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## Original Paper

# *ATM* Heterozygote Cells Exhibit Intermediate Levels of Apoptosis in Response to Streptonigrin and Etoposide

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**Ataxia-telangiectasia (A-T) is a rare recessive disease characterised by cerebellar ataxia, immunodeficiency, sensitivity to ionising radiation and increased cancer risk. Heterozygotes have an increased risk of cancer and may comprise 1% of the population. *In vitro*, A-T heterozygote cell lines show radiosensitivity intermediate between normal and A-T homozygotes. Furthermore, in A-T homozygotes, hypersensitivity to chemical agents which cause DNA damage, similar to that produced by ionising radiation, has been observed. To investigate the chemosensitivity of A-T heterozygote cell lines, we used TUNEL to analyse the level of apoptosis after drug treatment with etoposide and streptonigrin. Our samples included four normal, eight A-T heterozygote and 10 A-T homozygote lymphoblastoid cell lines. All cell lines were exposed to drugs for 24 h, then cultivated in fresh media for 0 and 72 h. The levels of apoptosis increased significantly in all cell lines, with the greatest increase in homozygote cells and an intermediate increase in heterozygote cells ($P$ values of $< 0.01$ for etoposide treatment and $< 0.02$ for streptonigrin treatment were obtained using the Kruskal–Wallis $H$-test). Our results indicate that A-T heterozygotes express intermediate sensitivity to etoposide and streptonigrin similar to that observed in response to ionising radiation.** 

**Key words: ataxia-telangiectasia, apoptosis, chemosensitivity, streptonigrin, etoposide, heterozygote**
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## INTRODUCTION

Ataxia-telangiectasia (A-T) is a rare, autosomal recessive, monogenic childhood disease characterised by a wide spectrum of clinical defects including telangiectasia, progressive cerebellar ataxia, immunodeficiency, sensitivity to ionising radiation, chromosome instability and increased risk of cancer [1, 2]. The A-T gene, *ATM*, is located on chromosome 11q23.1 [3]. A-T heterozygotes have no clinical phenotype, but they may have a slightly elevated risk of cancer [4, 5].

The role of the ATM protein in modulating programmed cell death has not been clearly demonstrated, although one of the characteristic features of A-T homozygote cells is enhanced cell killing by sensitivity to agents which directly induce DNA damage, such as ionising radiation and radiomimetic chemicals [6]. Meyn and colleagues reported that A-T cells exhibited enhanced apoptotic cell death in culture following low-dose exposure to radiation and streptonigrin [7] and incorporated this observation into a model for cellular responses to DNA damage: the Damage Surveillance Network [8].

*In vitro* cells from A-T heterozygotes exhibit several subtle defects intermediate between normal cells and homozygotes, including reduced survival, cell cycle defects and reduced p53 stabilisation [9–11]. Intermediate numbers of spontaneous and induced chromosome aberrations found in A-T heterozygote cells have been used to try to identify A-T heterozygotes in the population [12]. This *in vitro* phenotype suggests that A-T heterozygotes, in addition to their increased cancer risk, might be sensitive to DNA damaging agents used for cancer therapy. However, there is no obvious clinical radiosensitivity of A-T heterozygotes during cancer treatment, nor are A-T heterozygotes over-represented among breast cancer therapy over-reactors [13].

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In this study, we investigated the level of apoptosis of A-T heterozygote lymphoblastoid cell lines (LCLs) after exposure to two chemotherapeutic drugs, etoposide and streptonigrin, in comparison with normal and A-T homozygote LCLs, in order to establish their sensitivity to these agents. These two drugs cause DNA double-strand breaks, mimicking the effect of ionising radiation. This may enable us to identify A-T heterozygotes in the cancer population and to ascertain eventually whether they have clinically relevant chemosensitivity.

## MATERIALS AND METHODS

*Cell lines*

LCLs were kindly donated by Richard Gatti (University of California, Los Angeles, U.S.A.) (NPE, CRAT12-3 and WAR19) and Gilbert Lenoir (CIRC de Lyon, Lyon, France) (LNDP, iarc 1663, iarc 1665, FRAT 1-B, FRAT 2-B, iarc 2362, iarc 2364, iarc 2364, GM 3187, GM 3188, GM 3334, GM 3382, AT 01, AT 03, AT 05, AT 06, AT 09, AT 11, AT 12 and AT 13). LCLs were maintained as static suspension cultures in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 10% heat inactivated fetal bovine serum (all Gibco-BRL, Cergy Pontoise, France) at 37°C in 5% $CO_2$. The cultures were passaged every 3–4 days and were prevented from reaching 'confluency' (approximately $10^6$ cells/ml).

*Etoposide and streptonigrin treatment*

Etoposide and streptonigrin were purchased from Sigma (Saint Quentin Fallavier, France). Drug stock solutions were made in dimethyl sulphoxide (DMSO) and stored in small aliquots at −20°C. Etoposide and streptonigrin were added to the cell culture to a final concentration of 10 μg/ml and 1.5 ng/ml, respectively. T0 was the time at the beginning of manipulation, just before drug exposure. After 24 h of exposure, cells were washed in phosphate-buffered saline (PBS) twice and harvested (T24), or cultured in drug-free medium for an additional 72 h (T96).

*Detection of apoptosis*

Apoptosis staining was performed at the beginning of drug treatment and at T24 and T96. At the end of the cell culture, cells were centrifuged at 200 ***g*** for 10 min at room temperature (RT). The supernatant was discarded and the cell pellet was resuspended and fixed in 4% paraformaldehyde for 30 min at RT. A drop of the cell suspension was put on slides precoated with poly-L-lysine (Sigma) and air dried. Endogenous peroxidase was inactivated by covering the cells with 0.3% $H_2O_2$ for 30 min at RT. The cells were rinsed with PBS and the cell membrane was permeabilised with citrate buffer (0.1% sodium citrate and 0.1% Triton X-100). The apoptosis staining was performed by TUNEL (TdT-mediated dUTP biotin nick end labelling) [14], using the '*In situ* cell death detection kit' (Boehringer Mannheim, Cergy Pontoise, France) according to the manufacturer's recommendations.

*Apoptosis quantification*

For quantification, three independant assays were performed on separate cultures of each LCL. We counted the percentage of apoptotic cells observed in the calibration field of a Telaval 31 Ziess microscope. Each data point represented approximately 1000 cells. Debris fragments less than one half the size of live cells were not counted.

*Statistical analysis*

For the statistical analysis, we used Student's *t*-test where appropriate. When the distribution of data was non-Gaussien, we used the non-parametric Mann–Whitney *U*-test. *P* values derived from Student's *t*-test are noted ($t$: $P \leq 0.05$) and those derived by the *U*-test are noted ($U$: $P \leq 0.05$). Non-significant results were always non-significant by both tests. We used the Kruskal–Wallis *H*-test to compare the means. The statistical analyses were performed with SEM for PC.

## RESULTS

Figure 1(a) shows the effect of etoposide on each cell line, 24 and 96 h after the beginning of drug treatment. There was little change in the level of apoptosis for each individual cell line between the three independant trials. There was some heterogeneity among the different normal cell lines and among the heterozygote cell lines at both time points, as well as among the homozygote cell lines at 24 h. After 96 h, however, the different A-T homozygote cell lines exhibited significant differences in apoptosis, ranging from 20.1 ± 1.9% (WAR19), which was equivalent to the normal cell lines, to 69.4 ± 1.25% (AT11). The combined results from each *ATM* genotype showed that A-T homozygote cells were much more sensitive to etoposide than normal cells and that A-T heterozygote cells exhibited intermediate sensitivity (Figure 1b). With etoposide treatment, the difference between heterozygote cell lines and homozygote cell lines at T24 was not significant ($U$: $P > 0.05$), whereas the differences between normal cells and heterozygotes ($t$: $P < 0.01$) and normal cells and homozygotes ($t$: $P < 0.001$) were significant. At 96 h, there were significant differences in the level of apoptosis between all combinations of genotypes ($t$: $P < 0.01$).

Similar results were obtained for streptonigrin exposure, although the overall level of apoptosis was less (note the change in scale between Figures 1 and 2) and the variability between cell lines was greater. After 24 h of drug exposure, all the heterozygote cell lines exhibited more apoptosis than the normal cells. After an additional 72 h without drug (T96), apoptosis in heterozygotes overlapped both the normal and homozygote responses. There was again wide variation in the response of the homozygote cell lines, which was reliably reproduced. At 24 h, A-T homozygotes CRAT12-3 and WAR19 exhibited apoptosis in the normal range, although at 96 h their levels of apoptosis had surpassed the normal range. The combined results for each genotype group showed that A-T heterozygotes had an intermediate apoptotic response to streptonigrin (Figure 2b). At 24 h, there were significant differences between normal cells and heterozygotes and normal cells and homozygotes ($U$: $P < 0.001$), but not between heterozygotes and homozygotes ($P = 0.25$). At 96 h, the differences between all combinations were significant (normal cells and heterozygotes: $t$: $P < 0.001$; heterozygotes and homozygotes: $t$: $P < 0.05$; normal cells and homozygotes: $t$: $P < 0.001$).

## DISCUSSION

We studied the sensitivity to two drugs of LCLs with an alteration of one or both alleles of the *ATM* gene. Etoposide, a member of the epipodophyllotoxin class of cytotoxic drugs, is an inhibitor of topoisomerase II [15]. Drugs of this type cause DNA strand breakage attributable to stabilisation of the 'cleavage complex' formed by the covalent linkage of each of the two homologous subunits of the enzyme to the

5′-phosphoryl ends of DNA [16]. Streptonigrin is a radiomimetic antitumour antibiotic (NSC 45383) capable of causing chromosome-type aberrations following exposure of cells in $G_0$ and chromatid-type aberrations following exposure in $G_2$ [17]. Streptonigrin inhibits DNA synthesis and produces DNA strand breakage [18].

Our results show that etoposide and streptonigrin both cause an increase in apoptosis for A-T heterozygote and homozygote LCLs compared with normal LCLs. The sensitivity of A-T homozygote cells to these two drugs is well established in the literature [7]. In contrast, LCLs from A-T heterozygotes have not been extensively studied. A-T heterozygote fibroblasts have been shown to exhibit intermediate sensitivity to DNA strand-breaking agents [19] and our results for lymphoblastoid cells are consistent with these data.

The radio- and chemosensitivity of A-T homozygote cells is a reflection of the *in vivo* situation, where A-T patients exhibit extreme radio- and chemosensitivity [20]. The radio- and chemosensitivity of A-T heterozygote fibroblastoid cell lines and LCLs, however, is not obviously observed *in vivo*. There are no reports of A-T heterozygotes over-reacting to

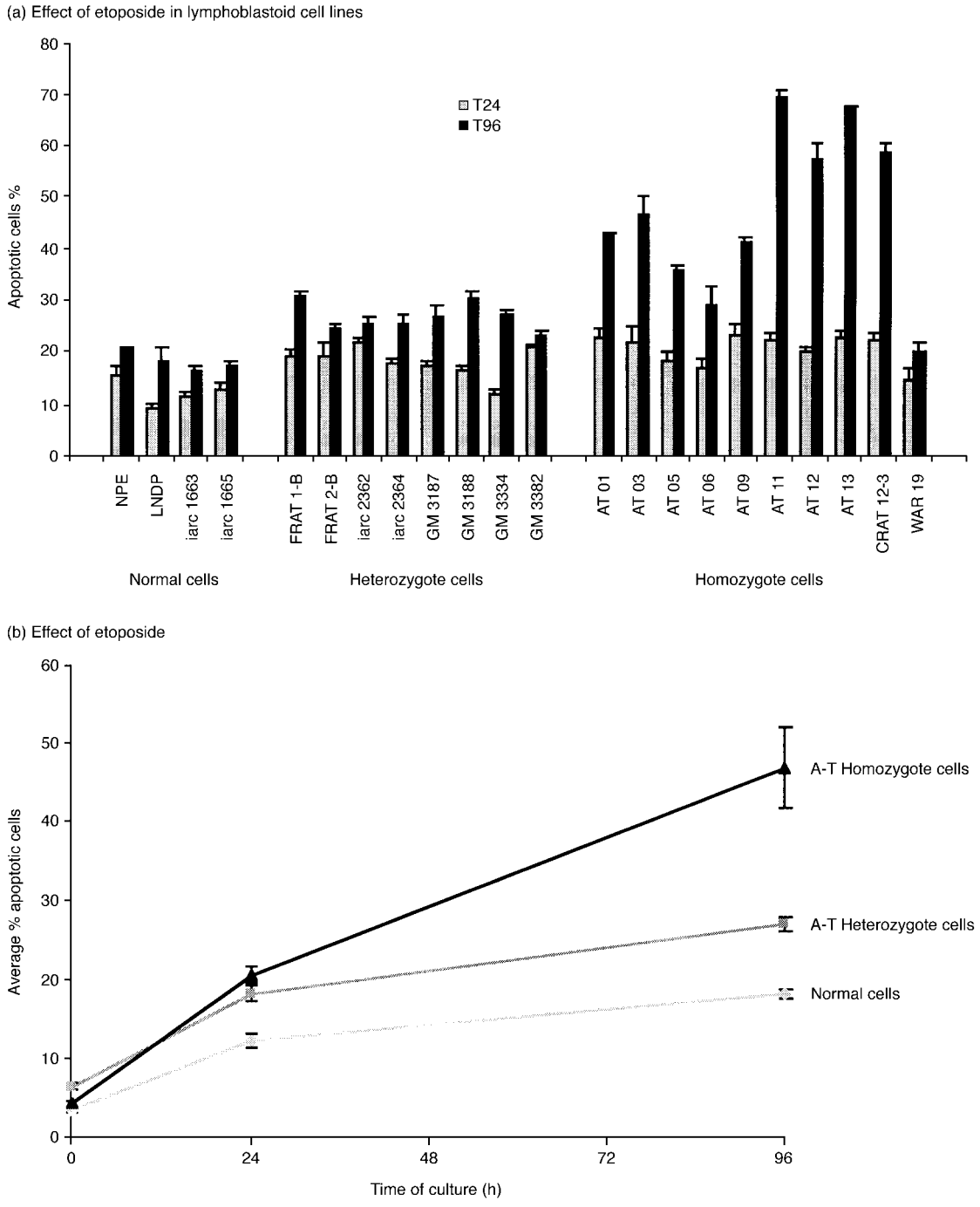


**Figure 1. Effect of etoposide on lymphoblastoid cell lines. (a) Apoptosis—individual cell lines. The percentage of TUNEL-positive cells among approximately 1000 cells is indicated as the average of three experiments. (b) Combined results for the three genotypes of cells. Bar: standard error of the mean (SEM).**

cancer therapy and analysis of radiation over-reactors has not revealed any constitutional *ATM* mutations [13]. One explanation for this difference seen in A-T heterozygotes is that the *in vivo* environment of the cells either modulates the amount of drug received or the drug is cleared from the body such that the duration of exposure is shorter. This supposed difference in dose, however, cannot account for the difference *in vivo* versus *in vitro* of radiation treatment. Therefore, either the *in vivo* environment supports survival after DNA damage and/or the intermediate level of cell death seen *in vitro* is insufficient to cause detectable pathology.

Although we observed differences in the level of apoptosis between the different groups of cells, there was considerable heterogeneity between the cells in each group, including one homozygote, WAR19, with a response to etoposide in the normal range. McConville and colleagues reported similar results for an A-T homozygote who exhibited normal *in vitro* radiosensitivity, possibly reflecting partial activity of ATM in these cells [21]. The complete genotype of WAR19 is unknown, although it has been established that WAR19 is a compound heterozygote for a splice-site mutation that deletes an 89 nt exon [22]. It is possible that this mutation allows for

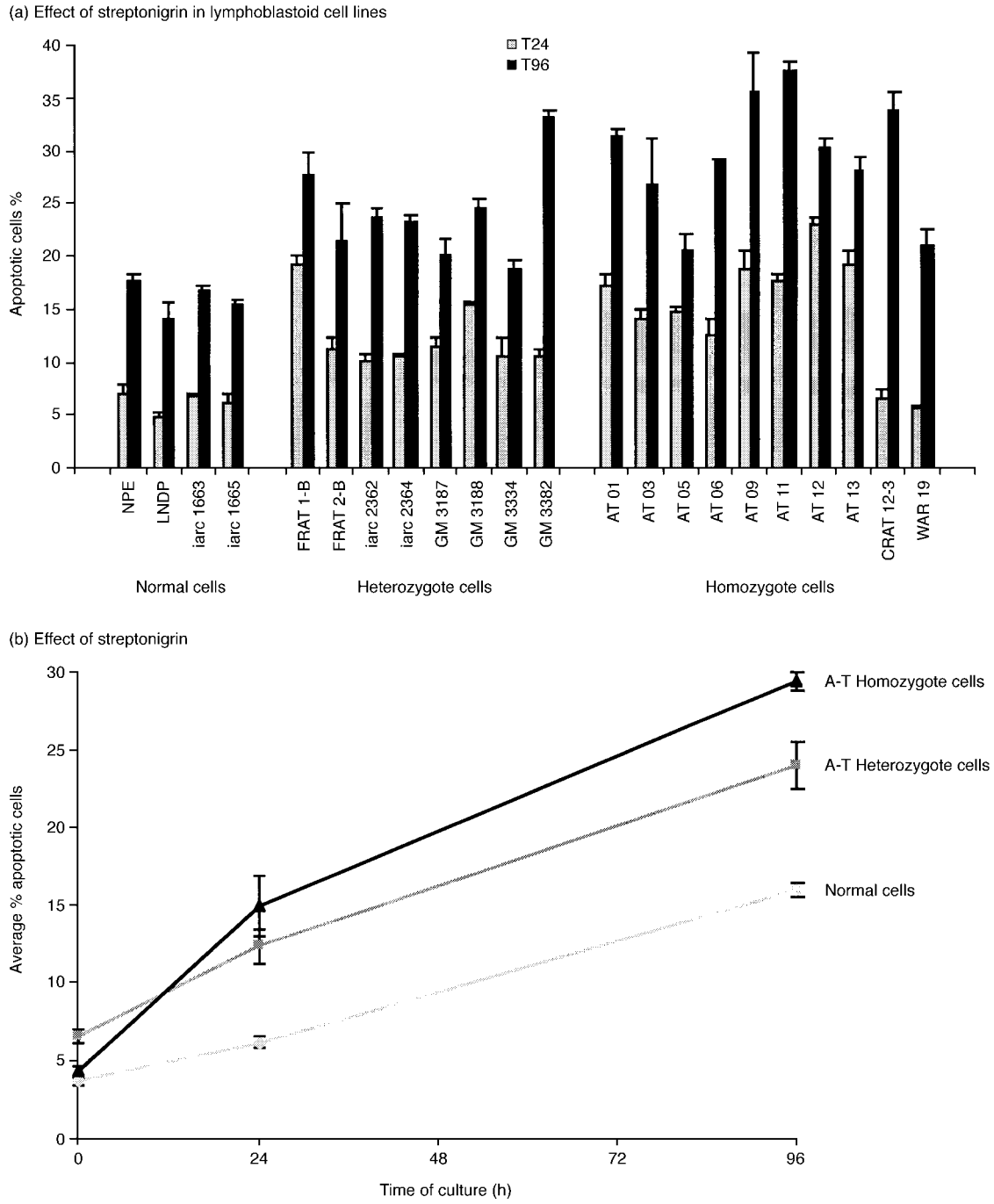


**Figure 2. Effect of streptonigrin on lymphoblastoid cell lines. (a) Apoptosis—individual cell lines. The percentage of TUNEL-positive cells among approximately 1000 cells is indicated as the average of three experiments. (b) Combined results for the three genotypes of cells. Bar: standard error of the mean (SEM).**

a small amount of full-length *ATM* mRNA to be made, which confers some resistance to radiomimetic drugs. The clinical phenotype of WAR19 patients seems to be classical, however.

All the other cell lines studied were also from classical A-T patients, one of whom (CRAT12-3) is known to be a compound heterozygote for two truncating mutations [22, 23]. Although sensitivity to DNA strand-breaking agents is a constant feature of classic A-T cells, the exact level and timing of apoptosis in the response to these agents may be modified by alleles of other genes.

The heterogeneity in the apoptotic response of the A-T heterozygote LCLs probably reflects differences in genetic background, but also whether the mutant *ATM* allele exerts a dominant-negative effect on the normal allele. Transfection experiments with a fragment of *ATM* containing the leucine zipper domain have shown that expression of this fragment can interfere with the function of normal ATM [24] and this means that A-T heterozygotes who express mutant protein may be more sensitive to DNA strand breaks than heterozygotes who do not express mutant protein. It has been shown, however, that most *ATM* mutations, regardless of where they occur in the gene, cause the protein to be unstable and then no mutant protein is detectable by Western blotting [25, 26]. Therefore, dominant-negative alleles are expected to be rare.

As a method for the detection of A-T heterozygotes, using the level of apoptosis observed after treatment with DNA strand-breaking agents may be difficult, due to the small difference between normal and heterozygote responses. Additional tests for A-T heterozygotes may have diagnostic value, although most authors find that the responses of some heterozygotes fall within the normal range, leading to false-negative results. The colony-survival assay (CSA) determines the cells' capacity for replication after irradiation [27]. In many cell types, and in transformed cultured cells in particular, increased apoptosis may be a direct cause of decreased clonogenic survival. The radioresistant DNA synthesis (RDS) assay measures the efficiency of the S phase cell cycle checkpoint, which is lacking in A-T cells and is reduced in A-T heterozygotes [28]. In addition, A-T heterozygote cells show an intermediate level of chromatid aberrations when irradiated in $G_2$ phase. This phenotype is detectable in fresh lymphocytes and has also been studied as a test for A-T heterozygotes, using fresh lymphocytes [12]. In addition to the problem of false-negative results, there are other genetic defects which may also lead to sensitivity to DNA damage that could be detected by this and other radiosensitivity assays. One example is heterozygosity for the Nijmegen breakage syndrome (NBS) gene. NBS homozygotes exhibit radiosensitivity similar to that of A-T [29] and NBS heterozygotes appear to exhibit subtle defects similar to A-T heterozygotes.

The identification of individuals sensitive to DNA damage *in vitro* is a first step towards correlating their genotype at loci such as *ATM*, *NBS* and others, and predicting their tolerance for radio- and chemotherapy. A-T heterozygotes may account for 1% of the population and if their relative risk of, for example, breast cancer, is even moderately elevated, they may account for a significant fraction of breast cancer cases [5]. It may be worthwhile to search for increased sensitivity to therapeutic agents in these individuals and alter their treatment if necessary.


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