Int. J. Radiation Oncology Biol. Phys., Vol. 44, No. 5, pp. 981–988, 1999
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0360-3016/99/$–see front matter

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PII S0360-3016(99)00108-X

CLINICAL INVESTIGATION **Normal Tissue**

SEQUENCE ANALYSIS OF THE *ATM* GENE IN 20 PATIENTS WITH RTOG GRADE 3 OR 4 ACUTE AND/OR LATE TISSUE RADIATION SIDE EFFECTS

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Purpose: Patients with ataxia-telangiectasia (A-T) show greatly increased radiation sensitivity and cancer predisposition. Family studies imply that the otherwise clinically silent heterozygotes of this autosomal recessive disease run a 3.5 to 3.8 higher risk of developing cancer. *In vitro* studies suggest moderately increased cellular radiation sensitivity of A-T carriers. They may also show elevated clinical radiosensitivity. We retrospectively examined patients who presented with severe adverse reactions during or after standard radiation treatment for mutations in the gene responsible for A-T, *ATM*, considering a potential means of future identification of radiosensitive individuals prospectively to adjust dosage schedules.

Material and Methods: We selected 20 cancer patients (breast, 11; rectum, 2; ENT, 2; bladder, 1; prostate, 1; anus, 1; astrocytoma, 1; Hodgkins lymphoma, 1) with Grade 3 to 4 (RTOG) acute and/or late tissue radiation side effects by reaction severity. DNA from the peripheral blood of patients was isolated. All 66 exons and adjacent intron regions of the *ATM* gene were PCR-amplified and examined for mutations by a combination of agarose gel electrophoresis, single-stranded conformational polymorphism (SSCP) analysis, and exon-scanning direct sequencing.

Results: Only 2 of the patients revealed altogether four heteroallelic sequence variants. The latter included two single-base deletions in different introns, a single-base change causing an amino acid substitution in an exon, and a large insertion in another intron. Both the single-base deletions and the single-base change represent known polymorphisms. The large insertion was an *Alu* repeat, shown not to give rise to altered gene product.

Conclusions: Despite high technical efforts, no unequivocal *ATM* mutation was detected. Nevertheless, extension of similar studies to larger and differently composed cohorts of patients suffering severe adverse effects of radiotherapy, and application of new technologies for mutation detection may be worthwhile to assess the definite prevalence of significant *ATM* mutations within the group of radiotherapy patients with adverse reactions. To date, it must be recognized that our present results do not suggest that heterozygous *ATM* mutations are involved in clinically observed radiosensitivity but, rather, invoke different genetic predisposition or so far unknown exogenous factors. **© 1999 Elsevier Science Inc.**

Ataxia-telangiectasia; ATM gene; Radiotherapy, Radiation sensitivity, Breast cancer.

INTRODUCTION

In radiation oncology, radiation dosage protocols are influenced by both the anticipated healthy tissue tolerance and the probability of tumor control. In a small percentage of patients treated by radiation doses that usually are well tolerated by the healthy tissues within the irradiated field or volume, unexpected severe acute and/or late adverse effects are observed (1). In the past 2 decades, the development of predictive methods to determine the degree of radiosensitvity of both tumor and healthy tissues has become of major interest in radiobiological research (2, 3). Ideally, dose prescriptions should be individualized pretherapeutically, dependent on the *in vitro* test results with the objective to prevent severe side effects in the small group of "overreactors" and to reach better local tumor control in the larger group of "normal reactors." Cellular *in vitro* test systems, such as the colony-forming assay or micronucleus induction, appear to be capable of discriminating between "strong" and "normal" *in vivo* reactors (4, 5), and show statistical correlation for the extent of observed clinical and cellular radiation sensitivity of patients, in particular those

This work was presented, in abbreviated form, as an oral contribution at the 40th Annual ASTRO Meeting, October 25–29, 1998, in Phoenix, AZ.

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Acknowledgements—This work was supported by a grant Op 71/1-1 from the Deutsche Forschungsgemeinschaft, Bonn, Germany. We are greatly indebted to Mrs. Gitta Emmert and Mrs. Renate Schakowski for excellent technical help.

Accepted for publication 15 March 1999.

with genetic disorders manifesting enhanced radiosensitivity (6–9). However, detecting slight differences in radiosensitivity using short-term cultures, for most lymphocytes, is neither practical nor reliable to assess A-T heterozygote status and, moreover, these global tests do not discern the type of risk factor.

In this respect, by far the most interesting inherited disease is ataxia-telangiectasia (A-T). A-T is an autosomal recessive genetic disorder. Homozygous individuals are found at a minimum frequency of 1:100 000 in the Western hemisphere (10, 11); clusters in Turkey, Italy, Israel, and elsewhere have been observed (12, 13). A-T patients show a progressive neurological disorder beginning in infancy, predominantly cerebellar ataxia, and generally become wheelchair-bound in the second decade of life (14). They also develop telangiectasia of the conjunctiva and sun-exposed skin areas. Other characteristic clinical and laboratory features observed in those affected include thymus and other organ dysplasia, immunodeficiency, frequent bronchopulmonary infections, hypersensitivity to ionizing radiation, chromosomal instability, and cancer predisposition (14–17). Enhanced radiation response is most consistent and has made A-T the classical model of a human disease of hypersensitivity to ionizing radiation (18).

Individuals who are heterozygous for A-T have been estimated to comprise about 1 to 2 % of the population (19), and interest has focused more on this much larger group than on that of those affected. Epidemiological studies imply that the gene is not entirely recessive because carriers of *ATM* gene mutations are believed to manifest at least two of the disease characteristics (20). Swift *et al.* (19) and Morell *et al.* (21) provided support for the idea that A-T heterozygotes are more prone to develop malignancies, especially cancer of the breast. This relative heterozygote risk finally was calculated to be 3.8-fold elevated in males and 3.5-fold in females for cancer of all types, and the risk of breast cancer in women to be 5.1-fold elevated compared to that in the general population (22). A-T carriers are suspected of running an increased risk of cancer being induced by regular mammography screening. They also have been claimed to represent a large proportion of radiotherapy patients with clinically enhanced radiation sensitivity (23). This latter idea gains support by mildly increased radiation sensitivity observed in cultured cells of different types from A-T heterozygotes (24–29) paralleling, at a lower level, the findings in cells from those affected by A-T who show high rates of chromosomal breaks and other indicators characteristic of elevated *in vitro* sensitivity to ionizing radiation (18, 30, 31).

The recent isolation and characterization of the *ATM* gene (32, 33) renders testing for mutations feasible in distinct groups of patients suspected of A-T heterozygous status. In this communication, we report *ATM* mutation screening in 20 patients who received standard radiation treatment and showed severe acute and/or late radiation side reactions.

METHODS AND MATERIALS

Patients

Tissue reactions were graded according to the RTOG acute and late radiation morbidity scoring criteria (34). Cancer patients were selected for participation in this study after radiation therapy had been initiated or completed, solely by the severity of acute or late, or both, tissue reactions due to irradiation, irrespective of the type of tumor (Table 1). Blood samples were obtained following informed consent of the patients.

Study outline

The present study was designed to be based on exon scanning automated sequencing. In 18 (90%) of the 20 patients (Patients 1 to 18), all *ATM* exons and adjacent intron regions were sequenced following agarose gel and single-stranded conformational polymorphism (SSCP) analyses. The remaining 2 patients (Patients 19 and 20) were included additionally and studied at the same time. However, the gels of the autosequencer would not fit more than 18 patient samples of each exon and, thus, for the rest, SSCP analysis was followed by manual sequencing only of the exons with fragment gel shifts.

PCR amplification

Genomic DNA was extracted from EDTA blood (35). A set of 68 PCR primer pairs were used to PCR-amplify all 66 exons and adjacent intron regions of the *ATM* gene from each patient sample. PCR amplifications were performed in 50-μl volumes containing 100 ng of genomic DNA, 0.2 mM of each dNTP, and 2 U of Taq DNA polymerase in the reaction buffer supplied by the manufacturer (Boehringer, Mannheim, Germany). Primer concentrations were 0.5 μM each. All primers and PCR conditions were exactly those specified by the German *ATM* Consortium (36). One-step precipitation of the templates with polyethylene glycol was used to remove excess primers, nucleotides, and most of the low-molecular weight truncated amplification fragments (37). Clean PCR products were stable at 4°C. Aliquots of the amplification products were examined by agarose gel electrophoresis for size, yield of DNA, and purity to check specificity of the PCR.

SSCP analysis

For pre-examinination using SSCPs (38), 2-μl aliquots of the original PCR product were used in each sample. These were denatured for 2 min at 94°C, allowed to reassemble for 3 min at room temperature, and finally quenched on ice. For electrophoresis in a Multiphor II chamber (Pharmacia, Freiburg, Germany), a manufactured kit of SSCP gels, buffers, and soaking solutions (ETC, Kirchentellinsfurth, Germany) was employed. Gels were stained using a DNA silver-staining kit (Pharmacia).

ATM sequence analysis

Direct cycle sequencing was performed on both strands using dye-terminator chemistry (Taq DyeDeoxy™ Termi-

Table 1. Patient and radiation therapy information

No.	Gender	Age (years)	Type of tumor	Irradiated area	Type,* quality, and energy of radiation	Single/total dose + boost (Gy)	Side effect in organ/tissue	Acute tissue reaction RTOG grade	Late tissue reaction RTOG grade
1	F	44	Mamma ca	Breast	P/5 MV, E/8 MeV	2/50 + 10	Skin, subcut. tissue	3, –	3, 2
2	M	50	Mamma ca	Thoracic wall	E/5 MeV	2/44	Skin	4	3
3	F	53	Mamma ca	Thoracic wall	P/10 MV, E/10 MeV	2.0 and 2.5/59.5	Skin, subcut. tissue	3, –	3, 2
4	M	75	Prostate ca	Sacral region	P/8 MV	2.5/30	Skin, subcut. tissue	–, –	2, 3
5	F	51	Mamma ca	Breast	P/8 MV, E/8 MeV	2/46 + 14	Skin	3	1
6	F	45	Mamma ca	Breast	P/5 MV, E/8 MeV	2/50 + 10	Skin	3	2
7	F	56	Mamma ca	Breast	P/5 MV, E/10 MeV	2/48 + 12	Skin, subcut. tissue	3, –	3, 2
8	F	69	Mamma ca	Thoracic wall	P/5 MV, E/8 and 12 MeV	2/50 + 8	Skin	3	Expired
9	F	72	Mamma ca	Breast	P/5 MV, E/10 MeV	2.0 and 2.3/49.6 + 10	Skin	3	3
10	F	74	Mamma ca	Thoracic wall	P/5 MV	2/46	Skin, lung	1, 3	–, 3
11	F	29	Astrozytoma	Brain	P/5 MV	2/60	Skin, CNS	1, –	–, 4
12	F	64	ENT ca	Neck	P/5 MV	1.8 + 1.5/69.3†	Skin, mucosal/subcut. tissue	4, 3, –	2, 2, 3
13	M	56	Rectum ca	Pelvis	P/8 MV	1.8/25.2	Lower GI tract	4	1
14	F	65	Mamma ca	Breast	P/5 MV, E/14 MeV	2/54	Skin	3	n.d.‡
15	M	69	ENT ca	Neck	P/5 MV	1.8 + 1.5/69.9†	Skin, mucosa, pharynx and esophagus	3, 3, 3	2, 3, 3
16	F	79	Mamma ca	Thoracic wall	P/8 MV, E/8 and 12 MeV	2/50	Skin, subcut. tissue, lung	2, –, 3	2, 2, 2
17	F	52	Hodgkin	Thorax, neck, abdomen	P/8 MV, P/8 MV, P/16 MV	2/30, 2/40, 1.8/30.6	Skin, salivary glands, heart	2, 2, –	2, –, 3
18	F	30	Anus ca	Pelvis	P/8 MV	1.8/54	Skin, mucosa	3, 3	3, 3
19	F	81	Bladder ca	Pelvis	P/8 MV	2/40	Skin	3	–
20	F	69	Rectum ca	Pelvis	P/10 MV	1.8/46.8	Lower GI tract	4	–

* P = Photon; E = Electron.
† Including Boost.
‡ n.d. = not determined.

nator Cycle Sequencing Kit, Perkin Elmer, Perkin Elmer, Langen, Germany). Whenever possible, the same primers as for PCR also were used for sequencing. Other sequencing primers were the same as reported by the German *ATM* Consortium (36). Cycle sequencing was done in a total volume of 15 μl, with an initial denaturing step for 60 s at 95°C followed by 25 cycles of amplification with denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and chain elongation for 4 min at 60°C. Samples were precipitated in a microtiter plate using an 1:4 (v/v) mixture of 7.5 M ammonium acetate and 96% ethanol in a volume of 130 μl. Pellets were washed twice with 200 μl of 70% ethanol and dried in a vacuum centrifuge. For automated sequencing, each sample was dissolved in 2 μl of formamide buffer. Sequence information was generated and filed using ABI 377 automated sequencers (Perkin Elmer). The data was assembled, compared to a backbone of reference sequences derived from genomic *ATM* clones, and edited twice using the XGAP program (39). Manual sequencing was performed using ^{33}P radioactive labeling in the Sequenase 2.0 Kit (USB, Braunschweig, Germany).

Reverse transcription—PCR

Total RNA was prepared from lymphoid cell lines (Micro RNA Isolation Kit, Stratagene, Amsterdam, The Netherlands) and reverse transcribed into cDNA using SuperScript II RNase H$^-$ Reverse Transcriptase (GibcoBRL, Karlsruhe, Germany). For subsequent PCR, we designed a set of 12 primer pairs corresponding to overlapping regions of the *ATM* cDNA, each spanning several exons. Forward primers included a 5′ T7 promotor sequence for transcription initiation and a Kozak consensus sequence for translation initiation. Reverse primers included a 5′ stop codon for translation termination. To examine cDNA from Patient No. 7, the PCR fragment spanning *ATM* cDNA nucleotide positions 7846 to 8929 was used.

Protein truncation assay

An aliquot of 100 ng of RT-PCR product was used directly as the template in a coupled transcription-translation reaction using rabbit reticulocyte lysate (TNT® T7 Quick Coupled Transcription/Translation System, Promega, Mannheim, Germany). The reaction was performed in a total volume of 12.5 μl using 6 μCi of ^{35}S-methionine (Amersham, Braunschweig, Germany). Translation products were separated on a 10% discontinous SDS-PAGE gel in a Mini-Protean II chamber (Bio-Rad, Munich, Germany) at 200 V for 1.5 h. Gels were fixed, dried, and exposed to x-ray film.

RESULTS

A total of four sequence variants in the *ATM* gene were detected in these patients. The sequence aberrations included two different single-base deletions, a single-base substitution, and a large insertion, all of which were heteroallelic and occurred in only 2 patients. Patient No. 7, with breast cancer and fractionated local irradiation amounting to a dose of altogether 60 Gy, showed Grade 3 acute and late skin reactions. SSCP analysis of the PCR product amplified from exon 25 along with the adjacent regions of introns 24 and 25 revealed band shift. Direct fluorescent autosequencing of this PCR fragment detected a sequence variant in intron 24, and the deletion of a T at position 3285–9 on one allele, whereas the other allele had retained this T. In the same patient, agarose gel electrophoresis of the PCR product from exon 56 along with the adjacent regions of introns 55 and 56 showed two types of fragments, in contrast to the expected single band. The two bands suggested similar amounts of DNA, but had different sizes, one being more than 300 bp larger than the other; the smaller apparently representing normal PCR product of 253 bp (Fig. 1). A corresponding finding was seen on SSCP analysis that demonstrated additional bands of slow mobility above the normal single-strand region and reduced amounts of common normal bands for this fragment (Fig. 2). The result of these studies was compatible with a large insertion on one allele. In fact, direct fluorescent autosequencing confirmed the presence of a 297-bp insertion at position 7927+23 in intron 56, in contrast to normal *ATM* sequence on the other allele. This insertion represented an *Alu* repeat flanked by an 11-bp direct repeat at its 5′ end. To examine if the insertion might cause aberrant splicing, resulting in splicing-in inserted sequence and/or disrupting proper splicing at the neighboring normal splice sites, RNA from this patient was reverse transcribed. PCR product of the region spanning *ATM* cDNA nucleotide positions 7846 to 8929 was amplified and studied in a coupled transcription-translation system. Normal protein fragment of expected 42 kDa and several minor bands were detected in Patient No. 7 that she shared, in similar fashion, with 2 other radiosensitive patients and 1 healthy control (Fig. 3). This finding suggests that the insertion is functionally irrelevant.

In Patient No. 10 with breast cancer, treated with fractionated irradation amounting to a cumulative dose of 46 Gy and subsequent Grade 3 pneumonitis, exon scanning auto-

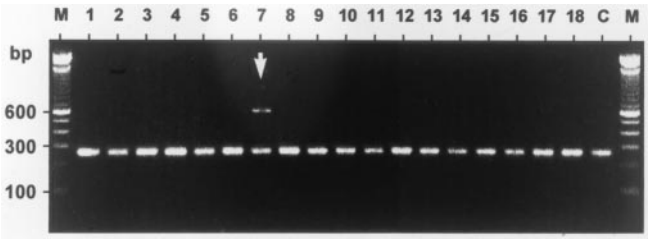

Fig. 1. Analysis of the PCR product of exon 56, including adjacent regions from intron 55 and 56, amplified from genomic DNA of 18 patients with severe side effects to radiation therapy. Agarose gel electrophoresis demonstrates normal 253-bp size of this amplification product (36) in Patients 1 to 6 and 8 to 18 (lanes 1 to 6 and 8 to 18, respectively) and in a normal Control (lane C). In Patient No. 7 (lane 7), an additional band of about 600 bp is detected (arrow), consistent with a heteroallelic insertion of 297+11 bp, as revealed by sequence analysis. A 100-bp ladder as a molecular size marker is shown in lanes M; sizes are given in the left margin.

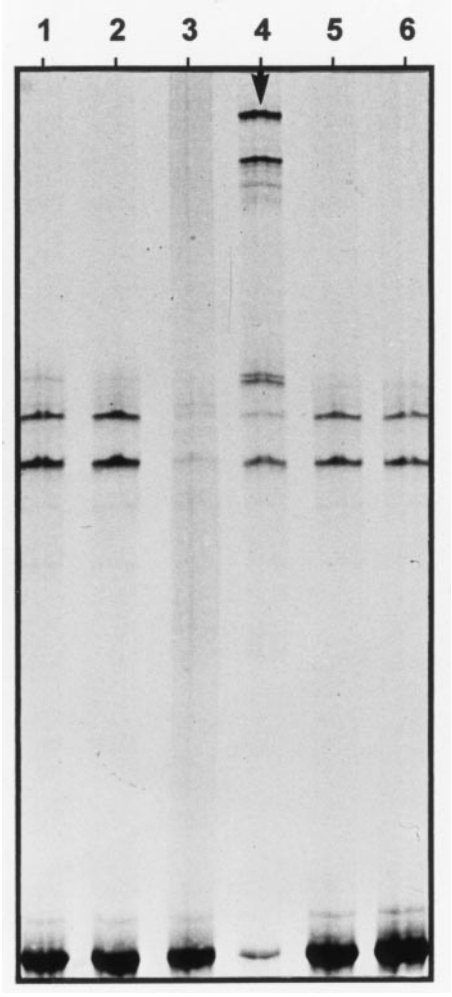

Fig. 2. Analysis of silver-stained, de- and renatured SSCP samples from PCR products of exon 56, including adjacent regions of the introns 55 and 56. On polyacrylamide gel electrophoresis, Patient No. 7 with severe side effects to radiation therapy (lane 4) shows several additional bands above the normal single-strand region (arrow) representing fragments of slower mobility, and reduced amounts of normal single- and double-strand bands as defined by normal Controls (lanes 1 and 2). Other patient samples (lanes 3, 5, and 6) conform to the control samples (lanes 1 and 2).

mated sequencing detected a C to G transversion located in exon 24 at nucleotide position 3161. This base exchange, again, was present only on one allele aside from normal

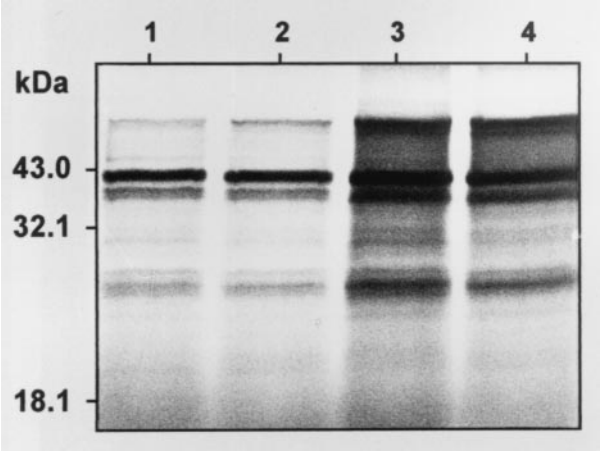

Fig. 3. Autoradiogram of the protein truncation assay representing *in vitro* transcription and translation of a PCR fragment comprising *ATM* cDNA nucleotides 7846 to 8929. Protein fragments from Patient No. 7 with the insertion 7927+23ins*Alu* in intron 56 (lane 1) include the expected major 42 kDa and other bands, and show the same pattern as those of other patients (lanes 2 and 3) and a normal Control (lane 4). Note that the concentrations are slightly different.

sequence on the other. This finding was confirmed on manual radioactive sequencing of the region (Fig. 4). This base transversion predicts an amino acid substitution of proline with arginine at codon 1054. In the same patient, direct fluorescent autosequencing of the PCR product from exon 26, including adjacent regions of the introns 25 and 26, detected a second sequence variant, namely the deletion of an A in intron 25. This single-base deletion was located at nucleotide position 3403–15 and was present on one allele, with the normal sequence observed on the other allele. All sequence variants detected are compiled in Table 2.

DISCUSSION

Predictive assays in radiobiology are aimed at defining highly radiosensitive patients pretherapeutically. Appropriate *in vitro* systems to determine intrinsic cellular radiosensitivity have been developed. Their possible clinical impact, however, is the subject of ongoing controversy (40). Genotypic characterization of patients prone to severe adverse

Table 2. *ATM* sequence variants identified in 20 patients with severe side effects to radiation therapy

Patient No.	ATM location	Variant designation*	Allele frequency†	Amino acid substitution
7	Intron 24	3285–9delT	0.18	none
7	Intron 56	7927 + 23insAlu	unknown	none
10	Exon 24	3161C→G	0.07	P1054R
10	Intron 25	3403–15delA	0.37	none

* Variants have been termed according to the nomenclature of Beaudet and Tsui 48. Nucleotide numbering follows the cDNA sequence reported by Savitsky et al. (32). (GenBank Accession No. U33841), with nucleotide Position 1 assigned to the first nucleotide of the ATG translation initiation codon in exon 4. Exon-intron boundaries and numbers are as defined by Uziel *et al.* (33).

†Allele frequencies are those reported by the German ATM Consortium (36).

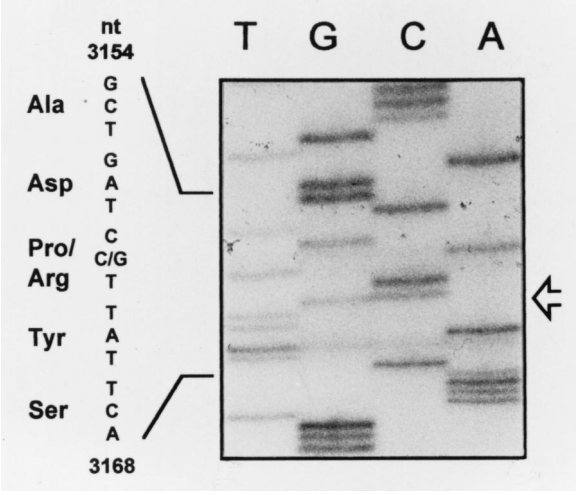

Fig. 4. Partial sequence of PCR amplified *ATM* exon 24 from genomic DNA of Patient No. 10. On the sense strand, a C to G transversion is shown on one allele, in addition to the normal allele with the C in cDNA position 3161 (arrow), resulting in heterozygosity for the amino acid substitution of proline with arginine. Strand orientation, base and amino acid sequence are depicted in the left margin.

radiation side reactions is a different approach. Appleby *et al.* (41) discussed this issue in view of the literature and calculated a theoretical proportion, or probability, in breast cancer patients treated with radiotherapy who develop severe adverse sequelae of radiation, of 80% to be A-T carriers. Thus, because heterozygosity for A-T is suspected of causing clinically increased radiation sensitivity, we screened unusually radiosensitive patients for mutations in the *ATM* gene.

No *ATM* gene mutations, specifically no A-T carriers, were found. Using a very similar technique, 46 different *ATM* mutations have been identified in 66 A-T patients (36). In the patients with severe side reactions to radiotherapy in the present study, we detected a total of four sequence variants, including two single-base deletions in different introns, a single-base substitution in an exon leading to an amino acid substitution, and a large insertion in another intron. The two single-base deletions (3285–9delT and 3403–15delA) do not affect any sequences known to be involved in splicing. Thus, it is probably reasonable to conclude that these changes do not affect *ATM* function, even though no attempt was made to look at splicing. Moreover, these two single-base deletions have previously been found in the screening of about 100 control chromosomes at rates of 0.18 and 0.37, respectively, and, thus, were classified as polymorphisms (36). Another change in the *ATM* gene detected in the present study (3161C→G) results in a nonconservative amino acid substitution. This base change, causing the amino acid substitution P1054R, was first reported by Vorechovsky *et al*. (42) as a rare polymorphism based on its occurrence at an allele frequency of 2.3% in a total of 224 control chromosomes in European Caucasians. Subsequently, it was found in similar numbers in 4 (3.2%) of 126 chromosomes in control females and in 4 (2.3%) of 176 chromosomes in patients with familial cancer, also emphasizing its nature of a functionally nonrelevant change (polymorphism) (43). Finally, this same change has independently been reported as a probable polymorphism or neutral variant, because it was found in 7% of 100 control chromosomes of anonymous donors (36). Formally, it cannot be ruled out that the 3161C→G base change could represent a frequent mutation allele, but the current view suggests the opposite. The last change reported in the present study is an *Alu* insertion in intron 56. The same insertion had previously been found in 1 of 125 breast cancer patients, but not in control alleles (Dr. B.H.F. Weber, personal communication). Large insertions in introns are known to cause disease in other genes by disrupting normal splicing, although type and location of the present insertion would rather argue against such an idea. To resolve this issue, RNA/cDNA from the patient was used for *in vitro* transcription/translation studies in the protein truncation assay including the *ATM* region spanning this insertion. As the result, the *Alu* insertion in intron 56 was demonstrated not to affect normal splicing and not to give rise to an abnormal gene product as compared to a healthy control and other radiosensitive patients.

Finding no heterozygous *ATM* carriers among the 20 highly radiosensitive cancer patients is consistent with recently published results from three other studies in patients with enhanced radiation reactions. No mutations were detected in 23 patients (breast cancer, 16; other cancers, 7) with considerably increased, predominantly early adverse reactions to radiotherapy in a study by Appleby *et al.* (41). These authors employed restriction endonuclease fingerprinting of *ATM* fragments with mutation sites described earlier in A-T patients. Also, no mutations were detected in 15 radiosensitive breast cancer patients by Ramsey *et al.* (44) using the protein truncation assay. With this method, they found mutations in 11 of 14 known A-T heterozygotes used as a group of positive controls. Other data bearing on this issue comes from a study by FitzGerald *et al.* (45), who screened 401 early-onset breast cancer patients for *ATM* mutations. Among these were 2 women with adverse skin reactions after radiotherapy severe enough to warrant interruption of treatment. Neither carried an *ATM* mutation. Only Hall *et al.* (46) claim in a recent preliminary report that they identified three significant *ATM* mutations in a group of 17 prostate cancer patients with severe late responses to radiation therapy, and calculated a proportion of 17.6% of these patients to be heterozygous for A-T. However, it is surprising that they only found missense mutations, whereas truncating mutations have been reported to be by far the most common in the *ATM* gene (20, 36). Because they do not specify the nucleotide positions of their mutations, it is not clear if, for example, their G to A base change in exon 39 resulting in the amino acid substitution of aspartic acid with asparagine reported as the significant mutation in their Patient 5 is the 5557G→A variant resulting in the D1853N amino acid substitution reported as a polyporphism in 8 of

his 23 patients by Appleby *et al.* (41) and in 18% of 100 control chromosomes by the German *ATM* Consortium (36). Unfortunately, Hall *et al.* (46) also did not screen a standard number of normal control alleles for their reported changes to discourage the idea that these may represent rare polymorphisms.

Mutation detection sensitivity below 100% with whatsoever technique remains a concern, especially in *ATM* mutation screening, considering the large size of this gene and the widespread distribution of mutations as evidenced by studies in A-T patients. Leaving alone the remote hypothesis of genetic heterogeneity, gross *ATM* gene rearrangements that would escape all PCR-based analysis, mutations deep in introns and in the promotor region are commonly reckoned with. In the end, detection rates between 50 and 90% appear realistic (36, 38) and may be taken into account for calculating the true frequency of A-T carriers among highly radiosensitive cancer patients, as soon as studied numbers of the latter are high enough to arrive at statistical significance. New developments, such as the DNA chip technology, may increase future patient throughput.

Meanwhile, other information on the question of how many patients with increased clinical radiosensitivity may be A-T heterozygotes comes from opposite approaches. Ramsey *et al.* (47) reported on a proven A-T heterozygote with bilateral breast cancer who received radiotherapy and developed only mild skin reaction and minimal late side effects, although fibroblasts and lymphoid cells showed elevated radiosensitivity on cellular *in vitro* testing. FitzGerald *et al.* (45) observed 2 *ATM* mutation carriers who received radiation therapy without adverse reaction. These findings demonstrate that heterozygous *ATM* mutations do not necessarily confer side reactions to radiotherapy. The conclusion is that A-T carrier cancer patients may not necessarily experience elevated healthy tissue radiosensitivity. Thus, if not all A-T heterozygotes are clinically radiosensitive, then we may, by chance, have not included A-T carriers in our analysis of clinically highly radiation-reactive patients.

Although the number we have screened is relatively small, our data and those of other studies demonstrate that not all patients owe their increased radiosensitivity to mutations in the *ATM* gene and that A-T heterozygotes may not constitute a major proportion of the patients with unexpected complications after radiation therapy. Nonetheless, the issue cannot yet be regarded as resolved if adverse effects to radiotherapy do occur as a consequence of genetically based radiosensitivity in a subset of this population. If such a subset of radiosensitive patients with a mutation on one allele of the *ATM* gene were to exist, and could be identified prospectively, their radiation treatment regimens might be revised for dose de-escalation that would spare a great deal of discomfort and suffering. Conversely, if most of the possible side effects to radiotherapy in a subset of patients, whatever they are defined by, were clearly identifiable, then dosage to the remaining population might be escalated, improving tumor control probability because dose schedules are governed by the overall incidence of complications. Thus, every effort must be made to continue to define any subset of patients with genetically determined clinical radiosensitivity. Extension of studies similar to ours to larger and differently selected cohorts of patients suffering severe adverse effects to radiotherapy, and application of rapidly evolving new technology, appear worthwhile to assess the definite prevalence of *ATM* mutations within the group of radiotherapy patients with adverse reactions.

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