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Advances in Brief

Modest Increased Sensitivity to Radiation Oncogenesis in *ATM* Heterozygous *versus* Wild-Type Mammalian Cells[1]

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Abstract

Subpopulations that are genetically predisposed to radiation-induced cancer could have significant public health consequences. Individuals homozygous for null mutations at the ataxia telangiectasia gene are indeed highly radiosensitive, but their numbers are very small. *Ataxia Telangiectasia* heterozygotes (1–2% of the population) have been associated with somewhat increased radiosensitivity for some end points, but none directly related to carcinogenesis. Here, intralitter comparisons between wild-type mouse embryo fibroblasts and mouse embryo fibroblasts carrying ataxia telangiectasia mutated (ATM) null mutation indicate that the heterozygous cells are more sensitive to radiation oncogenesis than their normal, litter-matched, counterparts. From these data we suggest that Ataxia Telangiectasia heterozygotes could indeed represent a societally-significant radiosensitive human subpopulation.

Introduction

A-T[3] is an autosomal recessive disorder characterized by cancer predisposition, radiosensitivity, and severe neurological and immunological abnormalities (1). In the general population A-T patients are rare, but approximately 1–2% of the United States population are A-T heterozygotes (2).

The *ATM* gene is located at 11q22–23 and has 66 exons coding a M_r 350,000 protein (3). The ATM protein is a sensor of DNA double-strand breaks and directly regulates multiple cell-signaling pathways involved in the response to this type of DNA damage by virtue of its protein kinase activity. Primary targets of this activity include p53, Mdm2, Chk2, BRCA1/Rad51 complex and nibrin/MRE 11/Rad50 complex.

A-T cells are extremely sensitive to ionizing radiation for many different *in vitro* end points. Cells heterozygous for *A-T* mutations have been reported to be somewhat more sensitive than the wild type (5–8), but no experiments with an end point of direct relevance to radiation-induced cancer have been reported.

Because of their large numbers of individuals involved, the background cancer rate in individuals heterozygous for *A-T* mutations has been studied extensively (9), but very little is known about their sensitivity to radiation-induced cancer. Several epidemiological studies of the relationship between radiation-induced breast cancer and *ATM* heterozygosity have been reported. Swift *et al.* (10) showed a positive relation in obligate heterozygotes that had undergone medical irradiation, but other reports (11–13) examined the *ATM* heterozygous prevalence in breast cancer survivors who had received large radiotherapy doses to the breast; no excess of A-T heterozygotes in the breast-cancer cases were reported, but an additional test for ATM heterozygosity (11) did yield the presence of "functional" A-T heterozygotes in 9% of the breast cancer cases.

All of these epidemiological studies lacked the power to detect relatively modest increase in radiation sensitivity (factors of 3 or less). Because of the societal importance of a significantly sized subpopulation with even a modest genetically-based enhanced sensitivity to radiation-induced cancer, these contradictory results point to the need for a model system where quantitative analysis of radiation oncogenicity can be achieved. In this study we adapted a mouse model, because fresh explants of rodent embryo cells have been used extensively as a quantitative model of oncogenic transformation (14). We report here the first direct (litter-matched) comparison of radiation oncogenesis in *ATM* heterozygotes (*ATM*+/−) compared with the corresponding normal wild type (*ATM*+/+).

Materials and Methods

Mice. The mice used in this study derive from two different ATM-knockout mice (ATM−/−), one generated at the NIH (15) and the other at Harvard Medical School (16). The NIH mouse is a 129/SvEv strain that was created in the laboratory of Dr. Anthony Wynshaw-Boris (15) and was purchased from The Jackson Laboratory (Bar Harbor, ME). The Harvard mouse has a mixed genetic background (129/SvEv × Black Swiss), and was created in the laboratory of Dr. Philip Leder (16). The two groups of mice were mated separately, and the transmission of the mutant allele followed a Mendelian inheritance pattern. The two mice have different deletions in the ATM gene, but there was no presence of full-length or truncated ATM protein in the *ATM* knockout embryos or mice from either type (15, 16; Fig. 1). Homozygous *ATM* mice from both strains displayed many of the characteristics of A-T, including growth retardation, infertility, defects in T-lymphocyte maturation, and extreme sensitivity to γ radiation. Most of the homozygotes developed thymic lymphoma between 2 and 4 months of age. Heterozygous mice displayed no detectable abnormalities through eight months of age.

Embryo Cell Preparation. A-T heterozygous mice were mated, and pregnant females were sacrificed on day 14 of the gestation period. The embryos were surgically removed and embryonic tissue prepared in culture. Each embryo was cultured separately, and during the 4 days necessary to amplify the MEF cells in mass culture, they were genotyped. *ATM* wild-type, heterozygous, and knockout cells were available from a single litter, which averaged 5–8 viable embryos with an average yield from an embryo of $\sim 3 \times 10^7$ cells.

Cell Transformation Assay. Exponentially-growing MEFs were irradiated with an acute dose of 2 Gy of ^{137}Cs γ-rays, and controls were sham irradiated. The MEFs were then plated in 100-mm plates at a density of 6,000 cells/plate over a feeder layer of 75,000 cells prepared from the same embryo but irradiated previously with a supralethal radiation dose. After 2 weeks of growth in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 air-humidified incubator, the cells were fixed, stained, and yields of transformed clones scored.

The scoring criteria was developed and examined by preliminary experiments, where embryo cells were irradiated and plated with the same density in plates. The clones appearing dense, having stellate-shaped cells, and cells piled were isolated with cloning cylinders. These clones were expanded and injected in nude mice. The ones that developed cancer in the mice were designated as transformed. Clones that matched their shape and dimensions were scored as

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[3] The abbreviations use are: A-T, Ataxia Telangiectasia; ATM, ataxia telangiectasia mutated; MEF, mouse embryo fibroblast; ROR, relative oncogenic radiosensitivity; CI, confidence interval.

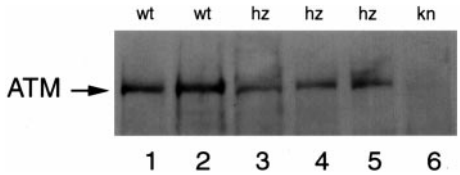

Fig. 1. Western blot analysis of ATM protein levels in *ATM* wild type (*wt*), *ATM* heterozygous (*hz*), and *ATM* knockout (*kn*) MEFs derived from embryos from same litter. Relative intensities of bands were measured, and corresponding numbers are given in parenthesis for each lane. *Lane 1*, embryo H6-B (17916); *Lane 2*, embryo H6-E (17000); *Lane 3*, embryo H6-C (8000); *Lane 4*, embryo H6-A (10600); *Lane 5*, embryo H6-G (10673); *Lane 6*, embryo H6-D (non reg).

transformed in the later experiments. Plating efficiency, cell surviving fractions, and the spontaneous and radiation-induced frequency of morphological transformation were determined.

Statistical Analysis. To directly compare the sensitivities to radiation oncogenesis of the wild-type MEFs with the corresponding *ATM* heterozygous cells, stratified 2×2 comparisons were used, *i.e.*, only litter-matched comparisons were made between the radiation sensitivities of *ATM* wild-type and heterozygous MEFs. This was done using a Monte-Carlo simulation of Zelen's exact test (17).

Expression of ATM Protein. Cells (5×10^6) were lysed directly in 0.5 ml of Laemmly sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% Glyderol, 2% SDS, 0.01% Bromphenol Blue) and boiled for 3 min. The cell lysate was subjected to SDS electrophoresis and Western blotting using the anti-ATM-2C1 antibody (GeneTex, San Antonio, TX). Estimation of the relative ATM protein quantities was performed using Kodak DC1 software.

Functional Assay for the ATM Protein. Cells (5×10^6) were irradiated with 2 Gy and lysed 1 h later in 20 mM Tris (pH 7.5), 250 mM NaCl, 1% NP40, and protease inhibitors. The same number of nonirradiated cells were used as control. The phosphorylation at ser18 (corresponding to ser15 in human cells) was detected on Western blots by using Phospho-p53 (Ser15) antibody (New England Biolabs, Beverly, MA), and p53 was detected using Ab-7 (Oncogene, San Diego, CA).

Results

Oncogenic Transformation. The end point of interest here is the induction of radiation-induced morphologically transformed clones in the freshly explanted MEFs. Whereas nontransformed normal colonies consist of rounded, contact-inhibited, monolayers of cells, transformed colonies contain elongated cells in parallel bundles that typically criss-cross each other and do not exhibit contact inhibition; these transformed colonies appear dark blue when stained with Giemsa in comparison with normal contact-inhibited cells because of the presence of multiple layers of abnormal cells. The transformed cells grow and demonstrate anchorage independence in semisolid agar and also produce tumors when injected into athymic nude mice, thus serving as a reliable marker of neoplastic transformation. The background (zero-dose) transformation rates were low ($<5 \times 10^{-4}$).

Litter-matched experiments were performed as outlined above to investigate the influence of ATM heterozygosity on radiation-induced oncogenic transformation of MEFs. A total of 13 intralitter comparisons were made between normal and A-T heterozygote embryos, 5 between pairs of embryos from the NIH mice and 8 between pairs of embryos from the Harvard mice (Table 1). Yields of transformed clones were measured both for zero-dose exposure and for exposure to a γ-ray dose of 2 Gy. Roughly 50,000 clones at zero-dose and 45,000 clones at 2 Gy were assessed for transformed morphology, and representative transformed clones were confirmed as neoplastically transformed by their ability to produce tumors when injected into athymic nude mice.

To directly compare the sensitivities to radiation oncogenesis of the wild-type MEFs with the corresponding *ATM* heterozygous cells, only litter-matched comparisons were made between the radiation sensitivities of *ATM* wild-type and heterozygous MEFs. We define the ROR as the yield of transformed clones per surviving *ATM* heterozygous MEFs exposed to a dose of 2 Gy relative to the yield of transformed clones per corresponding surviving wild-type MEFs also exposed to 2 Gy.

The RORs for the heterozygous *versus* wild-type MEFs were exactly estimated using standard maximum likelihood techniques (18) and the null hypothesis that the ROR was unity (no difference in sensitivity) subjected to a two-sided test. The results are shown in Table 2. For the NIH mice, the ROR (heterozygous *versus* wild type) was 1.48 (95% CI, 0.65–3.51; $P = 0.35$); for the Harvard mice, the

Table 2 *Litter-matched comparisons of radiation oncogenesis between heterozygous and normal wild-type MEFs*

To directly compare the sensitivities to radiation oncogenesis of the wild-type MEFs with the corresponding *ATM* heterozygous cells, stratified 2×2 comparisons were used, *i.e.*, only litter-matched comparisons were made between the radiation sensitivities of *ATM* wild-type and heterozygous MEFs. This was done using a Monte-Carlo simulation of Zelen's exact test (17).

	NIH mice	Harvard mice	All mice
Zalen test for homogeneity of ROR,[a]	$P = 0.68$	$P = 0.054$	$P = 0.19$
(99% confidence limits of *P*)	(0.68, 0.68)	(0.051, 0.058)	(0.18, 0.19)
Estimated ROR	1.48	1.89	1.74
(95% CI)	(0.65, 3.51)	(1.08, 3.42)	(1.11, 2.80)
(two-sided *P*)	($P = 0.35$)	($P = 0.024$)	($P = 0.016$)

[a] *ATM* heterozygotes *vs.* wild type.

Table 1 *Irradiated and transformed clones from irradiated ATM wild-type and heterozygote embryo cells*

Litter-matched wild-type and *ATM* heterozygous mouse embryo fibroblasts were irradiated with 2 Gy of γ-rays and the number and frequency of oncogenically transformed clones assayed.

Mouse no.	Embryo no.	Wild type (W)/ heterozygous (H)	Number of surviving clones	Number of transformed clones	Frequency of transformed clones (%)
NIH mice					
N1	A	W	1505	1	0.07
N1	B	W	2600	4	0.15
N1	C	H	3075	6	0.19
N2	A	W	2832	2	0.07
N2	B	H	2576	5	0.19
N2	C	H	1600	1	0.06
N2	D	H	1960	0	0.00
Harvard Mice					
H1	A	W	3000	2	0.07
H1	B	W	2250	2	0.09
H1	D	H	925	0	0.00
H1	E	H	1617	6	0.37
H6	B	W	1950	5	0.26
H6	E	W	2050	1	0.05
H6	A	H	4857	17	0.35
H6	G	H	3737	4	0.11

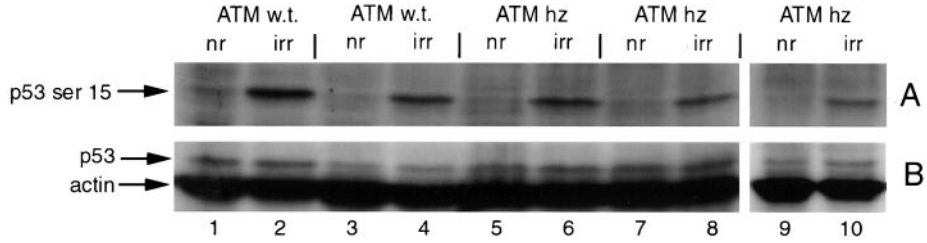

Fig. 2. Western blot analysis of p53 (ser18) phosphorylation before and 60 min after irradiation (5Gy) of *ATM* wild-type (*wt*) and *ATM* heterozygous (*hz*) MEFs derived from embryos from same litter. *A*, anti-p53 (ser18) antibody. *Lanes 1* and *2*, embryo H6-B; *Lanes 3* and *4*, embryo H6-E; *Lanes 5* and *6*, embryo H6-C; *Lanes 7* and *8*, embryo H6-A; *Lanes 9* and *10*, embryo H6-G. *nr*, nonirradiated, *irr*, irradiated cells. p53 was phosphorylated at ser18 in all MEFs. *B*, blot was stripped and reblotted with anti-p53 antibody and anti-actin antibody.

estimated ROR was 1.89 (95% CI, 1.08–3.43; $P = 0.02$), and for both animal strains combined the ROR was 1.74 (95% CI, 1.11–2.80). For the NIH mice, the null hypothesis could not be rejected that the wild-type and heterozygous cells have the same sensitivity to radiation oncogenesis. However, for the Harvard mice and for both strains of mice combined, the null hypothesis could be rejected ($P = 0.02$), *i.e.*, the *ATM* heterozygous mice were significantly more sensitive for radiation oncogenesis than were the corresponding wild-type animals by a factor of almost 2.

By contrast, the ROR at 2 Gy for *ATM*-deficient homozygous mice compared with the normal wild type was 10.5 (95% CI, 4.4–26.2; $P < 0.001$; 4 litter-matched comparisons made, data not shown).

ATM Status in the MEFs. To explorer the origins of the differences in the oncogenic transformation between wild-type and the heterozygous MEFs, we examined the ATM protein expression as well as its functional activity revealed by phosphorylation of p53 protein on ser18 in the H6 (see Table 1) embryo group. The results are shown in Figs. 1 and 2. A Western blotting with an anti-ATM antibody showed that the heterozygous cells synthesize about 40–60% less ATM protein than do the wild type, whereas the knockout cells showed no ATM protein expression. However, the ATM protein was active in both the wild-type and the heterozygous cells and stabilized p53 by phosphorylation at ser18, in contrast with the *ATM* knockout cells where no phosphorylation of p53 occurred. An interesting question is what are the kinetics of p53 phosphorylation on ser18 in *ATM* wild-type and *ATM* heterozygous cells. Radiation induced an increase in levels of phosphoserine 18 in both cell types with similar kinetics in contrast with ATM knockout MEFs where very little phosphorylation was detected (Fig. 3). As has been shown in other cases (16, 17), haploinsufficiency could be a factor in the radiation sensitivity of *ATM* heterozygous cells. The involvement of ATM protein in many different pathways may lead to dependence between its concentration and functional activity.

Discussion

This work is the first to demonstrate a statistically significant enhancement in sensitivity by almost a factor of 2 to radiation-induced oncogenic transformation in carriers of a heterozygous genetic mutation, in this case, *ATM*. By contrast, A-T homozygotes showed an increased sensitivity of about a factor of 10.

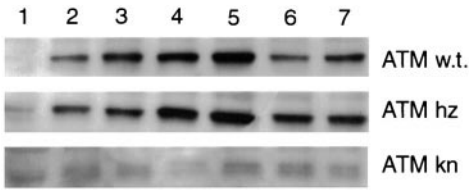

Fig. 3. Kinetics of p53 (ser18) phosphorylation after irradiation (10 Gy) of *ATM* wild-type, *ATM* heterozygous, and ATM knockout MEFs. Equal amounts of protein were subject of electrophoresis and Western blotting with anti-p53 (ser18) antibody. *Lane 1*, nonirradiated cells; *Lane 2*, 15 min after irradiation; *Lane 3*, 30 min after irradiation; *Lane 4*, 45 min after irradiation; *Lane 5*, 1 h after irradiation; *Lane 6*, 2 h after irradiation; *Lane 7*, 4 h after irradiation.

The data presented here are suggestive that the 1–2% of the human population that are heterozygous for the *ATM* gene may be genetically predisposed to radiation-induced cancer, though the relatively modest enhanced sensitivity would explain why past epidemiological studies have not been able to detect such an increase. It is of course quite likely that there are other genetically-based radiosensitive subpopulations, though whereas potentially radiosensitive low-frequency subpopulations such as *BRCA1/2* (prevalence $\leq 0.2\%$) have been studied (19), there are currently no other obvious high-frequency candidates.

The presence of comparatively large subpopulations that are genetically predisposed to radiation-induced cancer would be of relevance in several situations. First, the risk-benefit equation in mass-screening mammography would be altered (20), which is of particular relevance at younger ages, although the potential increase in the natural breast cancer rate in A-T heterozygotes also needs to be considered here. More generally, application of the results of epidemiological studies of radiation-induced cancer risks, such as at Hiroshima or Chernobyl, are currently premised on an essentially unimodal distribution of radiation sensitivity across the population; if a significantly-sized identifiable subpopulation were hypersensitive to radiation-induced cancer, a single radiation protection standard across the whole population would be of questionable relevance.

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