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Research Article

The novel human gene aprataxin is directly involved in DNA single-strand-break repair

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Abstract. The cells of an ataxia-oculomotor apraxia type 1 (AOA1) patient, homozygous for a new aprataxin mutation (T739C), were treated with camptothecin, an inhibitor of DNA topoisomerase I which induces DNA single-strand breaks. DNA damage was evaluated by cytogenetic analysis of chromosomal aberrations. The results obtained showed marked and dose-related increases in induced chromosomal aberrations in the patient and her heterozygous mother compared to the intrafamilial wild-type control. The alkaline comet assay confirmed this pattern. Moreover, the AOA1 cells did not show hypersensitivity to ionizing radiation, i.e. X-rays. These findings clearly indicate the direct involvement of aprataxin in the DNA single-strand-break repair machinery.

Key words. DNA single-strand-break repair; aprataxin; chromosomal aberration; camptothecin; inhibitor of DNA topoisomerase I.

Mutations of aprataxin (APTX), a novel human gene located in the 9p13 region, have been recently linked by two independent reports [1, 2] to ataxia-oculomotor apraxia type 1 (AOA1), an autosomal recessive syndrome characterised clinically by early onset cerebellar ataxia, oculomotor apraxia and late peripheral neuropathy. The same neurological features characterise ataxia telangiectasia (AT), another rare human autosomal recessive disorder also presenting with extreme sensitivity to ionising radiation (IR) and susceptibility to cancer, immunodeficiency, oculocutaneous telangiectasias, progressive neurodegeneration, growth retardation, developmental abnormalities and premature ageing [3–6]. The ATM gene, defective in AT patients, determines cellular responses for the recognition and processing of DNA double-strand breaks induced by IR and DNA-alkylating agents [7]. AOA1, however, is not sensitive to IR and does not show cancer proneness and genomic instability but is, rather, characterised by enhanced sensitivity to agents that cause DNA single strand breaks [8].

The APTX gene encodes a nuclear protein considered to be a member of the Hint (histidine-triad-nucleotide-binding) subfamily of the histidine triad (HIT) domain. These proteins are nucleotide hydrolases and transferases exhibiting approximately 30% homology with the active site of human and rabbit Hint1 which binds nucleotides and displays adenosine 5′-monophosphoramidase activity [9]. Althongh the structure and biochemical activities of these proteins have been fully described, their biological roles are still unknown. Similarly, nothing is known about the cellular involvement of aprataxin in preventing neurodegeneration in AOA1 patients.

In addition, there is a region of homology between the amino terminus of aparataxin and that of human PNK, a

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polynucleotide kinase involved in the repair of both 3′-phosphate and 5′-hydroxyl termini after interaction with XRCC1 and other proteins of the DNA single-strand-break repair (SSBR) pathway, thus suggesting a first link between APTX and SSBR [10].

More recently, aprataxin was shown to interact with the DNA repair proteins PARP-1, XRCC1 and p53, and to co-localize with XRCC1 along charged particle tracks on chromatin. Aprataxin influences the response to genotoxic agents as a component of the DNA SSBR complex with PARP-1, XRCC1 and p53 [8].

In the present paper, based on the suggested links between aprataxin and SSBR, our aim was to confirm the direct involvement of this protein in the SSBR machinery. We analysed the induction of chromosomal aberrations by camptothecin (CPT), an inhibitor of DNA topoisomerase I (topo I) which is a specific and powerful DNA single-strand-break inducer. CPT acts by stabilising the DNA-topo I complex (also known as the 'cleavable complex') generating DNA single-strand break, which, per se are not expected to finally result in chromosomal aberrations. After the induction of the damage, DNA single-strand breaks are produced by an enzyme-mediated process and being 'protein concealed' they can be detected by DNA filter elution methodology when the cell lysate is digested with a proteinase before elution [11–13]. CPT and its derivatives induce exclusively chromatid-type aberrations in human lymphocytes when the drug is present in the S and G_2 phases of the cell cycle; G_1 treatments have no effect [14]. Elevated frequencies of sister chromatid exchange (SCE) are also observed, provided that CPT is present during the S phase [15].

CPT has been suggested to induce chromosomal damage through the production of DNA double-strand breaks generated by the collision of the stabilised 'cleavable complex' in the topo I reaction and the replicative fork of DNA synthesis [16–19]. Accordingly, the induction of genetic damage by CPT is conditioned by DNA synthesis. However, this reaction is reversible since topo I can reseal the so called 'protein concealed' DNA single-strand breaks once CPT is removed.

Nevertheless, a number of CPT-induced topo I DNA single-strand breaks can become irreversible through collision of the 'cleavable complex' with the replication fork as described above.

Materials and Methods

Cell lines

Epstein Barr virus-transformed lymphoblastoid cell lines (LCLs) from a patient presenting clinically as AOA1 (738RM), her mother (739RM) and her brother (938RM) and additional non-obligate heterozygous carriers were cultured in RPMI 1640 medium (Gibco BRL) containing 10% fetal calf serum (Gibco BRL) 2 mM L-glutamine, 100 U/ml Hepes buffer (Fluka) and 100 U/ml streptomycin (Gibco BRL) and maintained in a 37 °C incubator (Forma Scientific) in a 5% CO_2 atmosphere and 100% nominal humidity.

Molecular analysis

Genomic DNA was extracted from peripheral blood lymphocytes and all the APTX exons were amplified by PCR using the primers designed by Moreira et al. [10]. Denaturing high-performance liquid chromatography (DHPLC) [20–22] was carried out on a 3500HT WAVE DNA fragment analysis system equipped with a DNASep column (Transgenomic). PCR products were examined for heteroduplexes: 10-µl aliquots of each amplified product were hybridized in a 1:1 ratio to control PCR product by heating to 95 °C and cooling slowly. DHPLC analysis of the PCR products was then performed using buffer gradient and temperature conditions calculated using WAVEmaker software (version 3.3; Transgenomic). Direct sequencing of the exon 5, showing an altered elution profile, was carried out using an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystem). Forward and reverse sequences were analysed and compared with the mRNA reference sequence. Nucleotide and amino acid numbers were given according to the long APTX transcript (GenBank accession no. FLJ20157).

Mutation analysis in the patient's relatives was also performed by DHPLC.

Determination of aprataxin level

The cells were washed with PBS buffer plus 0.1 mM Na_3VO_4, pelleted and lysed in Laemmli buffer (0.125 M Tris-HCl pH 6.8, 5% SDS) containing protease inhibitors. Lysates were boiled for 2 min, sonicated and quantitated by Bradford assay. Aliquots containing 40 mg/ml of protein plus 5% β-mercaptoethanol were size-fractionated on 7–10% SDS-PAGE and electroblotted onto PVDF membranes. After blocking with 5% non-fat dried milk in PBS plus 0.1% Tween 20, the membranes were incubated with specific antibody against the entire long form of aprataxin (isoform A in the NCBI database, kindly provided by Prof. M. Lavin) and vinculin for normalisation. After incubation with a peroxidase-conjugated secondary antibody the immunoreactive bands were visualised by ECL Supersignal on autoradiographic films. The same filter was reprobed with the anti-actin antibody after stripping the anti-aprataxin antibody.

Genotoxic treatments

The cultures were prepared at a density of 1×10^6 cells in duplicate to allow either the evaluation of chromosomal aberrations in cytogenetic preparations or the extent of DNA breakage analysed by alkaline Comet assay. The clastogenic agent CPT (Sigma), was dissolved in dimethylsulphoxide (DMSO) and 50 µl of appropriately

diluted solutions were added for 3 h to 5 ml culture at the final concentrations of 1, 2.5 and 5 nM, established after dose-range-finding experiments (data not shown). At the end of treatment, the cells were washed twice with PBS and left to recover in culture medium for further 21 h; in the last 3 h, colcemid (Gibco BRL) at a final concentration of 0.2 μg/ml was added.

To establish the extent of DNA breakage by 'comet assay', a time course evaluation of DNA breakage and its repair was performed at 0, 1, 2, 4, 8, 12 and 24 h from treatment with CPT.

Due to phenotypic similarities between AT and AOA1, we also checked for a possible hypersensitivity of AOA1 cells to IR (X-rays) [8]. X-irradiation (1.5 Gy) was performed using a Gilardoni X-ray generator operating at 250 kV and 6 mA at a dose rate of 0.75 Gy/min.

Cytogenetic analysis

Hypotonic treatment (KCl 0.075 M) and fixation of cells were carried out according to standard procedures. Air-dried preparations were stained with Giemsa (3%) for 5 min. For each experimental point, 100 metaphases were scored for chromosomal aberrations and were classified according to the description of Savage [23].

Mitotic indices were expressed as a percentage based on the number of metaphases present after a total of 1000 cells had been scored (interphases and metaphases).

The alkaline comet assay

Three independent experiments using the protocol for the alkaline 'comet assay' of Singh et al. [24] were performed. Briefly, 10 μl of cell suspension was mixed with 65 μl of 0.7% (w/v) low-melting point agarose (Bio-Rad) and sandwiched between a lower layer of 1% (w/v) normal-melting-point agarose (Bio-Rad) and an upper layer of 0.7% (w/v) low-melting-point agarose on microscope slides (Carlo Erba). Two slides were prepared from each individual treatment. The slides were then immersed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) containing 10% DMSO and 1% Triton X-100 (ICN Biomedicals), overnight at 4 °C. On completion of lysis, the slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH ≥ 13) and left in the solution for 25 min at 4 °C to allow the DNA to unwind and to express the alkali-labile sites. Electrophoresis was carried out at 4 °C for 25 min, 30 V (1 V/cm) and 300 mA, using a Bio-Rad power supply. After electrophoresis, the slides were immersed in 0.3 M sodium acetate in ethanol for 30 min. Microgels were then dehydrated in absolute ethanol for 2 h and immersed for 5 min in 70% ethanol. Slides were air-dried at room temperature. Immediately before scoring, slides were stained with 12 μg/ml ethidium bromide (Boehringer Mannheim) and examined at ×400 magnification with an automatic image analyser

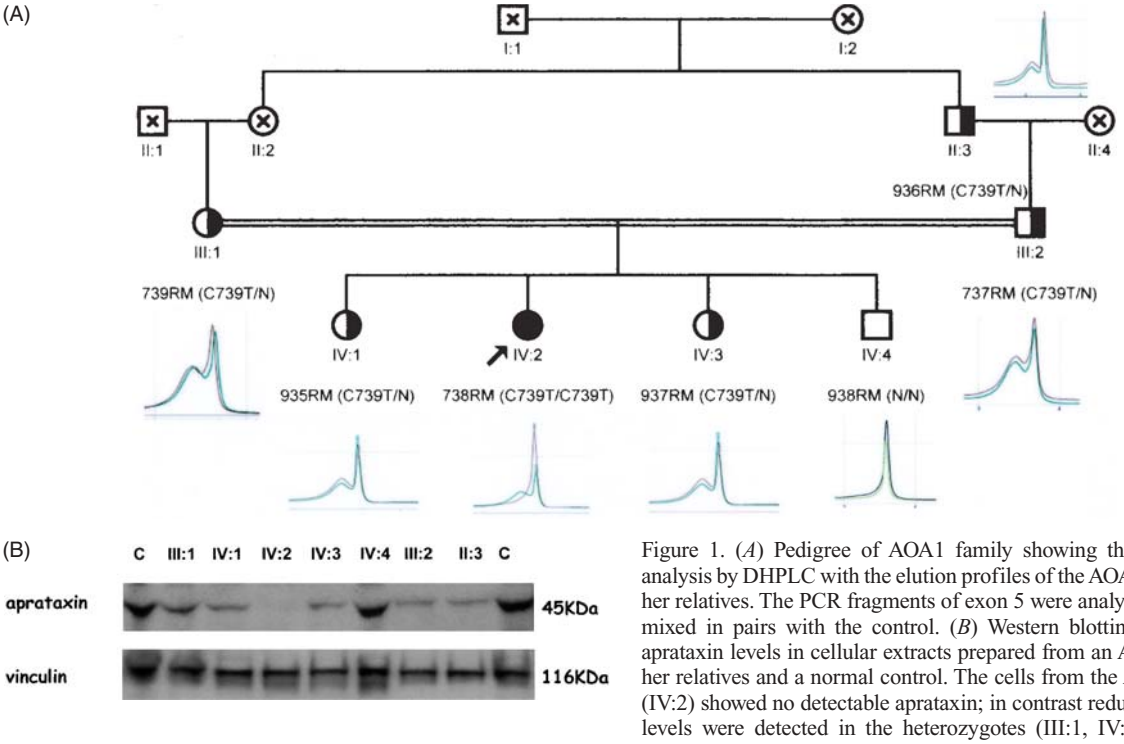

Figure 1. (*A*) Pedigree of AOA1 family showing the segregation analysis by DHPLC with the elution profiles of the AOA1 patient and her relatives. The PCR fragments of exon 5 were analysed alone and mixed in pairs with the control. (*B*) Western blotting analysis of aprataxin levels in cellular extracts prepared from an AOA1 patient, her relatives and a normal control. The cells from the AOA1 patient (IV:2) showed no detectable aprataxin; in contrast reduced aprataxin levels were detected in the heterozygotes (III:1, IV:1, IV:3, III:2 and II:3) and normal levels of aprataxin were present in the wild type brother (IV:4). I:1, I:2, II:1, II:2, II:4 were untested individuals; (C) normal control cell line

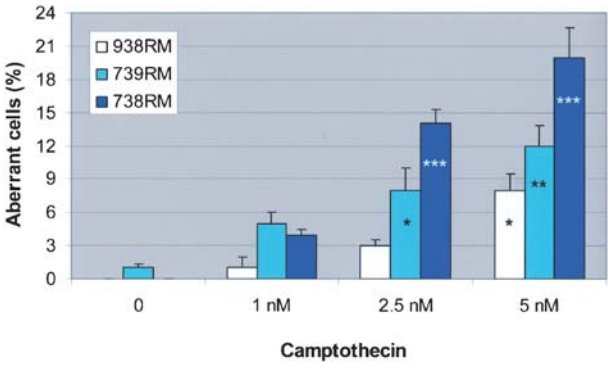

Figure 2. Frequencies of cells bearing aberrations induced by a 3-h treatment with different dose levels of CPT in the wild-type 938RM cell line, in the 739RM heterozygous cell line and in the 738RM homozygous cell line. Statistical significance was evaluated by Fisher's exact test: *p<0.05, **p<0.01, ***p<0.01.

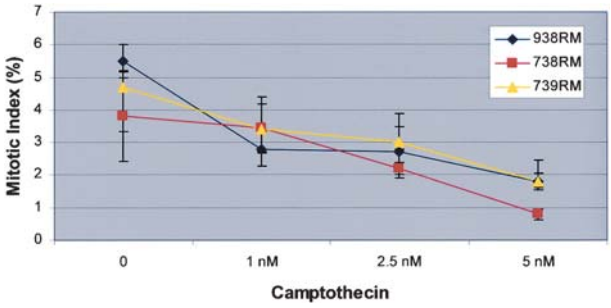

Figure 3. Mitotic index profiles following a 3-h treatment with CPT at 1, 2.5 and 5 μM in the homozygous 738RM cell line, in the heterozygous 739RM cell line and in the wild-type 938RM cell line.

(Comet Assay III; Perceptive Instruments) connected to a fluorescence microscope (Eclipse E400; Nikon). To evaluate the amount of DNA damage, computer-generated tail-moment values and percentage of migrated DNA were used. One hundred cells were scored for each individual treatment from two different slides.

Results

Mutation analysis

738RM was homozygous for the mutation C739T, a new mutation of the APTX gene leading to the nonsense variation R247X and to a truncated product with deletion of the HIT and zinc-finger domains of the protein. Mutation analysis in the relatives confirmed that the parents were heterozygous while the brother was wild type. Mutation analyses are summarised in figures 1, 2.

Aprataxin level

All human tissues and cell lines tested so far have shown the presence of two aprataxin transcripts, which correspond to the usage of alternative exon 3 acceptor splice sites. By Western blot analysis, a single band of 45 kDa corresponding to the long form of aprataxin was detected in normal control cells, as the short form is not detectable in this blot. LCLs from the AOA1 patient did not show a detectable amount of aprataxin; in contrast, reduced aprataxin levels were detected in her mother and normal levels of aprataxin were exhibited by her brother's cells (fig. 3).

Cytogenetic analysis

Following a 3-h treatment with CPT, marked and dose-related increases in chromosomal aberrations were observed in both the patient and the mother compared to the intrafamilial wild-type control (fig. 4). Statistical significance was achieved at the high and intermediate dose levels in both the homozygous patient and the heterozygote. In the normal individual, statistical significance was only achieved at the high dose level. In absolute terms, the homozygote showed approximately a doubling in the incidence of chromosomal aberrations at corresponding dose levels compared to the heterozygote values. Progression of cells towards mitosis monitored by the analysis of mitotic indices confirmed that 738RM is the more affected cell line at the high dose level (fig. 3).

Analysis of chromosomal aberrations induced by X-rays (table 1), which gives a mixture of lesions other than DNA single-strand breaks, including DNA double-strand breaks, base damage, and DNA-DNA and DNA-protein cross-links at any dose level, confirmed that cells from the patient were not hypersensitive to this agent. Although the total number of aberrant cells was very close between the normal, the homozygous and the heterozygous cell lines, the pattern of aberrations was different among them. As shown in table 1, the incidence of dicentric chromosomes was more than double in the normal cell line compared to the defective cells, while the incidence of chromatid exchanges was reversed, being double in the patient's cells versus the normal cells. The heterozygous cells showed intermediate values between normal and homozygous cells.

Comet assay

The accumulation of DNA strand breakage was studied by alkaline comet assay following the induction of 'protein-concealed' DNA single-strand breaks by CPT, and the kinetics of its repair recorded at 0, 1, 2, 4, 8, 12 and 24 h after the end of treatment in the 738RM, 739RM and 938RM cells (fig. 4). Following treatment with CPT at 5, 2.5 and 1 nM, marked and significant increases in the mean tail moment were observed between 0 and 1 h after treatment with 5 nM, between 1 and 2 h after treatment with 2.5 nM; and between 4 and 8 h after treatment with 1 nM in the homozygous cells compared to normal and heterozygous cells.

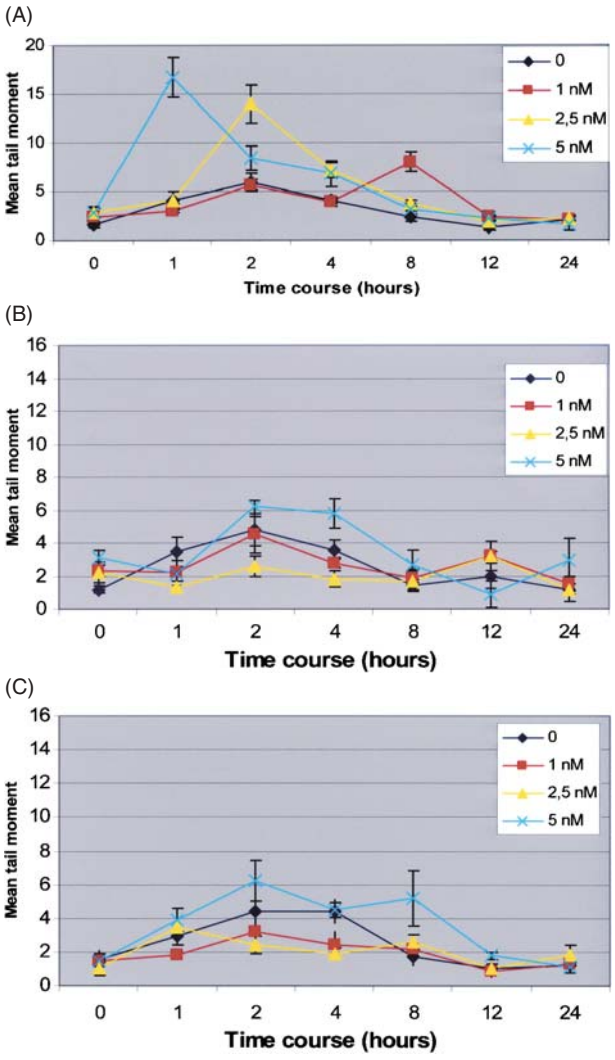

Figure 4. Alkaline 'comet assay'. DNA strand breakage (mean tail moment) and kinetics of its repair at 0, 1, 2, 4, 8, 12 and 24 h following treatments with CPT at dose-levels of 5, 2.5 and 1 nM in the homozygous 738RM cells (*A*), the heterozygote 739RMcells (*B*) and the wild-type 938RM cells (*C*).

Table 1. Frequencies of chromosome and chromatid-type aberrations induced by X-rays in the different LCLs cell lines.

Cell lines	Total number of aberrations (%)				Total aberrations	Aberrant cells (%)
	chromatid		chromosome			
	breaks	exchanges	breaks	dicentrics		
938RM						
0	0.0	0.0	0.0	0.0	0	0.0
3 Gy	30.0	24.0	38.0	52.0	144	84.0
738RM						
0	1.0	0.0	0.0	0.0	1	1.0
3 Gy	20.0	54.0	34.0	22.0	130	80.0
739RM						
0	0.0	0.0	0.0	0.0	0	0.0
3 Gy	24.0	44.0	42.0	38.0	148	80.0

Discussion

AOA1, an autosomal recessive ataxia characterised clinically by early onset of cerebellar ataxia, oculomotor apraxia and late peripheral neuropathy, shares some phenotypic similarities with AT, another rare human autosomal recessive disorder characterised by extreme sensitivity to IR and susceptibility to cancer. AT is caused by mutations in the ATM gene resulting after damage to abnormal responses for the recognition and processing of DNA double-strand breaks in DNA [5].

AOA1, however, is not sensitive to IR and does not show cancer proneness like AT, but is, rather, a syndrome specifically of the nervous system. The gene mutated in AOA1, APTX, has domains of homology with other proteins of the DNA SSBR system [1, 2].

Here, we showed that cells from an AOA1 patient with a new mutation in the APTX gene, T739C, resulting in a truncated product with the deletion of the HIT and zinc-finger domains of the protein, are hypersensitive in terms of the induction of chromosomal aberrations to the inhibitor of DNA topo I, CPT, a specific and powerful 'protein-concealed' DNA single-strand-break inducer.

As has been reported and already pointed out, CPT induces chromosomal damage through the production of DNA double-strand breaks generated by the collision of the stabilised 'cleavable complex' in the topo I reaction and the replicative fork of DNA synthesis [16–19]. Nevertheless, a number of CPT-induced topo I DNA single-strand breaks can become irreversible by collision of the 'cleavable complex' with the replication fork but can also be repaired by SSBR before becoming double-strand breaks. This process has been shown in cells with mutations in the XRCC1 gene which are hypersensitive to CPT even in the presence of the DNA polymerase inhibitor (APC), which protects them nearly completely from the lethality of CPT treatment. This protection is obtained through a reduction in DNA double-strand breaks formation in mammalian cells [25] or in cells over-expressing XRCC1 which are more resistant to CPT [26]. Our findings clearly indicate a direct involvement of APTX in the DNA SSBR machinery.

XRCC1 has recently been shown to interact with the N-terminal region of aprataxin containing the putative FHA domain and PARP-1 to bind to a region of the protein extending from the FHA to the HIT domains [8]. Both XRCC1 and PARP-1 play a central role in the repair of DNA single-strand breaks [27–29].

Our cytogenetic results overlap perfectly with the results obtained with the alkaline 'comet assay' where a clear dose-related and time-dependent accumulation of DNA damage induced by CPT was observed (fig. 4). In the patient, the result of the comet assay indicates the inability to 'process' CPT-induced DNA single-strand breaks, which are then converted into DNA double-strand breaks

by 'collision' of the cleavable complex with the replication fork and consequent formation of DNA double-strand breaks and chromosomal aberrations.

Interestingly, although the total number of aberrant cells following X-irradiation was very close between the normal, the homozygous and the heterozygous cell lines, the quality of the aberrations differed among them. As shown in table 1, in the normal cells, the incidence of dicentric chromosomes was more than double compared to that observed in the homozygote, while the incidence of chromatid exchanges was reversed, in the homozygote being double that in normal cells. The heterozygous cells showed intermediate values for these aberrations. These findings further corroborate our conclusion that APTX is directly involved in the machinery of DNA SSBR. In the wild-type cell line (938 RM), dicentric chromosomes are induced shortly after irradiation by fast repair of directly induced double-strand breaks. A slower component in the production of dicentrics is determined by repair of base damage by an excision process, for example, either through the action of a single-strand nuclease or as a consequence of damaged DNA bases close together on opposite strands of the DNA helix [30–32]. The absence of aprataxin in our patient's cells would probably affect the procession of base damage by efficient excision repair. Dicentric chromosomes could be generated only by fast repair of directly formed DNA double-strand breaks. The remaining lesions (base damages) persist up to DNA synthesis where they are repaired by homologous recombination with the consequent production of chromatid-type aberrations.

Finally, the data showing that the cells derived from the patient are not hypersensitive to the IR in terms of induction of chromosomal aberrations and radio-resistant DNA synthesis confirm previous results [8, 33]. Clements et al. [33], on the basis of the results of clonogenic assay, claimed for AOA1 a 'mild sensitivity' to IR. This result was not confirmed by Gueven et al. [8], suggesting that radiosensitivity might not be an intrinsic hallmark of AOA1 cells but rather a consequence of the different pattern of radiation-induced chromosomal aberrations on survival. In fact, in AOA1 cells we showed more chromatid- than chromosome-type aberrations compared to the wild type, indicating that the phenotypic homologies between AT and AOA1 patients underlie different etiopathogenetic mechanisms.

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