Heterozygosity for mutations in the ataxia telangiectasia gene is not a major cause of radiotherapy complications in breast cancer patients

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Summary Of patients being treated by radiotherapy for cancer, a small proportion develop marked long-term radiation damage. It is believed that this is due, at least in part, to intrinsic individual differences in radiosensitivity, but the underlying mechanism is unknown. Individuals affected by the recessive disease ataxia telangiectasia (AT) exhibit extreme sensitivity to ionizing radiation. Cells from such individuals are also radiosensitive in in vitro assays, and cells from AT heterozygotes are reported to show in vitro radiosensitivity at an intermediate level between homozygotes and control subjects. In order to examine the possibility that a defect in the *ATM* gene may account for a proportion of radiotherapy complications, 41 breast cancer patients developing marked changes in breast appearance after radiotherapy and 39 control subjects who showed no clinically detectable reaction after radiotherapy were screened for mutations in the *ATM* gene. One out of 41 cases showing adverse reactions was heterozygous for a mutation (insertion A at NT 898) that is predicted to generate a truncated protein of 251 amino acids. No truncating mutations were detected in the control subjects. On the basis of this result, the estimated percentage (95% confidence interval) of AT heterozygous patients in radiosensitive cases was 2.4% (0.1–12.9%) and in control subjects (0–9.0%). We conclude that *ATM* gene defects are not the major cause of radiotherapy complications in women with breast cancer.

Keywords: ataxia telangiectasia; *ATM*; radiation sensitivity; breast cancer

For most solid tumours. curative radiotherapy involves delivering a dose schedule at the limits of normal tissue tolerance. Most side-effects lead to moderate functional impairment. but occasionally these are severe and even life-threatening (Maher Committee. 1995). The severity of normal tissue reactions after a given course of radiotherapy varies widely from one patient to another. Severe reactions can often in part be explained by radiotherapy technique or by predisposing factors such as prior surgery. chemotherapy or diabetes. Nevertheless. even after allowing for known factors. considerable variation still exists. The clearest evidence for this is the work of Turesson et al (1989. 1990). They measured early and late manifestations of radiation skin damage under well-controlled conditions in breast cancer patients. some of whom have been followed up for over 10 years. A standard treatment protocol was found to produce very different degrees of telangiectasia. ranging from a barely detectable response to a severe reaction. Analysis of these clinical data by Tucker et al (1992) has suggested that variation in tolerance between patients is determined by differences in individual intrinsic radiosensitivity. even among patients who show no clinical symptoms of recognized radiosensitive syndromes. An understanding of the basis of these interpatient differences could lead to significant improvement in treatment by the individualization of the radiotherapy prescription.

Ataxia telangiectasia (AT) is an autosomal recessive disorder that is characterized by cerebellar ataxia. oculocutaneous telangiectasia and a predisposition to cancer (Boder and Sedgwick. 1958) Clinically. AT homozygotes exhibit marked hypersensitivity to ionizing radiation. and fibroblasts or lymphocytes from AT homozygotes are highly radiosensitive in various in vitro assays (Gotoff et al. 1967: Taylor et al. 1975: Weeks et al. 1991: Jorgensen and Shiloh. 1996). Although AT itself is a rare disease. it is estimated that approximately 1% of individuals in the general population are AT heterozygotes (Easton. 1994: Nagasawa et al. 1987). A number of in vitro studies have suggested that cells from AT heterozygotes may exhibit an intermediate level of radiosensitivity between AT homozygotes and controls (West et al. 1995). Moreover. cells from patients showing adverse normal tissue damage after radiotherapy have been shown to exhibit a degree of cellular radiosensitivity similar to that of AT heterozygotes (Johansen et al. 1996). Taken together these findings have led to the hypothesis that heterozygosity for AT may account for some of the radiation complications observed in clinical practice.

The AT gene (*ATM*) has recently been isolated (Savitsky et al. 1995). It is a large gene spanning approximately 200 kb of genomic DNA with a transcript size of approximately 10 kb encoding a predicted protein of 3056 amino acids. The mutations thus far discovered are highly heterogeneous. and are distributed throughout the entire extent of the gene. The majority are null mutations resulting in premature termination of translation (Byrd et al. 1996: Gilad et al. 1996). In this study. we examined the

Table 1 Treatment characteristics of 835 patients with post-operative baseline photographs

Radiotherapy to whole breast	50 Gy/25[a] 282	42.9 Gy/13[a] 270	39 Gy/13[a] 283	Totals 835
Radiotherapy to tumour bed (boost)				
Boost (non-randomized)	123	123	129	375
Boost (randomized)	79	74	78	231
No boost (randomized)	80	73	76	229
Treatment to axilla				
None	83	88	68	239
Radiotherapy (RT)	78	68	83	229
Surgery	103	91	113	307
RT + surgery	18	23	19	60
Adjuvant systemic therapy				
None	84	93	90	267
Tamoxifen	181	156	181	518
Chemotherapy (CT)	10	11	9	30
Tamoxifen + CT	7	10	3	20

[a]Fractions.

association between heterozygosity for *ATM* gene defects and the development of radiotherapy complications in breast cancer patients.

MATERIALS AND METHODS

Study population

Between January 1986 and July 1994. 915 patients were entered into a randomized trial comparing three fractionation regimens after breast-preserving surgery for early-stage operable breast cancer. All patients attended the Royal Marsden Hospital. Sutton. or the Gloucestershire Oncology Centre. Cheltenham. A total of 835/915 (91%) patients had baseline post-operative photographs of the breast. against which later radiation-induced changes scored from photographs were compared on an annual basis. The clinical and treatment characteristics of these 835 patients are summarized in Table 1. At the time of assessment. 735 of these had at least one follow-up photograph and made up the study sample.

Radiotherapy

The duration of whole-breast radiotherapy was 5 weeks in all arms. involving five treatments a fortnight for patients randomized to 13 fractions (3.0 Gy or 3.3 Gy per fraction) and five treatments per week for patients in the third arm (2.0 Gy per fraction). Patients were treated in a supine position and most patients were treated with 6-MV X-rays. The breast was encompassed by opposed tangential fields using 15–30° wedges as tissue compensators. Radiotherapy to the lymphatic pathways was included at the discretion of the clinician depending on disease stage and axillary surgery. An electron boost to the tumour bed of 14 Gy to the 90% isodose in seven daily fractions was given to all patients with cancer cells at the microscopic margins of resection. In patients with complete microscopic resection of the primary tumour. an option to randomize the boost (boost vs no boost) was offered with patient consent. A boost was otherwise given routinely.

Definition and assessment of end points

The primary end point of the trial. which was used in this analysis. relates to normal tissue responses in the breast as assessed by serial photographs. Frontal photographs of both breasts were taken after primary surgery and repeated annually for 5 years. All photographs were reviewed by three independent observers (two clinicians and one senior nurse) blind to patient identity. fractionation allocation and year of follow-up. Inclusion of the contralateral breast at each time point made it possible to distinguish radiotherapy effects from other time-related changes. e.g. weight gain. Changes in breast appearance caused by radiotherapy were scored on a three-point graded scale (none/minimal. 0: moderate. 1: marked. 2) based on change in breast size and/or shape. usually shrinkage. Inter- and intra-observer variability were monitored by comparing scores between observers. All discrepancies between observers were re-evaluated. Intra-observer variability was evaluated by assessing the reproducibility of scores for each observer by reassessing a random sample of photographs. Degree of agreement between scores was assessed using a weighted kappa statistic.

Case–control selection

Cases were defined as all individuals developing marked changes (grade 2) at any time between 1 and 5 years post radiotherapy or moderate changes (grade 1) scored for at least 3 years as assessed by clinical photographs. We identified 56 patients in these categories. 41 of whom were available for study. Control subjects were defined as individuals with 'no tissue reaction' (grade 0) at the same time since radiotherapy as the case experienced a reaction. We identified 39 control patients. matched as closely as possible for the factors listed in Table 2. Written informed consent for genetic testing was obtained from all patients (who remained alive) in the study.

Mutation detection

DNAs were isolated from peripheral blood leucocytes. All the individuals were screened for mutations using conformation sensitive gel electrophoresis (CSGE) (Ganguly et al. 1993) of polymerase

Table 2 Clinical factors matched as closely as possible in 41 cases with moderate or marked radiation damage and 39 control subjects without detectable radiation damage

Radiotherapy fractionation schedule (50, 43, 39 Gy)
Radiotherapy breast boost (yes, no)
Year of scoring a normal tissue response (1–5 years)
Location of treating hospital (Sutton, Cheltenham)
Breast size (small, medium, large)
Radiotherapy field separation (± 1 cm)
Width of tangential radiotherapy field to breast (± 1 cm)
Thickness of lung incorporated in tangential fields (± 0.5 cm)
Axillary radiotherapy (yes, no)
Tamoxifen (yes, no)
Adjuvant chemotherapy (yes, no)
Timing of chemotherapy in relation to radiotherapy (concurrent, sequential)

chain reaction (PCR) products covering the complete coding sequence and splice junctions of *ATM*. The primers used are shown in Table 3. For CSGE, both primers were radiolabelled using γ[^{32}P]ATP. Heteroduplexes were formed by heating the PCR products to 98°C for 10 min, holding at 60°C for 15 min and allowing to return to room temperature. Samples were electrophoresed through 6% polyacrylamide gels overnight at 4 W. Fragments showing an alteration in electrophoretic mobility were reamplified and directly sequenced, using the ABI 377 automated DNA sequencer and the ABI prism dye terminator cycle sequencing kit, with both forward and reverse primers.

RESULTS

Several sequence variants (summarized in Table 4) were observed in the course of the mutational screen of *ATM*. Of these, only one was predicted to generate a truncated protein. This mutation was an insertion of A at nucleotide (nt) 898 in exon 8 and was heterozygous. The predicted consequence is the production of a truncated protein including the N-terminal 251 amino acids, a product only 8% of the normal size. This variant was in a case with marked (grade 2) radiotherapy changes in breast appearance. No truncating mutations were detected in any of the 39 control subjects.

An additional radiosensitive case was heterozygous for a G→A transition at nt 4108 leading to substitution of Arg for Gly at amino acid 1306. This sequence variant was not found in the breast cancer control subjects or in 147 healthy women. Gly 1306 is conserved in mouse *ATM* (mouse *ATM* is 95% identical to human *ATM*) but is not within the kinase domain that shows substantial similarity to other members of this gene family. As 80–90% of *ATM* mutations result in truncated proteins, at present it is difficult to determine whether this is a rare innocuous polymorphism or a mutation deleterious to *ATM* function.

Three other sequence variants were observed in a single case but not in any of the 39 control subjects. Two of these are intronic, insertion T at nt 160–5 and G → A at nt 2438 + 80. Neither of these change consensual splice sequences and therefore are likely to be rare polymorphisms. The third alteration is a non-coding change, C → T at nt 7710 (Ala → Ala).

Seven sequence variants were detected in a single control but not in any of the 41 cases. These include: two intronic changes, G → T at nt 2088–39 and ins A at nt 3027 + 28: a variant in the 3' untranslated region, C → G at nt 9389; two non-coding changes, T → C at nt 5982 (Ala → Ala) and G → A at nt 7251 (Ala → Ala); and two missense coding variants G → A at nt 7572 (Arg → His) and C → T at nt 8683 (Arg → His).

The remainder of sequence variants was observed in both cases and control subjects and no substantial differences in heterozygote frequency (as ascertained from CSGE gels) between cases and control subjects were observed.

From these results the only sequence variant that is confidently predicted to alter *ATM* function is the heterozygous insertion of A at nucleotide 898 in exon 8.

DISCUSSION

A total of 80 patients (41 cases and 39 control subjects) selected from 735 evaluable women with early breast cancer randomized into a radiotherapy fractionation study were screened for mutations in *ATM*. One out of 41 cases showed a typical mutation that was predicted to generate a truncated protein (insertion A at nucleotide 898). This case had no other predisposing factors for radiation damage and developed marked breast shrinkage with moderate cutaneous telangiectasia following 39 Gy in 13 fractions (approximately equivalent to 46 Gy in 23 fractions of 2.0 Gy). No truncating mutations were detected in any of the 39 control subjects. It is likely that the mutational screening technique used will miss a minority of mutations, particularly of single base substitutions and large genomic rearrangements, and therefore the numbers reported may be underestimates. Nevertheless, the results suggest that *ATM* mutations are unlikely to account for a substantial proportion of patients with dose-limiting complications of radiotherapy (although a small contribution cannot be excluded). These results are consistent with previous reports of three AT heterozygotes who had radiotherapy for breast cancer without unusual reactions (Ramsay et al, 1996; Fitzgerald et al, 1997) and 16 breast cancer cases showing radiotherapy complications in whom *ATM* mutations were not detected (Appleby et al, 1997).

From studies of relatives of AT patients, there is evidence that AT heterozygosity may be associated with an increased frequency of certain types of cancer, particularly breast carcinoma (Swift et al, 1987, 1991; Pippard et al, 1988). Additional evidence supporting this hypothesis has recently been obtained by genetic linkage analyses of families of AT cases using markers in the vicinity of *ATM* on chromosome 11q (Athma et al, 1996). However, direct examination by mutational screening of the *ATM* gene revealed mutations in 2/401 women with breast cancer compared with 2/202 control subjects (Fitzgerald et al, 1997). Whereas these data do not exclude a role for *ATM* as a low-penetrance breast cancer susceptibility gene (Bishop and Hopper, 1997), they do not lend strong support either. Although the present study is not a formal test of this hypothesis because there is no matched control group and the numbers are small, detection of a single AT heterozygote in 80 breast cancer cases does not add further weight to the notion that *ATM* is a low-penetrance breast cancer susceptibility gene.

Radiotherapy-induced breast shrinkage and distortion changes in a proportion of women after radiotherapy are progressive, permanent and of clinical relevance to the patient. They are also clearly related to radiotherapy dose. In the clinical trial from which these patients are drawn, a 10% difference in randomized dose (42.9 Gy in 13 fractions vs 39 Gy in 13 fractions) was associated with roughly a twofold difference in the chance of breast shrinkage (Owen et al, 1994). It has been shown in this study that testing for AT heterozygosity does not appear to offer a worthwhile approach for the identification of the radiosensitive subgroup of breast cancer patients and the search for the genetic loci responsible should continue.

Table 3 Oligonucleotide primers for amplification of individual *ATM* exons

Exon	Nucleotide sequence (5'–3')	Size (bp)	Nucleotide sequence (5'–3')
2	CTAGCCCTTTTTTTGATTGGC	310	TGCTCATTCACTGATAGATGCA
3	CCTTTGACCAGAATGTGCCT	371	ATCTCGAATCAGGCGCTTAA
4	ATCTGCTTATCTGCTGCCGT	385	ATGCCAAATTCATATGCAAGG
5	GCTCTTTGTGATGGCATGAA	303	AAAAAAAAAAAAAACTCACGCG
6	AGTAGTTGCCATTCCAAGTGTC	345	AACTGTCAGGTCACTTGGGG
7	CTGCGACCTGGCTCTTAAAC	469	ATGGTCTTGCAAGATCAAAAGT
8	AGTTTGTACAGTTTGTTCCCCC	414	ATCAACCAGAGAAATCCAGAGG
9	GGTGTCTTCTAACGCTGATGC	342	CCCAAAATGCCCAGTTTAAA
10	GATACGAGATCGTGCTGTTCC	350	GGATTCCACTGAAAGTTTTCTG
11	TGTTAATGTGATGGAATAGTTT	501	AATGATCAGGGATATGTGAGTG
12	AAAGTCTTTGCCCCTCCAAT	339	AAATAAAGCCATCTGGCATCA
13	TTCTTTACATGGCTTTTGGTCT	238	TAAGATGCAGCTACTACCCAGC
14	TCAAAGTCCGAAGAAGAGAAGC	497	CCACCATCCTTGCTGTTTTT
15	AGCTATCCAGGATATGCCACC	499	TGCATGCTCTGCATCATGTA
16	TAAAAAGCAATACTAAACTA	280	CCAGGAGGTCAAGGCTACAATG
17	TCTGCCGAGAATAATTGTTTTT	248	TGTTGTGAGATGCATCCTTATT
18	TGACTACAGCATGCTCCTGC	306	CAATGAGGCCTCTTATACTGCC
19	ATATGGCTGTTGTGCCCTTC	347	TCAAAGACACCATGTGATTCTT
20	CGGCCTATGTTTATATACTT	226	GCTTAACAGAACACATCAGTT
21	TGTTCTTGAACTTCTGAAACCA	346	TGCATTCGTATCCACAGATAGC
22	GCAAGGTGAGTATGTTGGCA	349	TCAGCCTACGGGAAAAGAAC
23	GAATGGCCCTAGTAAATTGCC	335	TCTACTGCCATCTGCAGCAT
24	ATGCTTTGGAAAGTAGGGTTTG	250	TATGGGATATTCATAGCAAGCA
25	AAAAATGTGGAGTTCAGTTGGG	349	TGCCACTCAGAAAATCTAGCTT
26	TGTGTCAGATACTGTGCCAGTT	434	GTTGCTGGTGAGGGGACTT
27	GCTGATGGTATTAAAACAGTTT	396	GTTATATCTCATATCATTCAGG
28	TGCCTTTTGAGCTGTCTTGA	339	ATTACCTCAATTCAAAGGTGGC
29	AAATGGTTTTTGAATTTGGGG	452	GTGTCACGAGATTCTGTTCTCA
30	GTTTATTTTCTAGGATTCCTATC	299	TATGTTATTTACCTTTGGTTGA
31	ATGCTGAACAAAAGGACTTCTG	487	TGGACTACCTCTCCACTTCAGC
32	TTCGCAACGTTATGGTGGTAT	525	CAGGCTGGTCTTGAACTCC
33	TTTCACAGGCTTAACCAATACG	249	TCCCAAAATATTCTTTCCAAAA
34	CAAAAAGTGTTGTCTTCATGCT	203	TATGTGATCCGCAGTTGACTG
35	TTGACAACATTGGTGTGTAACG	234	GCCACATCCCCCTATGTTAA
36	ATGTATGATCTCTTACCTATGA	315	GCTTTAGTTACTGAGAATATCT
37	TTTGAAATTTTTTCAGTGGAGG	304	TTTAACAGTCATGACCCACAGC
38	GGAAAGGTACAATGATTTCCAC	350	AACAACAGTTTGAGTGGGGG
39	CGGGGCATGAAAATTTTAAG	336	TGGGATTCCATCTTAAATCCA
40	CTGGGACTGAGGGGAGATA	200	CATGTTAAAATTCAGCCGATAGTT
41	GGGGAAATGTGGTTTTTGG	350	ACCCTTATTGAGACAATGCCA
42	CAGGAGCTTCCAAATAGTATGT	225	GGCATCTGTACAGTGTCTATAA
43	CAGTTCAAACTCGTGTTGTTTG	350	AGCTTTGGGTTTTACACACACA
44	GGAGCCAGATAGTTTGTATGGC	345	TCTGGCTGTGTAAATATCCACC
45	TCTCTGGTTTTCTGTTGATATC	270	CAGTTGTTGTTTAGAATGAGGA
46	TTTGTCCTTTGGTGAAGCTATT	238	TTCAGAAAAGAAGCCATGACA
47	ATTTCCCTGAAAACCTCTTCTT	227	GGTAACAGAAAAGCTGCACTTT
48	CCGCATAGCATTTTGTAGGT	500	CCTCAGGCTTTCTGTTTTTTAA
49	GGTAGNTGCTGCTTTCATTATT	362	TTGCTAATTTCAAGGCTCTAAT
50	GGGCAGTTGGGTACAGTCAT	344	GTAACAATGTTTCACTCCACCC
51	CGTGGGTTGGACAAGTTTG	492	TAAGCCGACCTTTAGAGCTCC
52	TTTCCCTGGGATAAAAACCC	401	TACACGATTCCTGACATCAAGG
53	CCACTTGTGCTAATAGAGGAGC	320	TTCCATTTCTTAGAGGGAATGG
54	TGCAGGCATACACGCTCTAC	402	CCAGCCTTGAACCGATTTTA
55	AAAGGCACCTAAGTCATTGACG	489	GGGAATGTTGAAGCCATCAG
56	CTTGACCTTCAATGCTGTTCC	249	TGCCAATATTTAGCCAATTTTG
57	CACATCGCATTTGTTTCTCTG	340	CAAAATCCCAAATAAAGCAGAA
58	ATTGGTTTGAGTGCCCTTTG	299	ATTATGAATATGGGCATGAGCC
59	AGGTCAACGGATCATCAAATG	285	AGCTGTCAGCTTTAATAAGCCA
60	ATCCTGTTCATCTTTATTGCCC	339	CAAAAATAAAACCTGCCAAACA
61	CTCAACATGGCCGGTTATG	282	CAAACAACATTCCATGATGACC
62	TGAGGAAGGCAGCCAGAG	350	GTGCAAAGAACCATGCCC
63	TTGACAACATTGGTGTGTAACG	234	GCCACATCCCCCTATGTTAA
64	TCCCCCATCAACTACCATGT	324	GAACAGTTTAAAGGCCTTGGG
65	CAAGGCCTTTAAACTGTTCACC	309	TTGGCAGGTTAAAAATAAAGGC

Table 4 Summary of the *AT* sequence variants detected. Numbering is according to the cDNA sequence deposited in Genbank as U33841. Intronic variants are described as ± the number of nucleotides from the nearest exonic base in the cDNA sequence

Exon (E)/intron (I)	Location	AA change	No. of heterozygotes out of the 41 cases	No. of heterozygotes out of the 39 controls
I2	160 −5 insT	None	1	0
E3	A 201 G	Val 3 Val	15	21
I3	261−41 insAA	None	11	14
E4	C 335 G	Ser 48 Cys	3	1
E8	898 insA	Stop at codon 251	1	0
E8	C 924 T	Val 244 Val	1	1
I13	T 2088 −56 G	None	1	1
I13	G 2088 −39 T	None	0	1
I15	G 2438 +80 A	None	1	0
E18	T 2761 C	Phe 857 Leu	1	1
I19	3027 +28 insA	None	0	1
I21	T 3267 −80 C	None	12	16
E23	C 3350 G	Pro 1053 Arg	4	4
I23	3473 −13 delT	None	6	5
E27	G 4108 A	Gly 1306 Arg	1	0
E31	C 4767 T	Pro 1525 Pro	4	1
I37	T 5686 −8 C	None	5	6
E38	G 5746 A	Asp 1852 Asn	2	2
E40	T 5982 C	Ala 1930 Ala	0	1
E40	G 6010 C	Val 1940 Leu	3	1
I47	6997 −57 insATT	None	12	19
E49	G 7251 A	Ala 2353 Ala	0	1
E51	G 7572 A	Arg 2460 His	0	1
E52	C 7710 T	Ala 2506 Ala	1	0
E59	C 8683 T	Arg 2830 His	0	1
I62	A 9039 +60 G	None	10	17
E64	C 9389 G	None (3′ untranslated)	0	1

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