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

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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.

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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

STATEMENT OF WORK

Task 1 (Months 1-2): Identify all breast cancer radiotherapy patients treated during the five year period prior to initiation of the study at Mount Sinai and NYU who are eligible for participation using the criteria outlined in the proposal.

Task 2 (Months 1-36): Identify all new breast cancer patients at Mount Sinai and NYU eligible for the study.

Task 3 (Months 1-36): Obtain blood samples from breast cancer patients.

Task 4 (Months 1-36): Isolate DNA from blood lymphocytes of patients.

Task 5 (Months 1-36): Perform PCRs with these samples to amplify each exon of the *ATM* gene.

Task 6 (Months 1-36): Use DHPLC to identify PCR products that may possess mutations based upon the appearance of aberrant chromatograms.

Task 7 (Months 3-36): Sequence all PCR products that display aberrant DHPLC chromatogram profiles.

Task 8 (Months 6-36): Determine radiosensitivity of lymphoblastoid cell lines.

Task 9 (Months 6-36): Determine *ATM* protein levels in lymphoblastoid cell lines.

Task 10 (Months 6-36): Perform statistical analyses of the data generated in this project.

As described below, all ten tasks outlined in the approved Statement of Work have been accomplished.

A total of 162 women treated with radiotherapy were screened for variants in the *ATM* gene. The number of subjects with either grade 0 (none), 1, 2, or 3 late radiations were 46, 46, 46 and 24, respectively. It was found that 63 patients had at least one variant in one of the 62 coding exons of the gene or within 10 bases of the intron/exon boundary in a potential splice site region. The variants identified are provided in Table 1. The percentages of subjects with either any variant, any missense variant (change in an amino acid) or with the 5557 G>A (asp>asn) variant are listed in Table 2.

Table 1. *ATM* Variants Identified in Breast Cancer Patients

RTOG Grade Late Reaction	*ATM* Variant
0	378 T>A
0	5557 G>A
0	5558 A>T
0	1176C>G
0	2119T>C
0	2572T>C
0	378T>A; 1176C>G

0	378T>A; 6176C>T
0	4138C>T
0	IVS62+8 A>C
1	2362A>C
1	2572T>C; 2685A>G
1	378 T>A
1	378 T>A
1	1176 C>G
1	5557 G>A
1	5557 G>A
1	5557 G>A
1	5793
1	1810C>T
1	20+9
1	2614C>T, 2685A>G
1	3161C>G
1	378 T>A, 6176C>T
1	378T>A; 4578C>T
1	4578C>T
1	5557G>A
1	5557G>A
1	IVS5-7C>T; 378T>A
1	IVS5-7C>T; 378T>A; 4578C>T
1	IVS62+8 A>C
2	378 T>A
2	5557 G>A
2	5557 G>A
2	5557 G>A
2	5558 A>T
2	1636, 2614
2	2442C>A
2	378 T>A, 1176 C>G
2	378T>A; 1176 C>G; 4138C>T
2	378T>A; 5557G>A; IVS38-8T>C
2	5557 G>A
2	5557G>A
2	5557G>A
2	5557G>A
2	5557G>A
2	5558A>T
2	5793T>C

2	5793T>C, 9200C>G
2	IVS62+8 A>C
3	5557 G>A
3	5557 G>A
3	5557 G>A
3	5557 G>A
3	5557 G>A
3	5557 G>A
3	5557 G>A
3	IVS16-1A>T
3	2362A>C; 6088A>G
3	378 T>A, 2614C>T, 4278A>T
3	4138C>T; 4400A>G
3	IVS62+8 A>C
3	735C>T; 5557G>A; 7397C>T

Table 2. Percentage of Subjects Possessing Different *ATM* Variants

RTOG Grade of Late Radiation Reaction	Any Variant	Missense	5557 G>A
0	22 (10/46)	17 (8/46)	2 (1/46)
1,2 or 3	46 (53/116)	38 (44/116)	19 (22/116)
	p = 0.01	**P = 0.02**	**p = 0.01**
0 or 1	34 (31/92)	27 (25/92)	7 (6/92)
2 or 3	46 (32/70)	39 (27/70)	24 (17/70)
	p = 0.16	P = 0.17	**p = 0.01**
0, 1 or 2	36 (50/138)	30 (41/138)	11 (15/138)
3	54 (13/24)	46 (11/24)	33 (8/24)
	p = 0.15	p = 0.19	**p = 0.01**

An additional goal of this study was to perform 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 five subjects who did not exhibit late responses and did not possess *ATM* genetic alterations. 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 3.2+1.7 or 6.9+3.1, respectively. The results for the cell lines possessing variants are shown in Tables 3 and 4. 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 3. Functional Assays of Lymphoblastoid Cells Derived from Subjects Possessing *ATM* Variants

Cell Line	Radio-sensi-tive Yes/No	Nucleotide Change	Amino Acid Substitu-tion	ATM level	Phospho-p53 0.5 hr	Phospho-p53 2 hr	Normalized α-value
MS01-33	no	4138 C>T	1380 H>Y	1.1+0.6	5.1+4.4	5.4+3.0	1.2±0.2
MS01-30	No	IVS5-7 C>T 378 T>A 4578 C>T	N/A 126 D>E 1526 P>P	0.5+0.3	2.0+1.7	4.5+4.0	1.0±0.4
MS01-39	Yes	5557 G>A 5558 A>T	1853 D>N 1853 D>V	1.3±0.9	1.4±1.0	2.7±0.4	1.1±0.5
MS01-45	No	5557 G>A	1853 D>N	0.4±.04	1.4±.0.6	1.6±1.0	1.3±0.1
MS01-51	Yes	IVS5-7C>T 378 T>A	N/A 126 D>E	0.7±0.5	2.5±2.6	9.5±4.5	0.5±.2
MS01-37	Yes	378 T>A 1176 C>G 4138 C>T	126 D>E 392 G>G 1380 H>Y	1.6±0.2	2.1±1.2	2.0±1.2	1.4±0.4
MS01-67	Yes	4578 C>T	1526 P>P	0.5±.09	4.6±0.8	10.7±3.7	1.2±0.1
MS01-65	No	5557 G>A	1853 D>N	1.1±0.5	2.7±1.2	10.1+4.0	1.2±0.3
Ms01-53	No	378 T>A 1176 C>G	126 D>E 392 G>G	1.0±.0.1	2.5±0.8	6.5±2.1	0.8±0.3
MS01-07	No	4917 G>A 5557 G>A 5558 A>T	1639 P>P 1853 D>N 1853 D>V	0.8±0.5	0.9±0.7	2.0±1.7	0.5±0.3
MS01-37	Yes	378 T>A 1176 C>G 4138 C>T	126 D>E 392 G>G 1380 H>Y	1.6+0.2	2.1+1.2	2.0+1.2	1.4±0.2
MS02-13	YES	378 T>A 6176 C>T	126 T>A 2059 T>I	1.0+0.2	2.0+1.3	5.1+3.7	1.2±0.2
MS02-73	YES	IVS62+8 A>C	N/A	0.8+0.3	4.5+4.0	3.8+1.3	0.8±0.3
MS01-87	YES	5071 A>C	1691 S>R	1.0+0.5	2.3+1.3	5.0+2.0	0.8±0.1
MS01-03	NO	2614 C>T 2685 A>C	872 P>S 895 L>L	0.7+0.2	1.1+0.8	1.5+0.4	1.2±0.6
MS01-35	NO	1229 T>C	410 V>A	0.9+0.6	2.9+0.1	5.7+3.7	1.1±0.2
MS02-34	YES	915 G>C	25 R>P	2.0+1.5	1.5+0.6	1.8+1.1	1.3±0.5
MS02-05	YES	NONE	N/A	0.7+0.4	3.1+3.7	4.6+3.7	1.1±0.3
MS03-13	YES	NONE	N/A	0.7+0.1	3.6+1.2	7.9+3.8	0.9±0.5
MS03-48	YES	NONE	N/A	0.5+0.3	2.4+1.4	6.8+2.1	1.3±0.1

Table 4. Functional Assays of *ATM* Homozygoyte and *ATM* Heterozygote Lymphoblastoid Cell Lines

Cell Line	Homozygote or Heterozygote	ATM Level	Phospho p53 0.5 hr	Phospho p53 2 hr	Normalized α-value for radiation survival curve
8388	heterozygote	0.7±0.6	1.6±0.2	6.7±2.3	1.5±0.2
8925	heterozygote	0.7±0.8	1.9±0.4	5.1±0.1	1.4±0.2
8928	heterozygote	0.8±0.3	3.8±3.5	3.5±2.7	1.7±0.2
9579	heterozygote	0.5±0.3	2.3±1.3	2.6±0.3	1.1±0.3
2781	heterozygote	0.7±0.5	3.2±0.6	4.5±4.1	1.6±0.2
9588	heterozygote	0.5±0.5	6.1±4.0	6.9±2.8	1.2±0.3
8436	homozygote	0.04±0.06	2.9±1.2	2.8±0.4	1.8±0.3
9581	homozygote	0.08±0.02	1.5±1.7	4.0±1.6	2.0±0.3
9582	homozygote	0.05±0.02	2.0±4.4	2.1±0.4	2.2±0.3
2782	homozygote	0.08±0.05	2.1±3.1	3.1±1.3	2.1±0.3
1525	homozygote	0.05±0.02	2.6±1.1	3.1±1.2	1.8±0.2
11254	homozygote	0.09±0.06	1.8±0.1	2.5±0.9	2.3±0.3
9586	homozygote	0.24±0.22	1.7±1.0	4.3±1.9	1.8±0.4
13328	homozygote	0.13±0.09	0.6±0.5	2.1±1.3	2.1±0.3

The results for cells derived from AT patients clearly show a significantly lower level of ATM protein in these cells compared with wild type cells. In addition, the levels of p53 phosphorylation are consistently lower than those detected in wild type cells. The ATM levels are also consistently lower in the heterozygotes and the levels of phosphorylated p53 are also generally lower, although none of these values differed significantly from those obtained for wild type cells due to the variation in the results between experiments. As for the results obtained from the cell lines derived from the breast cancer patients analyzed in this study, there was a variation among the cell lines, but no clear pattern emerged that correlated either with the possession of an *ATM* variant (including the 5557 SNP) or whether the patient developed a late radiotherapy reaction. Hence, the results of this work suggest that neither measurement of ATM levels nor p53 phosphorylation can serve as a predictor as to whether the patient will develop late morbidity following radiotherapy for breast cancer.

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. The radiosensitivity of each cell line was estimated from the α-value ($S = e^{-\alpha D}$) normalized to the value obtained for wild type cells listed in Tables 3 and 4. The α-values for the cell lines derived from AT patients were all significantly greater than one. In addition, the α-values for the AT heterozygotes were consistently greater than one, although generally not significantly greater. In contrast, the α-values for the cell lines obtained from the breast cancer patients were variable and none was significantly greater than one.

This is not altogether surprising, since clearly none of the patients screened in this study manifested a radiation sensitivity approaching that displayed by a person suffering from AT. Any radiosensitive patients likely have only a mild radiosensitivity. However, even a slight radiosensitivty is probably sufficient to result the development of a late response since the dose used in treating breast cancer represents the tolerance dose. Hence, even just a 5-10% increase in radiosensitivity will make the difference as to whether a person will or will not develop a radiation complication. It is likely that the subtle changes in ATM protein function that result from the variants identified in this study are sufficient to cause these types of very mild changes in protein function. In contrast, it is impossible with the techniques currently available to detect such small changes in ATM function using the westerns performed in this work to measure ATM levels and p53 phosporylation. Hence, the results of this study indicate that the identification of genetic variants will serve as a far more important basis of a predictive assay for radiosensitivity compared with functional assays.

Key Research Accomplishments

- The percentage of women who are carriers of an *ATM* variant that develop late complications from the radiotherapy treatment of breast cancer is significantly greater than the percentage of women who are not carriers of a variant in this gene. Thus, the possession of an ATM variant is predictive for the development of late subcutaneous adverse effects in breast cancer patients treated with radiotherapy.
- The 5557 G>A SNP appears to be particularly correlated with the development of late radiation reactions
- No significant differences were detected in any of the functional end-points measured between patients who developed late complications compared with those that did not exhibit this type of radiation-induced morbidity. In addition, no significant differences in the results for the functional assays were identified for any *ATM* variant compared with wild type cells.

Reportable Outcomes

Andreassen, Christian N, Jens Overgaard, Jan Alsner, Marie Overgaard, Carsten Herskind, Jamie A. Cesaretti, David P. Atencio, Sheryl Green, Silvia C. Formenti, Richard G. Stock and **Barry S. Rosenstein.** *ATM* Sequence variants and risk of radiation-induced subcutaneous fibrosis after post-mastectomy radiotherapy. *International Journal of Radiation Oncology Biology Physics* 64:776-783, 2006.

Ho, Alice Y, David P Atencio, Sheila Peters, Richard G Stock, Silvia C Formenti, Jamie A Cesaretti, Sheryl Green, Bruce Haffty, Karen Drumea, Larisa Leitzin, Abraham Kuten, David Azria, Mahmut Ozsahin, Jens Overgaard, Christian N. Andreassen, Cynthia S. Trop, Janelle Park and **Barry S. Rosenstein**. Genetic Predictors of Adverse Radiotherapy Effects: The Gene-PARE Project. *International Journal of Radiation Oncology Biology Physics* 65:646-655.

Conclusions

The major result of this study is to provide definitive evidence that the possession of a genetic variant in the *ATM* gene is predictive for the development of late developing morbidity from radiotherapy. As for the specific results which resulted in statistically significant correlations, it is interesting to note that the possession of any *ATM* variant was correlated with the development of either a grade 1, 2 or 3 response, whereas this correlation was not significant if either grade 2 and 3 or just grade 3 reacting subjects were grouped. A similar result was obtained when subjects were grouped based upon the possession of a missense variant which results in a substitution of an amino acid as it might be thought that this type of genetic alteration would result in a greater impact upon protein function. However, a problem with the possession of either any variant or a missense variant as a basis of a predictive assay is that there would be a substantial number of patients incorrectly classified as being radiosensitive (false positives) as 22% and 17% of the subjects with either any variant or a missense variant did not develop a late radiation response. In contrast, the false positive rate was much lower for the patients with a 5557 G>A variant as only 2% of patients with such an alteration did not develop a late response. However, only 19% of the patients with this type of variant developed any grade of late response. The level of sensitivity of the test could be improved by either using this variant to predict either grade 2 or 3 or just grade 3 responses, but with a loss of specificity. Although the results of this study are of importance as they demonstrate that possession of an *ATM* genetic variant is associated with the development of late radiation morbidity associated with radiotherapy, it appears that screening of the *ATM* gene alone will not provide the level of sensitivity and specificity necessary to serve as an adequate basis for a predictive assay. Clearly, it will be necessary to identify additional genes and variants in these genes in future studies that along with *ATM* will serve as a basis for a predictive assay for the development of late radiation responses.

References

None

Appendices

Andreassen, Christian N, Jens Overgaard, Jan Alsner, Marie Overgaard, Carsten Herskind, Jamie A. Cesaretti, David P. Atencio, Sheryl Green, Silvia C. Formenti, Richard G. Stock and **Barry S. Rosenstein.** ATM Sequence variants and risk of radiation-induced subcutaneous fibrosis after post-mastectomy radiotherapy. *International Journal of Radiation Oncology Biology Physics* 64:776-783, 2006.

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ATM SEQUENCE VARIANTS AND RISK OF RADIATION-INDUCED SUBCUTANEOUS FIBROSIS AFTER POST-MASTECTOMY RADIOTHERAPY

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Running Title: *ATM* sequence variants and risk of radiation-induced fibrosis

Purpose: To examine the hypothesis that women who are carriers of genetic alterations in the *ATM* gene are more likely to develop subcutaneous fibrosis following radiotherapy for treatment of breast cancer compared with patients who do not possess DNA sequence variations in this gene.

Materials and Methods: DNA samples isolated from fibroblast cell lines established from 41 women treated with post-mastectomy radiotherapy for breast cancer were screened for genetic variants in *ATM* using denaturing high performance liquid chromatography (DHPLC). A minimum follow-up of 2 years enabled analysis of late effects to generate dose response curves and to estimate the dose that resulted in a 50% incidence of grade 3 fibrosis (ED_{50}).

Results: A total of 26 genetic alterations in the expressed portions of the *ATM* gene, or within 10 bases of each exon in regions encompassing putative splice sites, were detected in 22 patients. The ED_{50} (95% confidence of interval) of 60.2 (55.7-65.1) Gy calculated for patients without a sequence variation did not differ significantly from the ED_{50} of 58.4 (54.0-63.1) Gy for the group of patients with any *ATM* sequence abnormality. The ED_{50} of 53.7 (50.2-57.5) Gy for those patients who were either homozygous or heterozygous for the G→A polymorphism at nucleotide 5557, which results in substitution of asparagine for aspartic acid at position 1853 of the ATM protein, was substantially lower than the ED_{50} of 60.8 (57.0-64.8) Gy for patients not carriers of this sequence alteration. This resulted in an enhancement ratio (ratio of the ED_{50} values) of 1.13 (1.05-1.22,), which was significantly greater than unity.

Conclusion: The results of this study suggest an association between the *ATM* codon 1853 Asn/Asp and Asn/Asn genotypes with the development of grade 3 fibrosis in breast cancer patients treated with radiotherapy.

Key Words: *ATM*, Breast cancer, DHPLC, Fibrosis, Radiation sensitivity

INTRODUCTION

Radiation-induced fibrosis (1) constitutes an important potential complication after radiotherapy (2-3). The development of late normal-tissue reactions in breast cancer patients receiving radiotherapy shows considerable variation between individual patients. Although dosimetric variation or underlying medical conditions may be partly responsible for the morbidity, this explanation does not account for all differences between patients. Often, the adverse response is simply ascribed to unknown individual variations. However, evidence in support of genetic factors being responsible for inter-patient variation in radiosensitivity is emerging, such as an examination that was performed of radiation-induced telangiectasia in breast cancer patients (4). This study described a relatively large individual variation in the progression rate to development of telangiectasia for the same radiation treatment. It was concluded that 80-90% of the variation was due to deterministic effects related to the existence of possible genetic differences between individuals, whereas only 10-20% of the variation could be explained through stochastic events arising from the random nature of radiation-induced cell killing and random variations in dosimetry and dose delivery.

Substantial work has been performed in recent years in an effort to identify radiosensitivity candidate genes as well as the specific single nucleotide polymorphisms (SNPs) and rare genetic variants associated with the development of adverse responses to radiotherapy (5-6). The first gene to have received significant attention was the mutated in ataxia telangiectasia (AT) gene, *ATM,* as it was reported more than thirty years ago that patients suffering from the disease AT exhibit unusually severe and devastating responses to ionizing radiotherapy (7-8). The ATM protein functions primarily as a protein kinase involved in cellular stress responses, cell cycle checkpoint control and DNA repair (9). Evidence in support for the role of *ATM* genetic variants conferring radiosensitivity to breast cancer patients comes from a study (10) in which 46 breast cancer patients were screened for *ATM* sequence variations. It was reported that 100% (3/3) of the patients that developed a grade 3/4 subcutaneous reaction, manifested as either fibrosis or soft tissue necrosis, had *ATM* missense mutations. A second study reported a significant association specifically between homozygote carriers of the G→A transition at *ATM* nucleotide 5557 and adverse radiotherapy responses (11). In addition, evidence has been obtained demonstrating an association between *ATM* sequence variants with clinical radiosensitivity in prostate cancer patients (12-13).

The mutation screening technique used in this study, DHPLC (14-17), is a robust technique that can be used to screen any gene in a large population for SNPs, as well as small deletions and insertions. The advantage of DHPLC is that it enables the rapid, sensitive and accurate identification of genetic variants in an automated fashion. Of greatest importance is the evidence that DHPLC possesses a sensitivity and specificity for DNA sequence variant detection in *ATM* approaching 100% (18).

During the period 1978-1980, post-mastectomy breast cancer patients were treated in Aarhus, Denmark with a hypofractionated radiotherapy protocol. Due to a high incidence of late normal tissue complications, the fraction size was reduced to 2 Gy in 1980 (19). As a result, the majority of patients included in the present study received large doses per fraction. Skin biopsies were obtained from the patients and fibroblasts have been cultured (20) thereby providing a source of DNA for genetic analysis. Compared with most patients treated in recent decades who have been given standard radiotherapy protocols using 1.8-2.0 Gy fraction sizes, resulting in modest normal tissue biologic doses and a relatively low incidence of late subcutaneous tissue toxicities, this Danish patient cohort represents a unique population because of the relatively large biologic doses received and the availability of skin biopsies. Furthermore, all patients in the study cohort were scored for subcutaneous fibrosis in three independent treatment fields. Differences in the dose distribution between these fields as well as the diversity in fraction size used to treat the patients resulted in substantial intra- as well as inter-patient variation in biologically equivalent dose of 2 Gy per fraction, thereby permitting a dose response analysis of these data. The high incidence of patients with late effects provides an ideal population to identify genetic factors

associated with radiosensitivity since the doses used reached a level at which radiosensitive patients were likely to manifest a late radiation response. The relatively high biologic doses given to many patients in this cohort make this a relevant population to study in regard to treatment of tumors that require high doses to achieve control and therefore routinely result in normal tissue radiation doses in the 60-70 Gy range. In addition, the study cohort may be of particular interest considering the ongoing discussion about the ideal treatment technique (21) and fractionation regimen in post-operative radiotherapy for breast cancer (22-23).

MATERIALS AND METHODS

Treatment characteristics, dose and scoring of normal tissue reactions

Breast cancer patients were treated with post-mastectomy radiotherapy in the Department of Oncology, Aarhus, Denmark from 1978-1982 using two fractionation protocols as previously described (19, 24). The 41 patients screened in this study represent a portion of the cohort of 319 breast cancer patients given post-mastectomy radiotherapy during this period (25) and constitute the subjects for whom cultured fibroblasts were available (20). All patients were uniformly treated with a three-field technique comprising an anterior photon field, bolus area of the photon field and an anterior electron field (Figure 1). Thirty-four patients received 12 fractions to a minimum target dose of 36.6 Gy specified at the level of the mid-axilla or to an irradiated dose of 51.4 Gy irrespective of AP diameter. The other 7 patients were given a minimum target dose of 40.9 Gy in 22 fractions also specified at the mid-axilla. Every patient was evaluated for subcutaneous fibrosis in each individual treatment field at a single follow-up 2.3 to 5.4 years (median 4.0 years) after completion of radiotherapy. Fibrosis was graded using a four-point scale identical to that later used in the LENT-SOMA scoring system (26). Because of the large fraction sizes used for treatment of the majority of the patients, the biological doses were often relatively high (Table 1). Therefore, grade 3 fibrosis was detected in 37% of the individual treatment fields examined, with 56% of the patients exhibiting at least one field with this late effect.

ATM genetic screening

DNA samples were isolated from skin fibroblast cells using the Puregene^{TM} DNA Isolation Kit according to the manufactures protocols (Gentra Systems, Minneapolis, MN) PCR was used to amplify each of the 62 exons, and short intronic regions flanking each exon, that comprise the coding region of the *ATM* gene using primers previously described (18). DHPLC analysis was performed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) using buffer gradient and temperature conditions calculated using WAVEmaker software (version 3.3, Transgenomic) designed for this purpose. An example of a wild type and mutant chromatogram and resultant base pattern alteration is provided in Figure 2. Exons with an aberrant DHPLC chromatogram underwent DNA forward and reverse sequencing using an ABI PRISM 377 DNA Sequencer (Foster City, CA).

Statistics and dose response assessments

Based on exact dosimetric recordings, the physical dose absorbed at a dosimetric reference point of 4.1 mm was calculated in each field and converted into the biologically equivalent dose for 2 Gy per fraction using the linear quadratic model (27) with an α/β ratio of 1.9 Gy for late subcutaneous fibrosis. This parameter has previously been estimated from the same dataset as used in this study (28).

Dose response curves for patients with different *ATM* genotypes were fitted by logistic regression using the *fit model* procedure of the JMP statistical software package (SAS Institute Inc.). As part of this analysis, the Effect Likelihood Ratio was used to test whether the established dose response curves differed significantly from each other. In addition, the dose that resulted in a 50% incidence of grade 3 fibrosis (ED_{50}) was estimated by logit analysis and differences in

radiosensitivity were quantified in terms of enhancement ratios (ratios of the ED_{50} values). Ninety-five percent confidence intervals for these parameters were provided by the model (29).

The analysis was carried out for patients with any *ATM* alteration versus those without *ATM* alterations, for patients with two alterations each versus those with less than two alterations and for patients with and without the 5557G→A and IVS62+8A>C SNPs. The remaining sequence alterations could not be individually subjected to a meaningful statistical analysis as the carrier frequencies were too low to allow for dose response assessments.

RESULTS

Table 1 provides a list of the 26 genetic alterations in the expressed portions of the *ATM* gene, or within 10 bases of each exon in putative splice site regions, that were detected in 22 of the 41 screened breast cancer patients treated with post-mastectomy radiotherapy. In addition, this table lists the dose given to each field and whether grade 3 fibrosis developed.

Figure 3 displays the dose response for patients found to harbor any *ATM* sequence variant compared with the group of patients who did not possess an *ATM* sequence alteration. These curves did not differ significantly form each other (p=0.57) The ED_{50} (95% confidence of interval) was 58.4 (54.0-63.1) Gy for the group of patients with any *ATM* sequence abnormality and 60.2 (55.7-65.1) Gy for patients without a sequence variation. This corresponded to an enhancement ratio of 1.03 (0.97-1.20). A similar analysis was performed for the patients with two *ATM* variants each (six patients, including two being homozygous for the 5557 G→A polymorphism), compared to those with less than two alterations each. There was a trend that the dose response curves for these groups differed from each other (p=0.14) (dose response curves not shown). The ED_{50} value for patients with two sequence alterations was 54.8 (51.3-58.5) Gy opposed to 60.5 (56.7-64.5) for those with less than two alterations each. The corresponding enhancement ratio was 1.10 (1.03-1.19).

With regard to the 5557 G→A SNP, the dose response curve for the seven patients who were either homozygous or heterozygous for the G→A transition polymorphism was significantly different compared to the curve derived from patients without the polymorphism (p=.0.03) (Fig. 4). For these two groups, ED_{50} values of 53.7 (50.2-57.5) and 60.8 (57.0-64.8) Gy respectively were found, leading to an enhancement ratio of 1.13 (1.05-1.22). By contrast, no significant difference was found between the dose response curves from the six patients with the IVS62+8A>C SNP polymorphism and those without (p=0.41) (dose response curves not shown), or between the ED_{50} values 56.4 (50.9-62.5) and 59.9 (56.3-63.8) Gy respectively, yielding an enhancement ratio 1.06 (0.96-1.17)).

DISCUSSION

Post-mastectomy breast cancer patients treated with two different radiation protocols, resulting in a range of 2 Gy equivalent doses from 34-69 Gy to three fields, were screened for genetic alterations in *ATM*. Statistically significant results were obtained when the patients were analyzed with respect to the possession of the 5557 G→A SNP. Regarding the possession of two

ATM **sequence variants, a statistically significant result was found when the analysis was based on the ED_{50} estimates and enhancement ratios provided by logit analysis whereas only a trend towards significance was found when the dose response curves were compared by logistic regression. For these two types of sequence alterations enhancement ratios of 1.13 and 1.10 respectively were found. A further analysis revealed a high degree of concordance between the group of patients with two sequence alterations each and those harboring the 5557 G→A SNP (5 out of 6 patients with two alterations had the 5557 G→A SNP and 5 out of 7 patients with the 5557 G→A SNP had two alterations each)(Table 1). Based on these observations it seems plausible that the enhanced fibrosis risk observed among patients with two alterations was mediated by the possession of the** ***ATM*** **5557 G→A SNP. Thus, the results suggest that women who were carriers of the 5557 G→A polymorphism developed grade 3 subcutaneous fibrosis at lower doses compared with patients who did not possess this type of genetic alterations. In contrast, the findings of this work do not support an association between the development of fibrosis and any other** ***ATM*** **variant detected in the group of patients screened. However, we emphasize that this study provided limited statistical power to detect associations for alterations with low carrier frequencies.**

It should be noted that although multiple comparisons were made in this study, a Bonferroni correction (30) was not applied to the calculated p-values as the purpose of this study was exploratory and it will be necessary to confirm the results of this work in a larger study. In addition, the mathematical model used to construct the dose-response curves treats the assessed radiation fields as independent data points. This approach may result in an overestimation of the statistical significance as some intra-individual association may exist between the outcomes. To address this potential problem, an analysis was performed that restricted the observations to only the bolus-covered part of the photon field (Fig. 1). This field was chosen for analysis as it had the largest range in absorbed radiation dose and provided the highest number of responses (Table 1). Even with this limitation to just one field per patient, the dose response curves for those with or without the 5557 G→A polymorphism, remained significantly different from each other when analyzed by logistic regression (p=0.02)(figure 5). However, due to the reduced number of observations and a smaller range in absorbed radiation dose, ED_{50} values and enhancement ratios with confidence intervals could not be determined by logit analysis.

It has previously been reported that both the incidence and severity of late normal tissue reactions after radiotherapy increase with time of follow-up (28). Although this might potentially constitute a problem, the mean follow-up time for carriers of the 5557 G>A SNP (1345 days) was nearly the same as for those patients who did not possess this variant (1399 days). Thus, the observed difference in fibrosis risk could hardly be attributed to differences in length of follow-up.

Approximately 15-20% of the general population (31) possesses an adenine in place of a guanine at nucleotide position 5557 in ***ATM*** **resulting in substitution of asparagine for aspartic acid at amino acid 1853 in the encoded protein. The results of this study are consistent with Angele et al. (11) who reported an association between possession of the 5557 G→A polymorphism with radiosensitivity, although the correlation found in this study was for patients homozygous for this polymorphism. In a recently published study, a non-significant over-representation of the** ***ATM*** **5557 A allele was found among breast cancer patients with marked alterations in breast appearance after post-lumpectomy radiotherapy (32). In addition, an association, which did not achieve statistical significance due to the small sample size, was reported between this SNP and late morbidity in prostate cancer patients (12).**

Although there is now substantial evidence supportive of ***ATM*** **as a gene associated with clinical radiosensitivity, it is nevertheless highly likely that this is not the only gene whose alteration is responsible for adverse radiotherapy responses. Among the additional radiosensitivity candidate genes that have been identified as having an association with enhanced radiation responses are** ***TGFB1, XRCC1***, ***XRCC3***, ***SOD2*** **and** ***hHR21***. **In a previously published study based on the same patient cohort as used in the present investigation, it was observed that the risk of radiation-induced fibrosis was positively associated with the Pro/Pro genotype at codon 10 and the T/T genotype in position –509 of** ***TGFB1.*** **In addition, the** ***SOD2*** **codon 16 Val/Ala,** ***XRCC3*** **codon 241 Thr/Thr and** ***XRCC1*** **codon 399 Arg/Arg genotypes were associated with enhanced radiosensitivity (29). Two separate studies examined polymorphic sites in** ***TGFB1*** **and also found**

an association between the –509 T/T and codon 10 Pro/Pro genotypes with the development of late normal tissue damage (32, 40). Another study screened three SNPs in *XRCC1* and detected an association with radiosensitivity for patients possessing either the codon 194 Arg/Trp alone or in combination with the codon 399 Arg/Gln genotype (41). It has also been reported that a T→C transition at position 1440 of the open reading frame of *hHR21* was found in 6 of 19 radiation-sensitive cancer patients (42). It should be noted that an important distinction between the patient population reported upon in this paper compared with those in other studies, is that the Danish patients were not selected for screening based upon the development of late effects. Generally, it is difficult to screen unselected populations as the incidence of late effects is too low to provide a sufficient number of cases to yield statistically significant results. Because many of the patients in this study were treated with high biologic doses, there was an adequate number of subjects who developed late effects without specifically selecting patients based upon their radiation response.

As described above, associations with risk of radiation-induced fibrosis have previously been detected for SNPs in the *TGFB1, SOD2, XRCC1* and *XRCC3* genes within the 41 patients screened in the present study. Founded on this observation, a model for estimation of fibrosis risk based on multiple SNPs was established. According to this model, the ED_{50} values for grade three fibrosis correlated with the total number of 'risk alleles' harbored at six polymorphic sites in these genes (29). Considering the current indications that the *ATM* 5557 G→A (codon 1883 Asp/Asn) polymorphism may also influence risk of radiation-induced fibrosis, we incorporated this SNP in a similar analysis of multiple SNPs. In the original model (29) three *TGFB1* polymorphisms (position -509, codon 10 and codon 25) were included. However due to the existence of tight genetic linkage between these SNPs, they segregate into a limited number of well-defined haplotypes (6). Therefore these three SNPs should probably not be regarded as independent risk factors. Furthermore, recent *in vitro* data has suggested a functional impact of the codon 10 SNP on the TGFβ-1 secretion rate (43). Consequently, the analysis was restricted to this *TGFB1* SNP in the current model. Thus, the Asn, Arg, Thr, Ala and Pro alleles in *ATM* codon 1853, *XRCC1* codon 399, *XRCC3* codon 241, *SOD2* codon 16 and *TGFB1* codon 10, respectively, were defined as putative 'risk alleles'. The patients were grouped according to the total number of risk alleles they possessed. ED_{50} values were calculated for patients with 2-3, 4-5 and 6-7 'risk alleles' (Fig. 6). The patients were grouped in this way to achieve approximately the same number of subjects in each group. Due to the fact that the patients segregated differently with respect to the number risk alleles harbored, this new model could not be directly compared to the original version. However, this analysis supports the hypothesis that clinical normal tissue radiosensitivity is determined by the combined influence of multiple genetic alterations (44). Furthermore, it is noteworthy that the model identified a subset of patients characterized by a high degree of radioresistance. Nonetheless, it should be stressed that this analysis was based on a limited number of subjects and that confirmation in independent studies is needed before reaching definitive conclusions concerning a possible subpopulation of radioresistant patients.

CONCLUSION

Based upon the results of this study, a hypothesis can be formulated, which will be tested in a larger cohort of patients, that the *ATM* 5557 G>A polymorphism, resulting in the codon 1853 Asn/Asp and Asn/Asn genotypes, is associated with the development of grade 3 subcutaneous fibrosis in breast cancer patients following post-mastectomy radiation treatment.

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Table 1. *ATM* Genetic Status, Dose and Fibrosis in each of the 41 patients.

		Photon Field[1]		Electron Field[2]		Bolus covered Part of Photon Field[3]	
ATM Variant	Amino Acid	Dose[4]	Fibrosis[5]	Dose	Fibrosis	Dose	Fibrosis
	Change						

5557G>A	1853D>N	43	0	52	0	56	1
5557G>A	1853D>N	52	0	62	1	69	1
5557G>A (h)[6]	1853D>N	42	0	52	1	56	1
5557G>A (h)[6]	1853D>N	38	0	41	0	49	0
IVS38-8T>C; 5557G>A	1853D>N	55	0	61	1	69	1
IVS38-8T>C; 5557G>A	1853D>N	42	0	41	0	50	0
735C>T; 5557G>A	245V>V; 1853D>N	57	1	61	1	69	1
378T>A	126D>E	43	0	52	0	56	0
2614C>T; 3161C>G	872P>S; 1054P>P	36	0	41	0	47	0
4258C>T	1420L>F	39	0	45	0	53	0
4258C>T	1420L>F	45	0	52	0	58	0
4258C>T	1420L>F	53	0	62	0	69	1
4578C>T	1526P>P	51	0	50	0	65	0
4578C>T	1526P>P	38	0	41	0	48	0
4578C>T	1526P>P	50	0	61	0	68	0
IVS10-6T>G	n/a	41	0	51	1	52	1
IVS62+8A>C	n/a	46	0	52	0	59	0
IVS62+8A>C	n/a	34	0	41	0	45	0
IVS62+8A>C	n/a	54	0	57	1	69	1
IVS62+8A>C	n/a	36	0	41	0	47	0
IVS62+8A>C	n/a	54	0	62	1	69	1
IVS62+8A>C	n/a	54	1	62	1	69	1
None	n/a	36	0	41	0	47	0
None	n/a	53	1	62	1	69	1
None	n/a	52	1	62	1	69	1
None	n/a	54	0	61	0	69	0
None	n/a	52	0	62	1	69	1
None	n/a	55	1	61	1	69	1
None	n/a	51	0	58	0	69	0
None	n/a	53	0	62	1	69	1
none	n/a	53	0	61	0	69	0
none	n/a	54	0	62	0	69	1
none	n/a	53	0	62	1	69	1
none	n/a	52	0	61	1	69	1
none	n/a	53	0	62	1	69	0
none	n/a	53	0	62	1	69	1
none	n/a	56	0	62	0	69	1
none	n/a	52	0	62	0	69	1
none	n/a	50	1	60	1	67	1
none	n/a	41	0	51	0	54	0
none	n/a	43	0	51	0	55	0

[1]**Anterior photon field including supra/infraclavicular region and axillary region**

[2]**Anterior electron field**

[3]**The part of the anterior photon field covered by a 5 mm wax bolus**

[4]**Equivalent dose of 2 Gy per fraction**

[5]**0=no fibrosis, 1=fibrosis**

[6]h=homozygote, all other variants were present in the heterozygous state

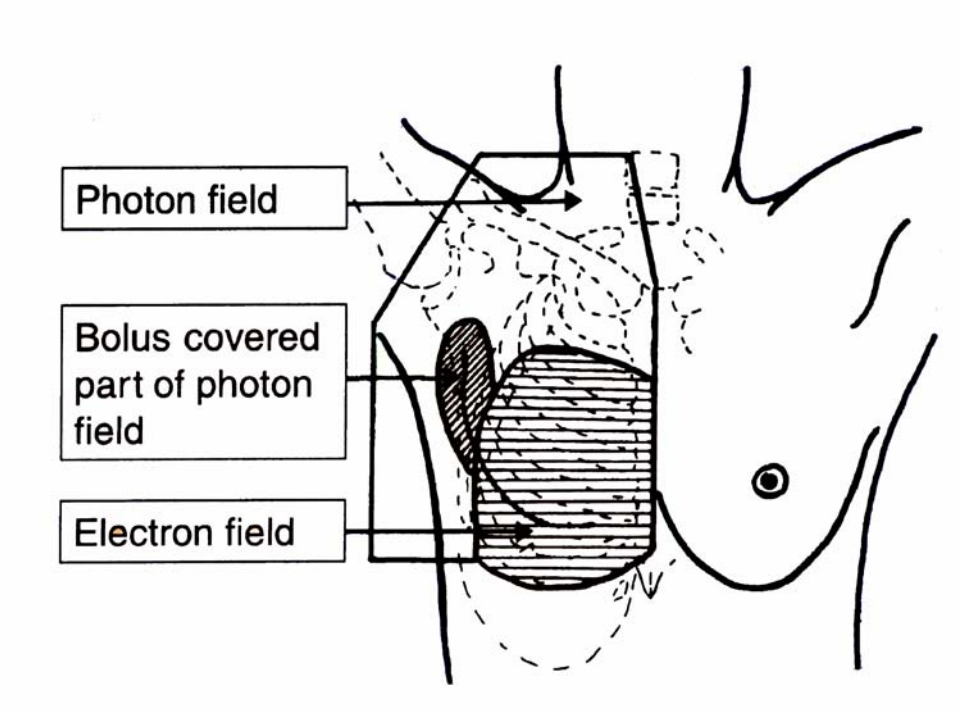
Photon field
Bolus covered part of photon field
Electron field

Figure 1

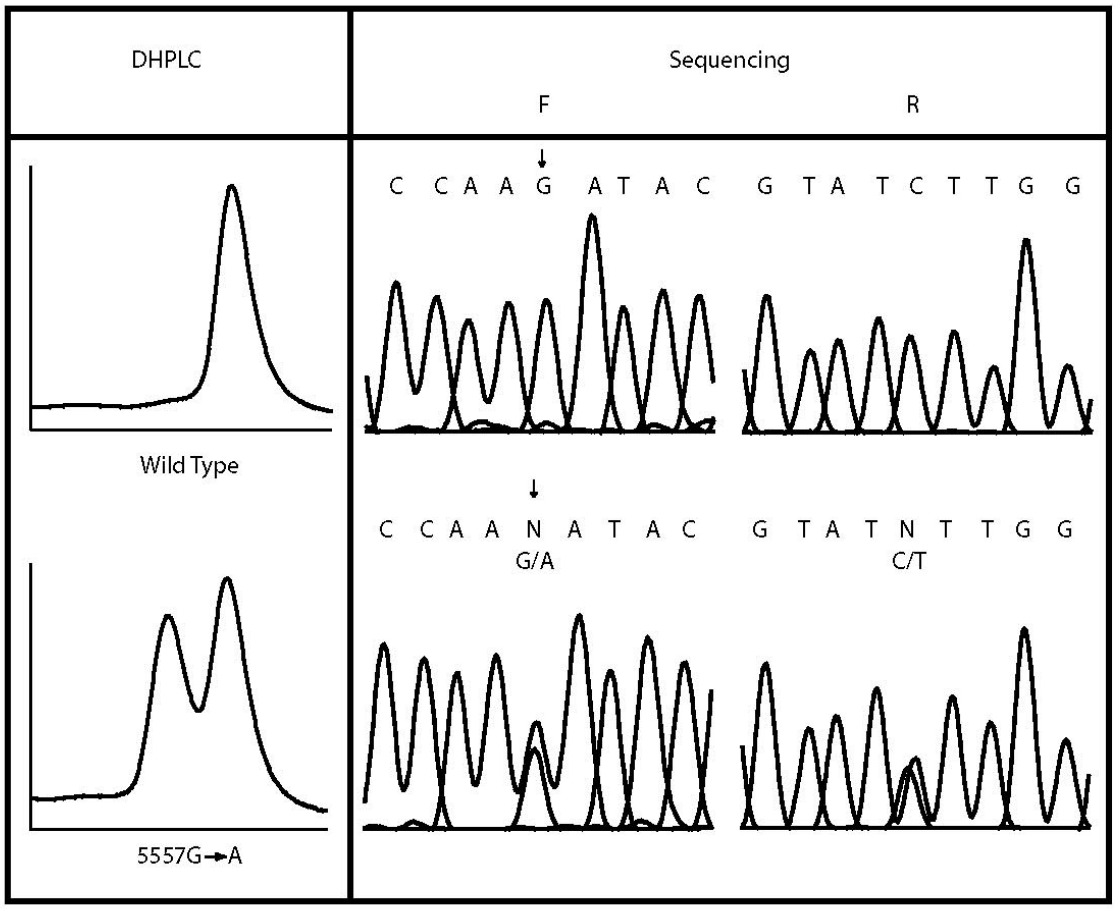
DHPLC
Sequencing
F
R
C C A A G A T A C G T A T C T T G G
Wild Type
C C A A N A T A C G T A T N T T G G
G/A
C/T
5557G→A

Figure 2

Figure 3

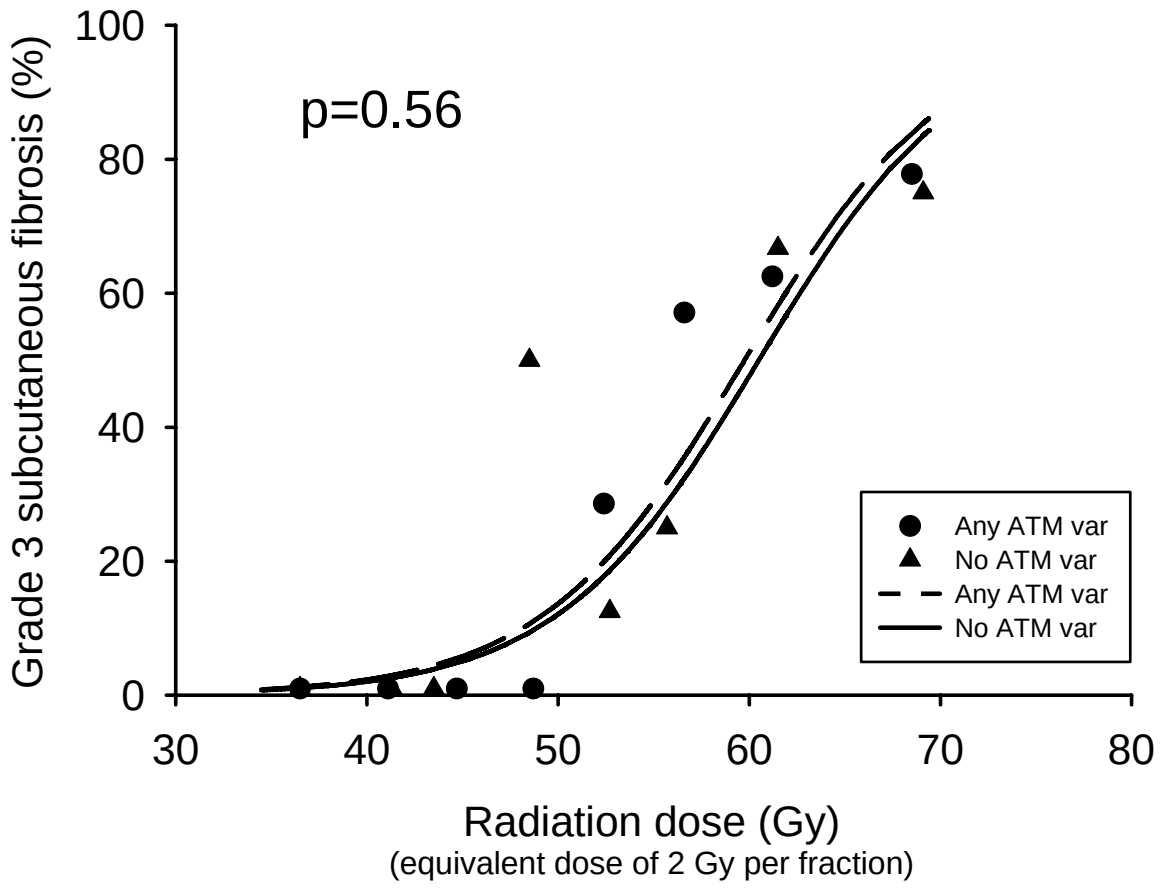
p=0.56
Grade 3 subcutaneous fibrosis (%)
100
80
60
40
20
0
30
40
50
60
70
80
Radiation dose (Gy)
(equivalent dose of 2 Gy per fraction)
Any ATM var
No ATM var
Any ATM var
No ATM var

Figure 4

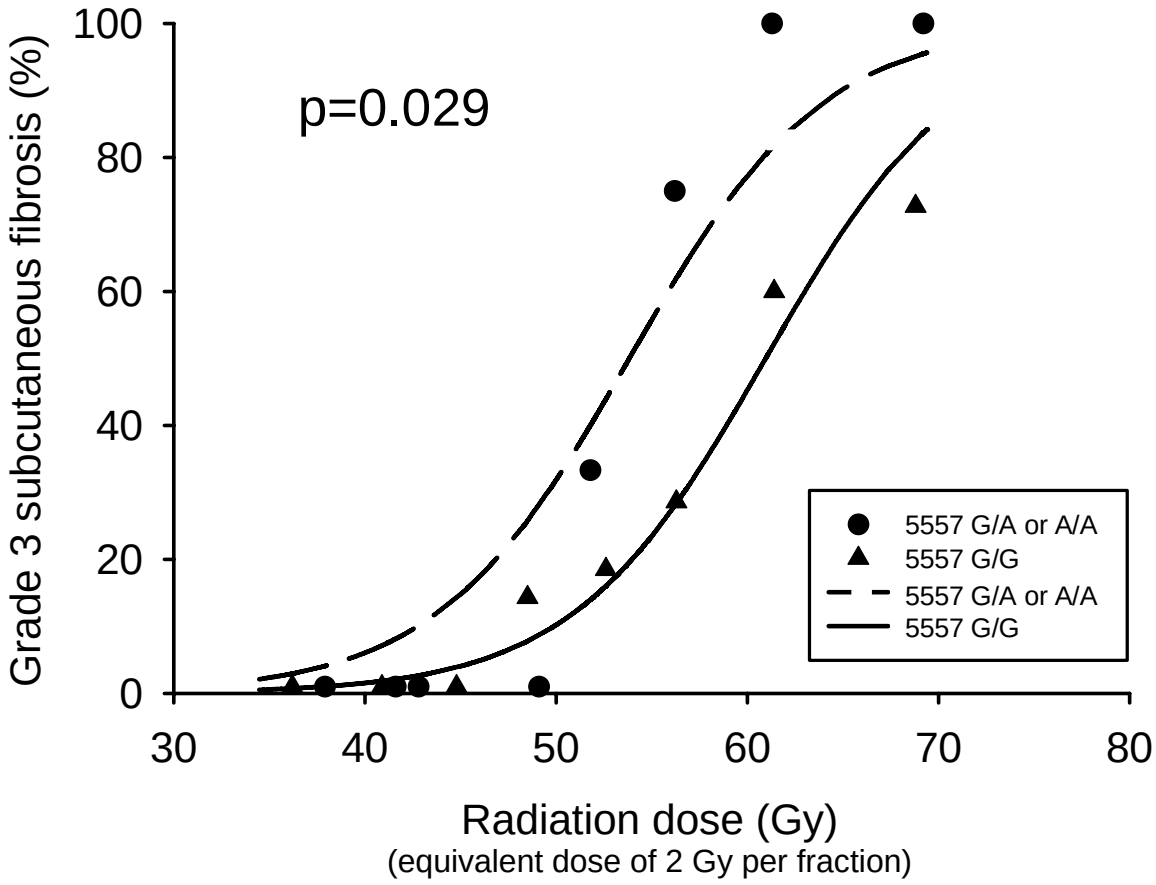
Grade 3 subcutaneous fibrosis (%)
100
80
60
40
20
0
p=0.029
30
40
50
60
70
80
Radiation dose (Gy)
(equivalent dose of 2 Gy per fraction)
5557 G/A or A/A
5557 G/G
5557 G/A or A/A
5557 G/G

Figure 5

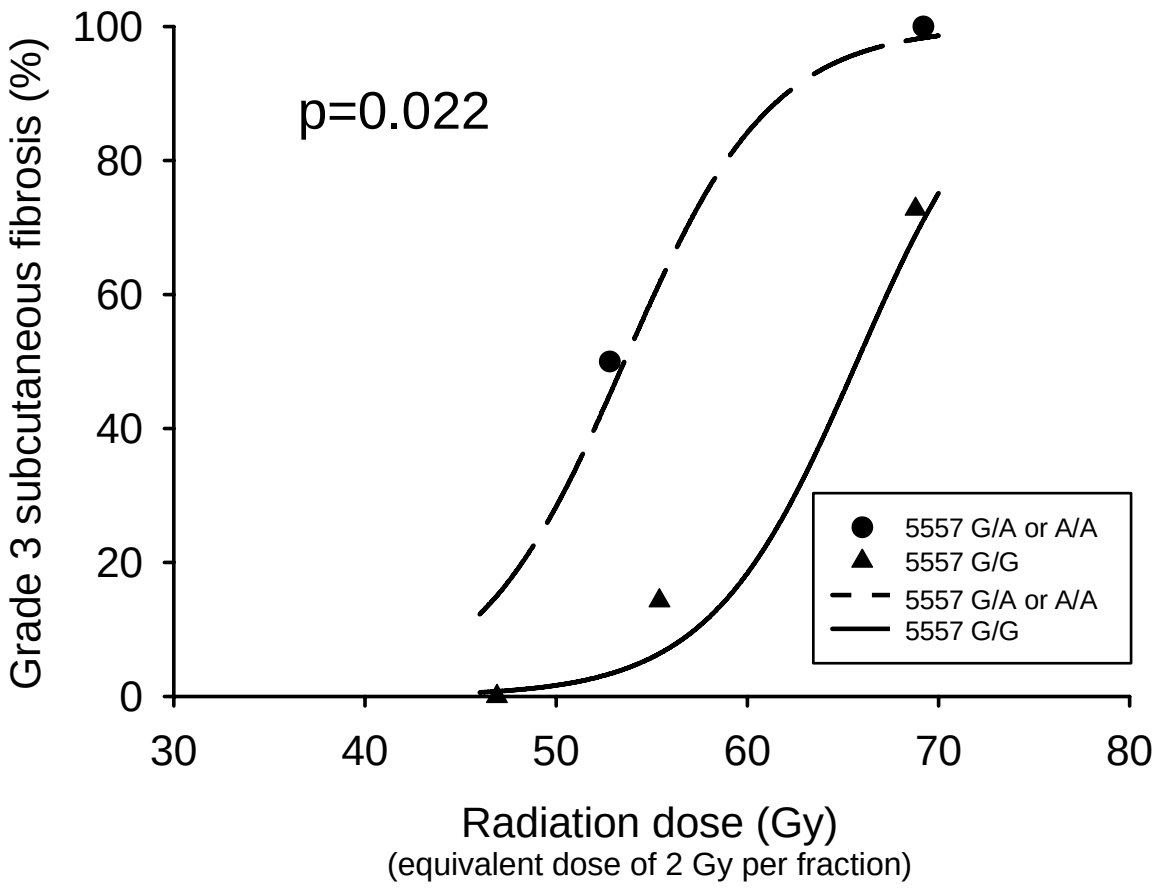
Grade 3 subcutaneous fibrosis (%)
100
80
60
40
20
0
p=0.022
30
40
50
60
70
80
Radiation dose (Gy)
(equivalent dose of 2 Gy per fraction)
5557 G/A or A/A
5557 G/G
5557 G/A or A/A
5557 G/G

Figure 6

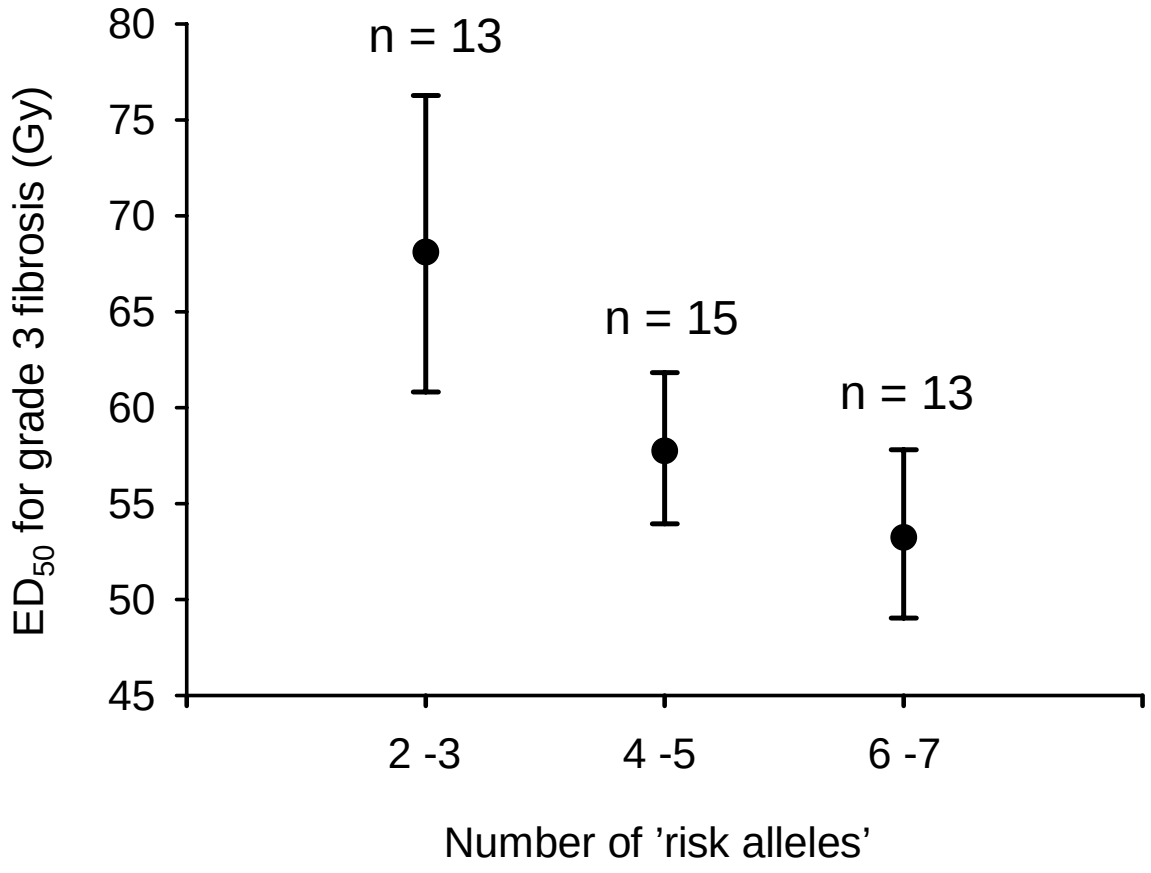

FIGURE LEGENDS

Figure 1. Treatment field arrangement for post-mastectomy radiotherapy in Aarhus 1978-1982. All patients screened in this study were treated with this technique.

Figure 2. Examples of wild-type pattern and genetic variant DHPLC chromatograms. The double peak is indicative of a change in base pair sequence and subsequent amino acid substitution.

Figure 3. Dose response curves for subcutaneous fibrosis in patients with either any *ATM* variant or no alteration in this gene.

Figure 4. Dose response curves for subcutaneous fibrosis in patients with either the G→A polymorphism at nucleotide 5557 or not possessing this alteration.

Figure 5. Dose response curves for subcutaneous fibrosis in patients with either the G→A polymorphism at nucleotide 5557 or not possessing this alteration when the analysis was exclusively based on observations form the bolus covered part of the photon field (i.e. one observation per patient).

Figure 6. ED_{50} values for patients with different numbers of 'risk alleles'. Error bars indicate 95% confidence intervals.

Genetic Predictors of Adverse Radiotherapy Effects: The Gene-PARE Project

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Running Title: The Gene-PARE project

Key Words: genetic predictors; adverse radiotherapy effects; breast cancer; prostate cancer

Purpose: **The development of adverse effects resulting from the radiotherapy of cancer limits the use of this treatment modality. The validation of a test capable of predicting which patients would be most likely to develop adverse responses to radiation treatment, based upon the possession of specific genetic variants, would therefore be of value. The purpose of the Gene-PARE project is to help achieve this goal.**

Materials and Methods: **A continuously expanding biorepository has been created consisting of frozen lymphocytes and DNA isolated from patients treated with radiotherapy. In conjunction with this biorepository, a database is maintained with detailed clinical information pertaining to diagnosis, treatment and outcome. The DNA samples are screened using denaturing high performance liquid chromatography (DHPLC) and the Surveyor nuclease assay for variants in *ATM, TGFB1, XRCC1*, *XRCC3*, *SOD2* and *hHR21.* It is anticipated that additional genes, which control the biologic response to radiation, will also be screened in future work.**

Results: **Evidence has been obtained that possession of variants in genes whose products play a role in radiation response is predictive for the development of adverse effects following radiotherapy.**

Conclusions: **It is anticipated that the Gene-PARE project will yield information that will allow radiation oncologists to use genetic data to optimize treatment on an individual basis.**

The term “adverse radiation effects” can generally be defined as undesirable clinical and physiological responses secondary to radiation treatment. In an effort to balance the eradication of clonogenic tumor cells with minimization of damage to surrounding normal tissues, the mechanisms underlying adverse responses to radiation therapy have been studied by both basic scientists and clinicians (1-5). In this article, both the historical and current literature examining genetic factors in adverse radiation response will be reviewed. In addition, current efforts and techniques utilized in the Gene-PARE project will be discussed as well as future directions for developing genetic predictors of radiation-induced morbidity.

Genetic Factors and Radiosensitivity

A variety of patient, tumor, treatment, cellular and molecular factors contribute to the variability in severity of normal tissue reactions exhibited following radiotherapy. Patient characteristics including age, nutritional status, medications, body habitus and coexisting morbidities, such as diabetes or the presence of recent surgery, all may contribute to radiation toxicity (6). Tumor-related factors such as size, histology and tumor grade may also affect the reaction to radiotherapy. Variation in treatment-related parameters including treated volume, field size, anatomic prescription point, total dose, dose per fraction and use of concomitant chemotherapy may also contribute to response heterogeneity. Due to the steep dose-response relationship for normal tissues, a small difference in dose could produce divergent outcomes (7, 8). In addition, it has been hypothesized that individual genetic variations may also influence the development of adverse radiation responses (9-14). Evidence in support of this theory was obtained through a study (15) which examined the incidence and time to development of radiation-induced telangiectasia in a cohort of breast cancer patients. A wide range of values was reported for this patient population despite uniform radiation treatment. Consistent with the results of previous analyses of radiotherapy patients (8, 16, 17) it was estimated that approximately 80-90% of the variability was attributed to deterministic effects, possibly arising from potential individual genetic differences, whereas only 10-20% of the variation resulted from stochastic events associated with the random nature of radiation-induced cell killing in addition to random variations in dosimetry and dose delivery.

Efforts to Develop Predictive Assays for Normal Tissue Radiosensitivity

The development of an *in vitro* radiosensitivity assay capable of predicting the extent of normal tissue damage in radiotherapy patients represents a long sought after goal (18). Despite limited success, the effort to achieve this objective continues since an assay capable of predicting susceptibility for the development of adverse radiation effects would allow customization of radiotherapy protocols on an individual basis. By doing so, it has been estimated that a significant improvement in the therapeutic index could be achieved (16, 19). This work is also reflective of the new era of “individualized” or “personalized” medicine (20-22). The goal is therefore to develop a robust, specific assay to enable individual dose adjustment based upon the response of each patient to this test (16, 19, 23, 24).

Numerous assays have been proposed to provide the clinician with information that predicts the outcome after irradiation and thus guide treatment prescription, but none have become established in daily practice. Major difficulties limiting the success of these assays are lack of sensitivity and specificity, technical burden of the procedures, poor characterization of the assayed cells and the complexity of normal tissue radiobiology (25).

Skin fibroblast SF_2 assays

Several studies have attempted to define the relationship between *in vitro* radiation response and clinically evident effects by correlating fibroblast radiosensitivity with the development of acute and late radiation damage. The underlying hypothesis of these studies is that genetic differences may account for much of the unanticipated severity of acute and chronic radiation reactions exhibited by some radiotherapy patients. Several studies have reported a correlation between dermal fibroblast radiosensitivity quantified by clonogenic survival assays, measuring the SF_2 (the surviving fraction following exposure to 2 Gy of X-rays), and the severity of both early and late effects (26, 27). In addition, it has been reported that in vitro fibroblast proliferation post-irradiation may be a useful predictor of wound healing morbidity for soft tissue sarcoma patients who received preoperative radiotherapy (28). However, in contrast to these positive results, several studies have reported a lack of correlation between dermal fibroblast SF_2 with either early

or late skin reactions (29). Taken together, these studies indicate that skin fibroblast sensitivity correlates only weakly with assessment of radiation-induced skin injury.

Lymphocyte Assays

For assays of normal tissue radiation response, blood is considered the tissue of choice due to the ease of collection in a standardized, patient-convenient manner. However, initial lymphocyte radiosensitivity studies (30-33) were disappointing with respect to experimental variation which confuted the predictive power of this assay. As the various lymphocyte cell-types display different radiation responses, fluctuations in the relative frequency of lymphocyte types cause an apparent shift in radiosensitivity resulting in large experimental variation (30, 31). However, by taking into account cell-type specific radiosensitivities, it has been reported that CD4 and CD8 T-lymphocyte radiosensitivity can discriminate differences in radiation-induced cytotoxicity between individuals (32-36), although it is premature to use such an approach as a predictive assay.

Chromosomal aberrations and micronuclei

Additional attempts to find suitable assays include analysis of fibroblast chromosomal aberrations (37). However, this technique is time-consuming and allows examination of only a limited number of cells. Thus, it is considered impractical for cell types that exhibit slow growth and low mitotic indices. Micronucleus induction analysis is another means of detecting chromosomal damage. Although this assay has a well-established role in genetic toxicology (38) for biomonitoring of human populations (39) and as a biologic indicator of radiation damage (40-42), efforts to predict radiosensitivity have been inconclusive (43, 44).

Molecular approaches

Despite multiple and various attempts to develop a assay capable of predicting which patients are susceptible to developing adverse radiotherapy effects, none of the assays examined to date has proven to be consistently sensitive and accurate for the prediction of side effects among patients receiving radiation (45). However, new technologies in molecular biology may promote novel strategies for developing a predictive assay with clinical applicability. The use of gene expression arrays that could predict the variation in normal tissue radiation sensitivity between individuals based upon the expression patterns of different genes is currently under investigation. Several studies have demonstrated the predictive power of pretreatment expression profiling for human tumors (46-51) but similar large-scale studies on normal tissues to assess the extent of radiation-induced toxicity have yet to be reported. In addition, a few studies have demonstrated meaningful correlations with morbidity by focusing primarily on cytokine responses (52). Another new molecular approach involves analysis of DNA end-binding complexes that form at DNA double strand breaks following irradiation. It has been reported that the levels of ATM-containing complexes correlated with cellular radiosensitivity as measured by the SF_2 (53). Although these new molecular approaches appear promising, it is undetermined whether any will have clinical applicability.

Goal of the Gene-PARE project

In order to develop an alternative approach to establish an assay predictive of which patients are most likely to experience radiation-induced complications, a research program has been initiated to identify the genetic factors associated with clinical radiosensitivity. To achieve this goal, a broad international effort has been organized comprising investigators from radiation oncology departments in the United States, Israel, France and Switzerland, to create the Gene-PARE (*Gene*tic *P*redictors of *A*dverse *R*adiotherapy *E*ffects) project. Through the studies currently active in Gene-PARE, over two thousand radiotherapy patients will be screened for genetic variants. The primary objective of Gene-PARE is to establish the genetic alterations whose presence may confer increased susceptibility for developing an adverse response to radiotherapy. Although the subjects screened to date are primarily breast and prostate cancer patients, the Gene-PARE tissue biorepository is not exclusive to these two types of cancers as it is open to tissue samples from patients diagnosed with any form of cancer treated with radiation. For all patients accrued into Gene-PARE studies, a blood sample is obtained for lymphocyte isolation and DNA extraction. In addition, frozen lymphocytes from patients exhibiting clinical radiosensitivity or notable genetic characteristics have been used for EBV transformation to create permanent cell lines, which are being employed in assays examining the functional significance of specific variants.

By identifying genetic factors associated with radiosensitivity, the goal of Gene-PARE is to develop a means to predict which patients are at increased risk for complications secondary to radiation treatment. In this sense, we are attempting to “pare away” those individuals from the general patient population who are most likely to suffer pronounced radiation-induced normal tissue damage. Although these radiosensitive patients may be better suited to a surgical treatment approach, paradoxically, these individuals could alternatively represent a subset of patients who are actually optimal candidates for radiotherapy, given that their cancers should harbor identical sequence alterations associated with radiosensitivity. This highlights the potential for radiotherapy dose modification as radiosensitive tumors theoretically should require lower total treatment doses than their genetically non-variant counterparts. Conversely, for the vast majority of patients who do not possess genetic variants associated with radiosensitivity, it may be possible to dose escalate and potentially achieve a larger number of cancer cures.

Inclusion of African-American patients

A unique feature of Gene-PARE that distinguishes it from its European counterpart, the GENEPI (genetic pathways for the prediction of the effects of irradiation) project (10, 54), coordinated through ESTRO, as well as the developing Japanese RadGenomics (55) and the British RAPPER (Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy) and RACE (Radiation Complications and Epidemiology) studies (54), is the inclusion of a substantial number of African-American patients. Based upon currently funded Gene-PARE studies, it is anticipated that at a minimum, approximately 500 African-American subjects will be screened for genetic variants associated with clinical radiosensitivity. Screening of these samples may allow identification of important genetic predictors specific for this population, as genetic alterations that contribute to enhanced radiosensitivity could differ among ethnicities. Initial results of Gene-PARE studies suggest that substantial differences exist between the genetic factors associated with the development of adverse radiotherapy effects for African-Americans compared with variants correlated with radiosensitivity in the general population (56). This preliminary finding is consistent with accumulating pharmacogenomic evidence indicating that African-Americans have a

significantly different spectrum of polymorphisms in genes associated with drug metabolism, compared with the general population (57).

Distinction between Mutations, SNPs and Rare Variants

Several semantic issues deserve mention. Throughout this review, the word "mutation" is generally avoided as this term is often employed to signify a particular DNA sequence variation that exerts a functional impact upon the protein encoded by the gene. Instead, the term "SNP" (single nucleotide polymorphism) is used to indicate a sequence variation in which the less common or minor allele occurs at a population frequency greater than 1% (58). The expression "rare variant" is employed to mean a sequence variation for which the minor allele occurs with a frequency less than 1%. Hence, these terms only refer to the prevalence of a minor allele and do not imply whether or not a particular genetic variant possesses functional or pathologic significance. The terms "DNA sequence variation" or "genetic variant/alteration" are utilized to signify SNPs and rare variants. The use of "mutation" is limited in order to avoid any suggestion as to the functional impact upon the protein encoded by a gene possessing a particular variant allele.

The Role of ATM in Clinical Radiosensitivity

During the initial years of the Gene-PARE project, substantial attention was devoted to study of the *ATM* gene and its relationship to radiosensitivity, which has pioneered the way for examination of other genetic variations as predictors of adverse radiation responses. The ATM protein functions as a protein kinase involved in cellular stress responses, cell cycle checkpoint control and DNA repair (59-62). Loss of these functions may subsequently lead to a diminished DNA repair ability and defective cell cycle checkpoint control. The clinical association between patients producing non-functional ATM protein and the subsequent devastating responses to ionizing radiotherapy have been described (63, 64). In addition, cells derived from individuals who were heterozygous for a mutation in *ATM* exhibited a radiosensitivity intermediate between people diagnosed with AT and individuals who were not *ATM* carriers (65-70).

The initial studies examining the role of *ATM* variants in clinical radiosensitivity failed to find a positive correlation between *ATM* mutation status and the development of enhanced normal tissue damage in breast cancer patients (71-75). However, all of these studies utilized a protein truncation test, which only detects genetic alterations that cause protein truncations. Subsequent to these reports, evidence was obtained that missense mutations, which result in amino acid substitutions rather than protein truncation, are more prevalent in cancer patients and therefore serve as a more appropriate type of DNA alteration to measure for ascertainment of *ATM* mutational status (76-78).

In the first Gene-PARE study examining the role of *ATM* mutations in susceptibility to radiotherapy-induced morbidity, 46 breast cancer patients were screened for *ATM* sequence variations (79). It was reported that 100% (3/3) of the patients who developed a grade 3/4 subcutaneous reaction, manifested as either fibrosis or soft tissue necrosis, had *ATM* missense variants. In contrast, only 7% (3/43) of the patients who did not develop this form of severe toxicity harbored this type of *ATM* alteration. In a separate study, DNA samples isolated from 41 post-mastectomy patients who were treated with

either a hypofractionated or standard radiotherapy fractionation protocol were screened (80). Since many of these patients received a hypofractionated treatment, radiation-induced skin fibrosis was relatively common in this cohort. Based on a logistic regression model, a dose response utilizing the ED_{50} (the dose that resulted in a 50% incidence of grade 3 radiation-induced fibrosis) was generated for these patients. The findings of this study suggest a correlation between possession of the 5557 G>A variant in *ATM* and radiosensitivity as the ED_{50} for women who were carriers of this SNP was 52 Gy, compared to an ED_{50} of 61 Gy for patients who did not possess this genetic alteration. These results are consistent with Angele et al. (81), who found a significant association between homozygote carriers of the G>A transition at *ATM* nucleotide 5557 and adverse radiotherapy responses as well as a separate study that reported a non-significant over-representation of the *ATM* 5557 A allele among breast cancer patients with marked alterations in breast appearance after post-lumpectomy radiotherapy (82). In addition, an association, which did not achieve statistical significance due to the small sample size, was reported between this SNP and late morbidity in prostate cancer patients (83).

Further evidence supporting the relationship between *ATM* sequence variations and radiosensitivity has been obtained for prostate cancer patients treated with ^{125}I brachytherapy (84). The samples for these patients were obtained from the Mount Sinai Prostate Cancer Patient Tissue Biorepository, which represents a critical resource for Gene-PARE. This biorepository maintains DNA and frozen blood lymphocytes derived from the approximately 2,400 prostate cancer patients treated with radiotherapy and followed at this medical center over the past 15 years. A pilot study involving *ATM* screening reported that 10/16 (63%) of the subjects shown to possess sequence variants exhibited at least one form of adverse response, defined as erectile dysfunction, late rectal bleeding or severe urinary bother. In contrast, of the patients who did not harbor an *ATM* sequence variation, only 3/21 (14%) manifested radiation-induced adverse responses. Nine of the patients with sequence alterations specifically possessed missense mutations, which encode for amino acid substitutions, and are therefore more likely to possess functional importance. For this group, 7/9 (78%) exhibited at least one form of adverse response. In contrast, among the 28 patients who did not have a missense alteration, only 6/28 (21%) displayed any form of adverse response to the radiotherapy.

Additional Radiosensitivity Candidate Genes Under Study

Although there is now evidence supportive of *ATM* as a gene associated with clinical radiosensitivity, it is nevertheless likely that this is not the only gene whose alteration is responsible for adverse radiotherapy responses. Additional radiosensitivity candidate genes that have been linked to enhanced radiation responses include *TGFB1, XRCC1*, *XRCC3*, *SOD2* and *hHR21*. TGFβ1, the protein encoded by *TGFB1*, is a key cytokine involved with the regulation of cell growth and immunosuppressive activities. It is also associated with the deposition of extracellular matrix proteins and plays a central role in radiation-induced fibrosis (85). The primary function of the XRCC1 protein is to coordinate the activities of the enzymes that perform base excision repair of radiation-induced damage. Cells lacking a functional XRCC1 protein have demonstrated a hypersensitivity to radiation (86, 87). XRCC3 is involved in recombinational repair of radiation-induced DNA double strand breaks (88). *SOD2* encodes the manganese superoxide dismutatse which represents an important line of cellular antioxidant defense against the reactive oxygen species induced by irradiation (89). *hHR21* is the human homologue of the yeast *rad21* (90) whose encoded protein is involved with repair of DNA double stand breaks (91), sister chromatid cohesion and apoptosis (92).

To summarize this work, a correlation between radiosensitivity and the presence of a Pro/Pro at codon 10 and the T/T genotype in position –509 of *TGFB1* has been reported (82, 93). A relationship has also been demonstrated between the *SOD2* codon 16 Val/Ala, *XRCC3* codon 241 Thr/Thr and *XRCC1* codon 399 Arg/Arg genotypes and an increased risk of radiation-induced fibrosis (94). Another study screened three SNPs in *XRCC1* and detected an association with radiosensitivity for patients possessing either the codon 194 Arg/Trp alone or in combination with the codon 399 Arg/Gln genotype (95). Lastly, a T>C transition at position 1440 of the open reading frame of *hHR21* has been found in 6 of 19 radiation-sensitive cancer patients (96).

In aggregate, these studies support the general hypothesis that genetic factors play a significant role as predictors of adverse radiotherapy responses. It is also important to note that the postmastectomy radiotherapy breast cancer patients that were screened through Gene-PARE for *ATM* variants, have also been examined for SNPs in the additional genes cited above (94). From the results obtained, it appears that susceptibility to the development of radiation-induced fibrosis depends critically upon the total number

of genetic variants possessed rather than on any single genetic alteration or gene affected (80). These findings suggest that clinical normal tissue radiosensitivity should be regarded as a complex genetic trait that is dependent on the effect of multiple DNA sequence variants.

Cellular Radiosensitivity and Possession of Genetic Variants

The Human Genome Project is a well-publicized example of the increasing effort to unravel the genetic variation underlying complex diseases and traits by illustrating the genetic differences existing between individuals (97). The role of SNPs and rare variants, which constitute approximately 90% of naturally occurring sequence variations, is of particular importance (98-100). SNPs and rare variants are known to potentially affect phenotype, although they have often been regarded as genetic changes without functional significance. However, these sequence alterations may in fact have an important biologic impact as genetic variants located within regulatory regions could affect gene expression, while amino acid substitutions resulting from variants present in exons may alter protein function. Even SNPs present within non-coding regions could be of significance through their affect upon RNA stability or splicing mechanisms (58).

The "allelic architecture" of complex traits has received significant attention (101-104). Susceptibility to adverse radiotherapy responses can be conceptualized through the two competing theories for the genetic basis of complex traits (105). The first theory, the so called "common disease/ common variant hypothesis" suggests that the inherited basis of complex traits is most likely the result of genetic variants characterized by relatively high allelic frequencies (106). According to this theory, common SNPs in a limited number of genes are responsible for the inheritance of complex traits. However, this approach to identify genes associated with complex traits has achieved only modest success. Therefore, the alternative "rare variant" hypothesis has been proposed, which suggests that a large pool of alleles is accountable for the development of complex traits (107). The most realistic model for complex genetic traits likely incorporates aspects of both theories, with predisposing alleles of varying population frequencies present in the same and different genes. The Gene-PARE project will not be limited by either of these theories, since the approach being used in the studies that constitute this project routinely involves screening the entire coding portion of each candidate gene.

A question also arises as to the types of mutations that may be associated with clinical radiosensitivity. The studies reporting the results of *ATM* screening lend support to an association between minor sequence alterations, such as SNPs and rare variants, with susceptibility to adverse effects of radiotherapy (79-84). In contrast, evidence has been provided (72, 75, 108, 109) that patients who were carriers of pathogenic truncating mutations, which are typically the type of mutation found in individuals with AT (110), appear not to have been radiosensitive. It is possible that the presence of a null mutation in one copy of the *ATM* gene does not confer clinical radiosensitivity, whereas, possession of a functional, but altered ATM protein may result in an increased risk for the development of an adverse response to radiation treatment.

Radiosensitivity and Tolerance Dose

The question may also be raised as to whether a small difference in cellular survival associated with possession of genetic variants that confers a relatively small increase in cellular radiosensitivity could account for an increased severity in radiation response. In fact, the performance of a simple calculation demonstrates that this is a likely outcome. For example, an SF_2 for cells from an individual not possessing variants associated with radiosensitivity may be 0.5, while for a person possessing genetic variants causing mild radiosensitivity, the SF_2 could be 0.3. Considering a protocol involving the use of twenty-five 2 Gy fractions, at the completion of treatment, cellular survival would be approximately $3x10^{-8}$ for normal patients whereas it would be $8x10^{-14}$ for patients possessing radiosensitivity alleles. This effectively represents the biological impact of an 88 Gy total treatment dose for radiosensitive patients compared with 50 Gy for the patients not harboring such genetic alterations. This large biologically effective dose could certainly account for adverse effects from the radiation treatment. In fact, when taking into account the relatively steep increase in the complication curves for normal tissue responses and the practice of treating to normal tissue tolerance, only a small increase in radiosensitivity could result in a large increase in the probability of normal tissue radiation-induced toxicity.

It is also important to note that this small increase in radiosensitivity may be difficult to detect through routine cellular radiosensitivity studies considering the limitations in accuracy and precision of in vitro assays. Thus, when taking into account the steep slope of the normal tissue dose-complication

curves, it is likely that a relatively modest, and possibly undetectable effect upon ATM protein function, resulting in mild cellular radiosensitivity, could still substantially increase the probability for an adverse clinical response. Thus, it may prove difficult or impossible to detect through functional assays, such as p53 phosphorylation, ATM protein levels, H2AX phosphorylation, chromosomal aberrations or cellular clonogenicity, the functional impact of a genetic variant that causes clinical radiosensitivity.

Denaturing High Performance Liquid Chromatography (DHPLC) and the Surveyor nuclease assay

The principle screening techniques for identification of genetic variants in the Gene-PARE project are DHPLC and the Surveyor nuclease assay. These are both robust techniques that can be used to screen any gene in a large population for single nucleotide substitutions as well as small deletions and insertions (111-113). The main advantage of DHPLC lies in its rapid and accurate identification of polymorphisms and rare genetic variants in an automated fashion with a high level of sensitivity and specificity (114-122). The samples obtained through Gene-PARE are also being screened using a complementary methodology that employs Surveyor nuclease (Trangenomic, Inc.) which is a mismatch-specific DNA endonuclease. It is a member of the CEL nuclease family of plant DNA endonucleases. Surveyor nuclease cleaves with high specificity at the 3' side of any mismatch site in both DNA strands, including all base substitutions and insertion/deletions up to at least 12 nucleotides. When mutant and wild-type alleles are mixed, heated and then cooled to form heteroduplexes, Surveyor nuclease cleaves the heteroduplex fragments. The cleavage products are then analyzed using the same HPLC platform used for DHPLC, but performed under non-denaturing conditions. This assay is performed under high sensitivity conditions in which the DNA is stained with a fluorescent probe and detected using a fluorescence detector. Hence, use of this approach permits the recognition of certain variants that are difficult to identify using DHPLC which may require samples to be run at multiple denaturing temperatures in order to be detected. A further advantage in the use of the Surveyor nuclease assay is that it provides information not only as to the presence of a genetic alteration, but also it’s relative position in the DNA fragment being analyzed (123-126). While genotyping assays designed to detect common SNPs may be less costly to perform, these assays are limited to detection of already known SNPs and are not designed to discover new sequence variants. Of greatest importance, DHPLC and the Surveyor assay are capable of detecting virtually all variants in a gene, rather than just specific SNPs.

Conclusions

The goal of the Gene-PARE project is to identify the genetic sequence variants that are predictive for the development of adverse effects resulting from radiotherapy. In order to accomplish this objective, a clinical database and biorepository of frozen lymphocytes derived from cancer patients treated with radiation have been established. DNA isolated from each tissue sample is being screened for variants in genes associated with radiation responses. It is expected that the results of Gene-PARE will enable the greater utilization of data generated as part of the Human Genome Project and the emerging field of radiogenomics. In addition, Gene-PARE will enable radiation oncologists to take greater advantage of the increasingly powerful and inexpensive methodologies to sequence DNA in anticipation of the day when patients diagnosed with cancer arrive at their initial radiation oncology consultation armed with their full

genome sequenced (127, 128). By identifying genetic predictors of radiosensitivity, Gene-PARE may help cancer patients avoid serious complications that lead to severe morbidity, or even mortality, arising from organ damage secondary to radiotherapy. In addition, it could be discovered through this work that there exists a small radiosensitive portion of the population and that standard treatment doses are effectively being limited by their radiation tolerance. If these individuals can be identified through genetic screening, it may then be revealed that the vast majority of people are more resistant to radiation than generally assumed. This finding might permit radiation oncologists to be more aggressive and dose escalate, which could translate not only into an improved clinical outcome for radiotherapy patients, but also an ability to more often safely treat relatively radioresistant cancers. Thus, the results of the research conducted under Gene-PARE will help in the development of a predictive test that will provide critical information to individuals faced with a diagnosis of cancer, and their doctors, necessary to reach optimal treatment decisions.

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Table 1: Gene-PARE Studies

Funding Agency	Treated Cancer Site	Country Where Patients Are Accrued	Specific Targeted Ethnic Group	Period of Study	Screened Genes	Number of Subjects to be Screened	Adverse Effects
DOD-BCRP[a]	Breast	U.S	African-American	2002-2006	***ATM***	150	Telangiectasia, Fibrosis
DOD-PCRP[b]	Prostate	U.S	None	2004-2009	***ATM***	200	ED[c], UTM[d], Proctitis
NY State Dept. of Health	Breast & Prostate	U.S	None	2005-2007	*ATM, TGFB1 XRCC1 XRCC3, SOD2, hHR21*	100	Telangiectasia, Fibrosis ED, UTM, Proctitis
ACS[e]	Prostate	U.S	African-American	2005-2009	*ATM, TGFB1 XRCC1 XRCC3, SOD2, hHR21*	225	ED, UTM, Proctitis
VA[f]	Prostate	U.S	None	2005-2010	*ATM, TGFB1 XRCC1 XRCC3, SOD2, hHR21*	350	ED, UTM, Proctitis
Danish Cancer Society	Breast, Head& Neck	Denmark	None	2004-unlimited	***ATM, TGFB1 XRCC1 XRCC3, SOD2, hHR21***	41	Fibrosis, Telangiectasia
	Breast	Israel	None	2005-2006	***ATM***	150	Telangiectasia, Fibrosis
Swiss Cancer League	Breast, Head & Neck	Switzerland	None	2005-2006	*ATM, TGFB1 XRCC1 XRCC3, SOD2, hHR21*	28	Telangiectasia, Fibrosis
COHORT	Breast	France& Switzerland	None	2005-2007	***ATM***	1012	Telangiectasia, Fibrosis (Concomitant Letrozole Therapy

[a]Department of Defense Breast Cancer Research Program
[b]Department of Defense Prostate Cancer Research Program
[c]Erectile Dysfunction
[d]Urinary Tract Morbidity
[e]American Cancer Society
[f]U.S. Veterans Affairs Administration