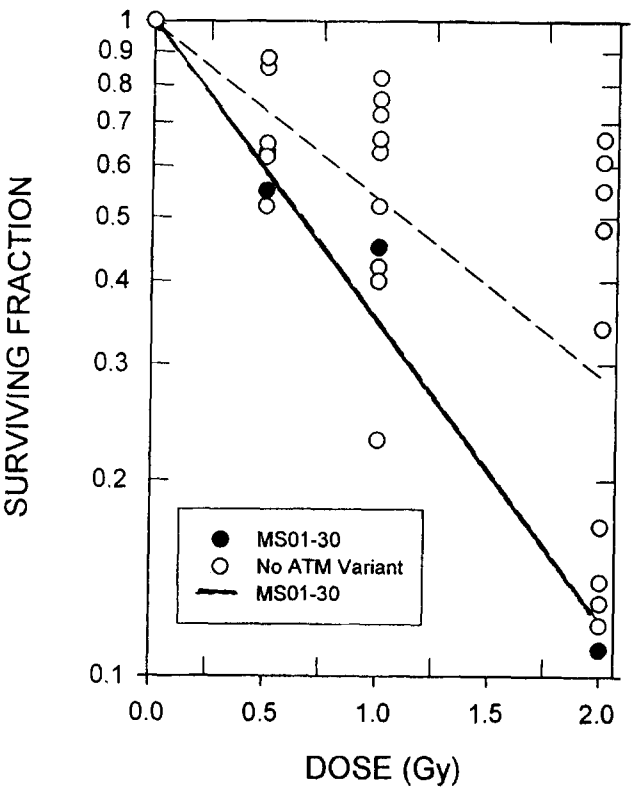
The radiosensitivity of each cell line was also determined from the growth response of cells irradiated with either 0, 0.5, 1.0 or 2.0 Gy of X-rays by extrapolating the growth curve to the intercept at zero time. An example is shown for a 4 Gy dose to wild type cells and the dose response based upon each growth curve is then provided. One cell line, MS01-30, appeared to be moderately radiosensitive. The patient from whom these cells were derived exhibited an adverse radiotherapy response.


Normalized Number of Cells
100
10
1
0.1
0
2
4
6
8
10
12
14
16
18
Days Following Irradiation
0 Gy
4 Gy

MS01-07



MS01-30



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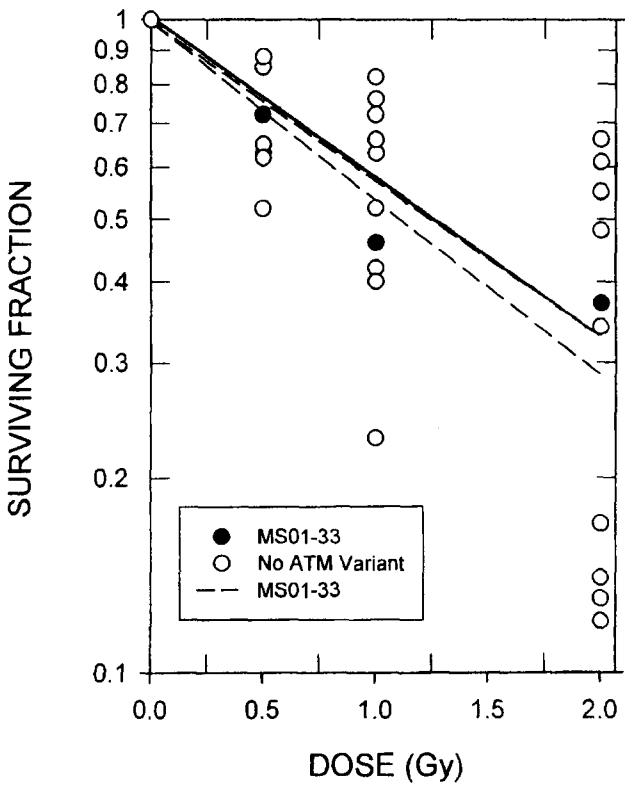

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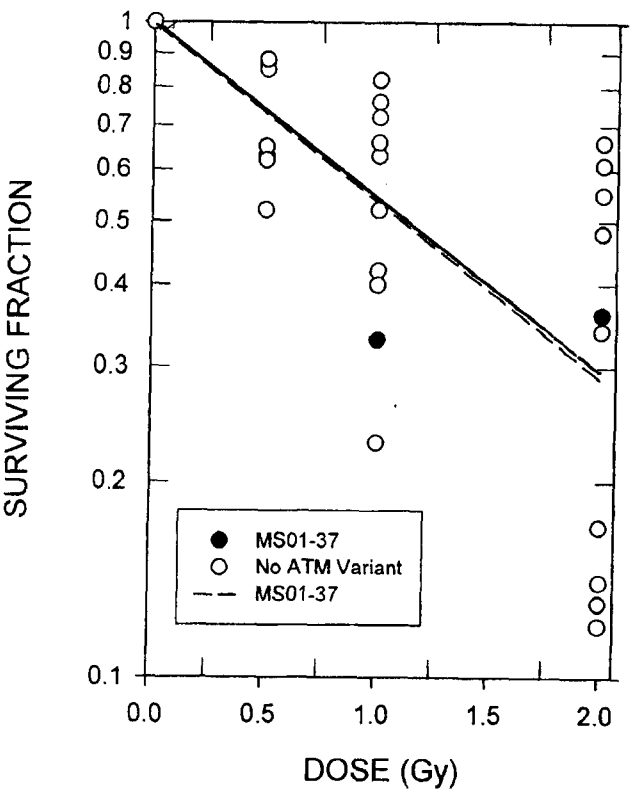

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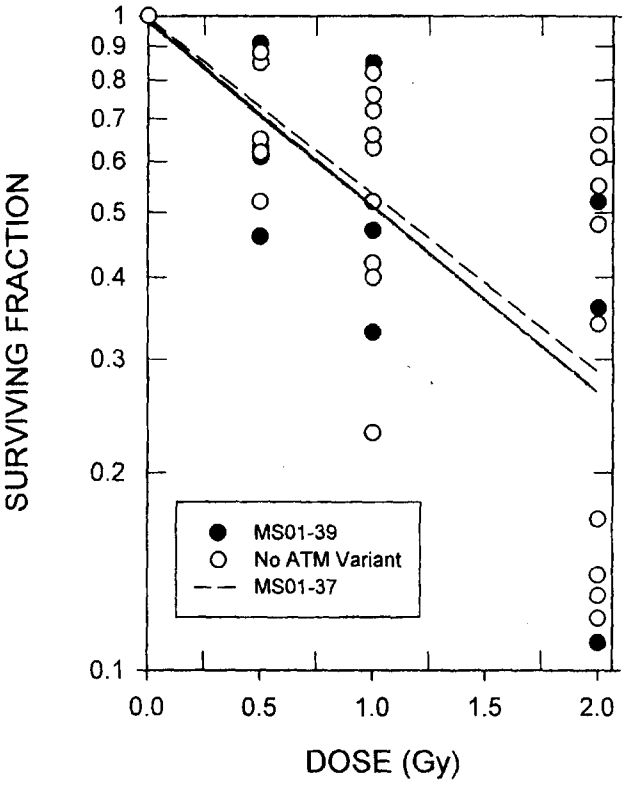

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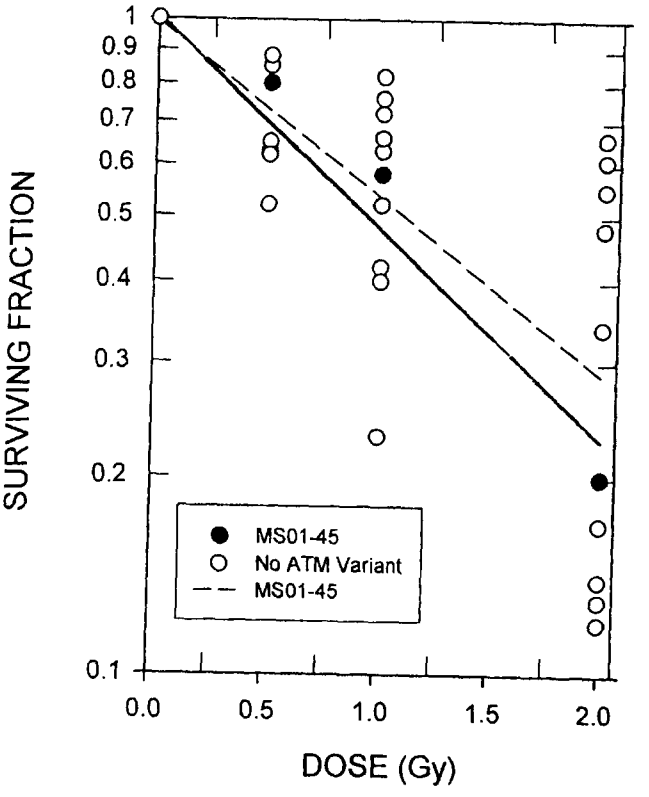
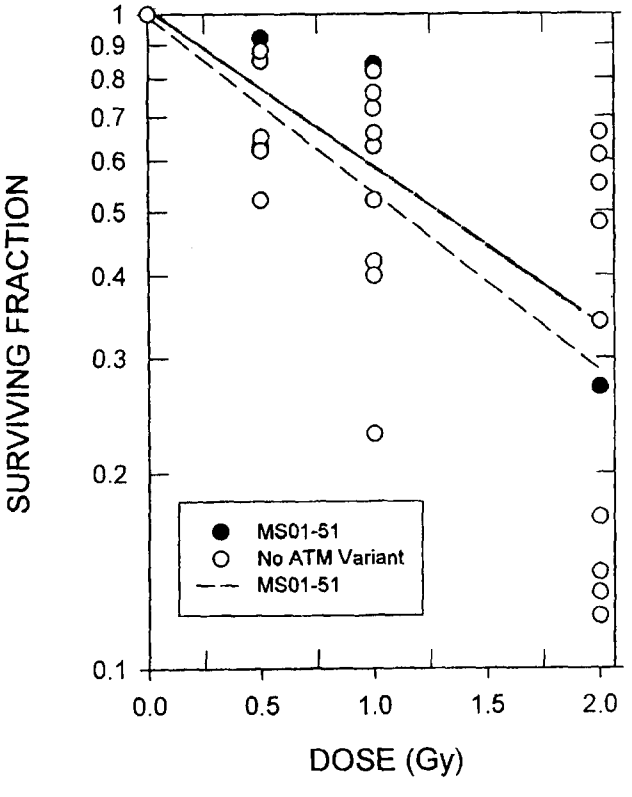
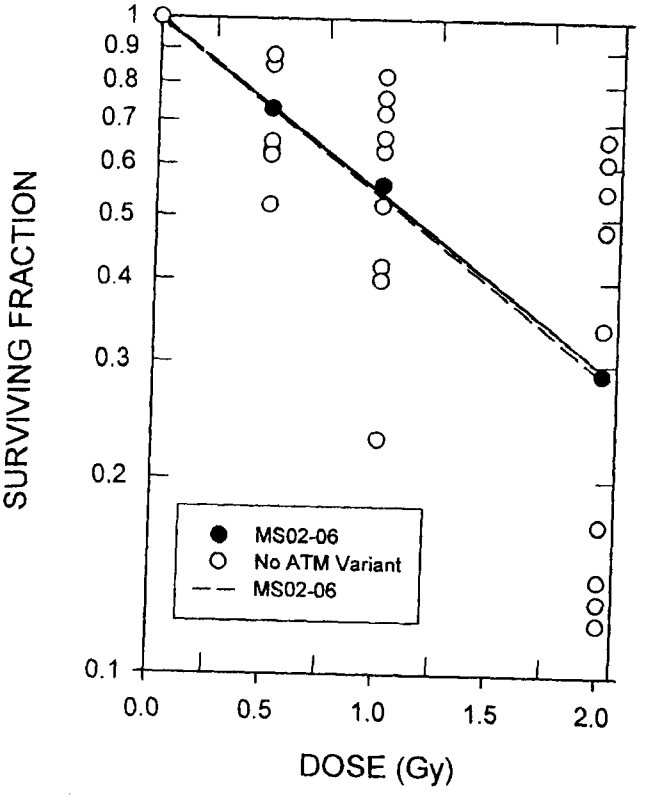


MS01-51



MS02-06



Key Research Accomplishments

- Accrual and complete DHPLC screening of the *ATM* gene in a total of 125 breast cancer patients who received standard radiotherapy.

- Measurement of ATM functional activity through performance of western blots to measure ATM protein levels and p53 ser-15 phosphorylation with a series of wild type and *ATM* variant cell lines.

- Determination of cellular radiosensitivity in five wild type and eight *ATM* variant cell lines derived from breast cancer patients treated with radiotherapy.

Reportable Outcomes

None

Conclusions

- From the initial results obtained during the past year it appears that possession of an *ATM* variant or the manifestation of clinical radiosensitivity was not routinely associated with an abrogation of ATM functional activity based upon either ATM protein levels, p53 phosphorylation or cellular radiosensitivity. If upon testing of additional cell lines during the final year of this project this result is maintained, this finding may suggest that although relatively small changes in the ATM protein could be sufficient to cause clinical radiosensitivity, they are not adequate to produce a measurable impact upon ATM function as measured in cellular assays

- The sequence variants possessed by African-American patients are distinctly different from those detected in non-African-Americans. Hence, it is critical to screen African-American patients to identify the genetic predictors of radiosensitivity in this population.

- Possession of *ATM* sequence variants is associated with the development of late skin fibrosis in breast cancer patients treated with a standard radiotherapy protocol. However, having an *ATM* alteration by itself may not be adequate to predict clinical radiosensitivity. Therefore, *ATM* could be just one of a battery of radiation response genes, whose inheritance in a variant form, is predictive for the development of adverse radiation effects.

- For the final year of this project, additional patients who exhibited grade 3 fibrosis will be screened. Functional assays will also be performed with additional patients who either did or did not exhibit clinical radiosensitivity.

References

None

Appendices

None