

## Clinical Short Communication

# Novel compound heterozygous mutations in a child with Ataxia-Telangiectasia showing unrelated cerebellar disorders



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## ABSTRACT

We report the case of a 6-year-old female patient with Ataxia Telangiectasia, an extremely rare condition, who developed in addition a left cerebellar astrocytoma and a right cerebellar infarction, considered as two independent events. Children with AT have an increased risk of developing cancer, but only few cases of glioma are reported and, at our knowledge, no other case of unrelated cerebellar glioma and cerebellar infarction in with the same AT patient have been described. The molecular analysis of *ATM* (Ataxia Telangiectasia Mutated) gene showed that the patient is compound heterozygote for two previously unreported mutations: c.3291delC (p.Phe1097fs) at exon 25 and c.8198A>C (p.Gln2733Pro) at exon 58. The role of the identified *ATM* gene mutations in the pathogenesis of Ataxia Telangiectasia and the coexisting cerebellar disorders is discussed.

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## 1. Introduction

Ataxia Telangiectasia [AT] is a rare, progressive neurodegenerative disorder caused by biallelic mutations in the *ATM* gene, located at 11q22–23 [1]. Homozygosity or compound heterozygosity for *ATM* mutations results in a multisystemic disorder, mainly involving nervous and immune system. AT patients showing the classical phenotype present with early onset ataxia, oculocutaneous telangiectasias, immunodeficiency, recurrent sinopulmonary infections, radiosensitivity and increased proneness to cancer, ranging from 10% to 25% [2,3,4]. The mechanism of malignancy in AT is considered primarily related to impaired DNA damage repair and genomic instability; however, the role of the various functions of *ATM* remain unclear. The most common malignancies in AT are hematologic tumours, with a temporal difference in the incidence of various cancers: acute lymphoblastic leukemias and Hodgkin lymphoma occur up to age 20 years, T-cell prolymphocytic leukemia and also solid tumours (breast, gastric, liver and thyroid) occur from age 20 years onward; the non-Hodgkin lymphoma shows a

continuous pattern of occurrence, starting before 10 years of age [5]. Only few cases of CNS cancer have been reported [6,7,8]. Here we describe the case of a 6-year-old girl with Ataxia Telangiectasia who developed a left cerebellar astrocytoma and a contralateral cerebellar infarction, the latter a previously unreported feature of AT. The possible coexistence of the two pathologies in relation to the *ATM* mutations identified in the proband is discussed.

## 2. Material and methods

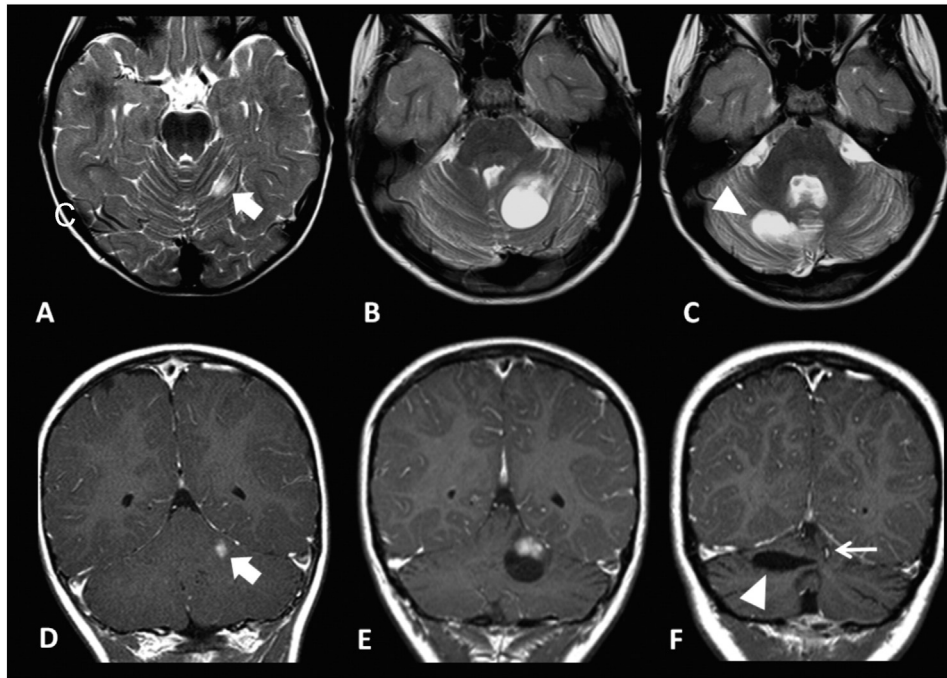
## 2.1. Case study

A 2-year-old female, who had uneventful antenatal and birth history, was referred to the Unit of Child Neurology of Spedali Civili, Brescia, Italy, for gait disturbance. On neurologic examination, ataxic gait and oculomotor apraxia were noted; laboratory examination revealed elevated serum  $\alpha$ -fetoprotein level (20 IU/ml). Following the clinical suspicion of AT, *ATM* gene sequencing was performed; the detection of a compound heterozygous genotype c.[3291delC];[8198A>C] confirmed the clinical diagnosis of Ataxia Telangiectasia. A brain magnetic resonance imaging (MRI) showed a small intraxial lesion in the cortex of the left cerebellar hemisphere, approximately 1 cm in diameter, with nodular enhancement after i.v. contrast medium administration and without mass effect (Fig. 1A and D); the lesion appeared stable on short-term MRI follow-up (2 months). The main diagnostic hypotheses

**Abbreviations:** AT, Ataxia Telangiectasia; *ATM*, Ataxia Telangiectasia Mutated gene; *ATM*, Ataxia Telangiectasia Mutated protein; CNS, Central Nervous System; LCLs, lymphoblastoid cell lines; MRI, magnetic resonance imaging; PBS, Phosphate Buffer Solution; PCR, Polymerase Chain Reaction; WB, Western Blot; IRIF, Irradiation-induced Foci.

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**Fig. 1.** Brain MRI, axial T2-weighted images (A, B, C) and coronal T1-weighted images after i.v. paramagnetic contrast medium administration (D, E, F). The first examination (September 2008, images A and D) shows a small intraxial lesion (thick arrows) in the cortex of the left cerebellar hemisphere, without mass effect, and with nodular enhancement after i.v. contrast medium administration. The main diagnostic hypotheses are a glial tumour, an inflammatory lesion, or a slow flow vascular malformation (capillary telangiectasia). In the second examination (September 2012, images B and E) the nodular enhancement in the left cerebellar hemisphere has grown and it is associated to a cyst with CSF-like signal: the findings are typical for pilocytic astrocytoma. In the post-surgery brain MRI (May 2013, images C and F) the pilocytic astrocytoma in the left cerebellar hemisphere is no longer visible (the small signal abnormality with faint enhancement [thin arrow] is a normal post-operative finding). A new lesion with CSF-like signal intensity, sharp margins, no mass effect and no enhancement after i.v. contrast medium administration appeared in the contralateral cerebellar hemisphere and adjacent vermis: the findings are consistent with focal cerebellar malacia, possibly due to a previous infarction.

were a glial tumour, an inflammatory/demyelinating lesion, or a slow flow vascular malformation (capillary telangiectasia). Longitudinal neurological and immunological evaluations with no signs of disease progression were performed up to the age of 6 years, when a follow-up brain MRI showed the enlargement of the lesion, with appearance of a cystic component and clear mass effect (Fig. 1B and E), consistent with a diagnosis of low-grade glioma. The patient underwent a complete surgical resection and a histologic diagnosis of pilocytic astrocytoma was achieved. The child quickly recovered from surgery, but 5 months later acute dysarthria and a worsening in ataxia, spontaneously resolved in 3 days, were noted. Brain MRI performed 1 month later revealed a malacic area in the right cerebellar hemisphere, contralateral to the resected glioma, consistent with a previous ischemic stroke (IS) (Fig. 1C and F). Common risk factor for IS, both genetic and acquired, were excluded.

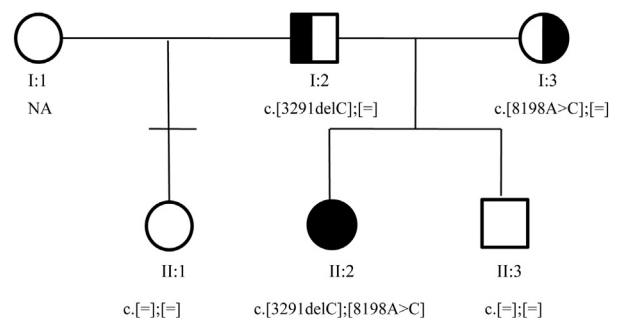
## 2.2. Molecular study

Genomic DNA was extracted from peripheral blood lymphocytes using standard organic extraction procedures. To screen for mutations in the *ATM* gene, all the exons and splice junctions were amplified using the primers designed by Bernstein et al. [9]. PCR reactions for direct sequencing of 50 ng of genomic DNA were done in 25 µl volumes containing 100 mM each of dNTPs, 0.15 mM of each primer and 0.70 units of Taq DNA polymerase (Perkin-Elmer/ABI) in PCR buffer. Purified PCR products were sequenced using the BigDye Terminator chemistry and an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). Mutations were named based on cDNA reference sequence U82828, with 1 + being the A of the initiation start codon. Prediction of mutations pathogenicity was performed using PolyPhen-2 (Polymorphism Phenotyping v2: <http://genetics.bwh.harvard.edu/pph2/>), SIFT (Sort in intolerant from tolerant: <http://sift.jcvi.org>), Mutation Taster (<http://www.mutationtaster.org>) and Align-GVGD (Align-Grantham Variation Grantham Deviation: [http://agvgd.iarc.fr/agvgd\\_input.php](http://agvgd.iarc.fr/agvgd_input.php)).

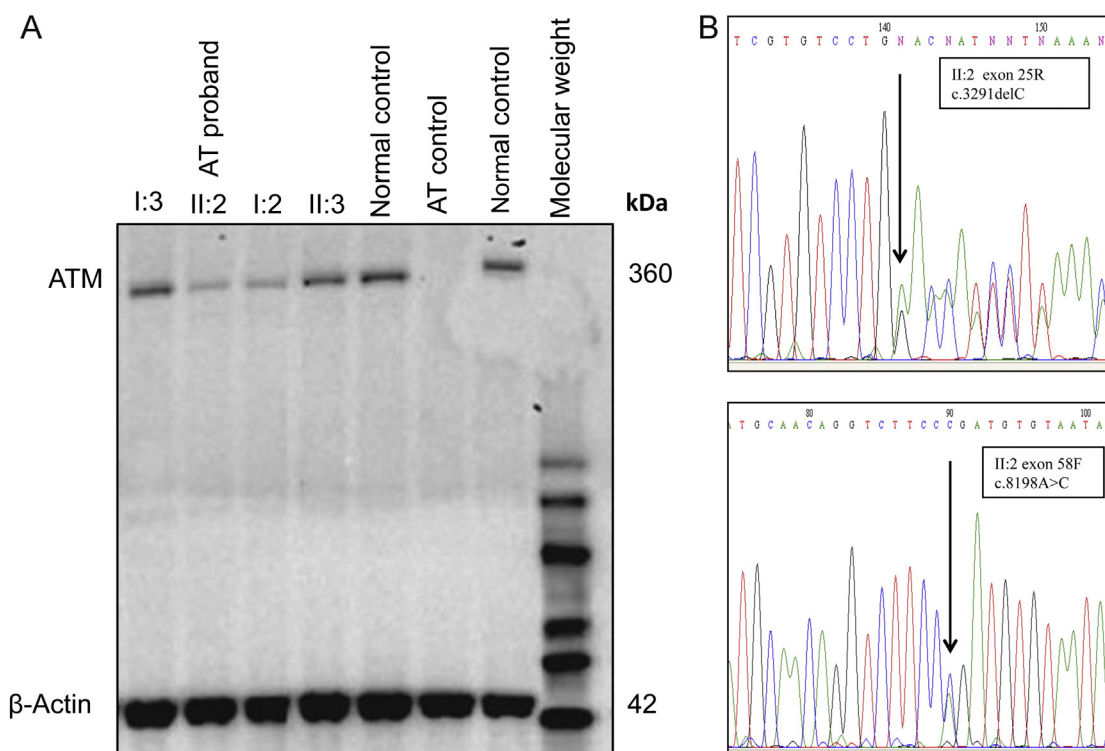
<http://sift.jcvi.org>), Mutation Taster (<http://www.mutationtaster.org>) and Align-GVGD (Align-Grantham Variation Grantham Deviation: [http://agvgd.iarc.fr/agvgd\\_input.php](http://agvgd.iarc.fr/agvgd_input.php)).

## 2.3. ATM level and kinase assay

ATM level and function were determined by Western Blot analysis (WB). Protein lysates were obtained from Epstein Barr virus-transformed lymphoblastoid cell lines (LCLs) of the AT proband, parents and control. LCLs were cultured in RPMI 1640 medium containing 20%



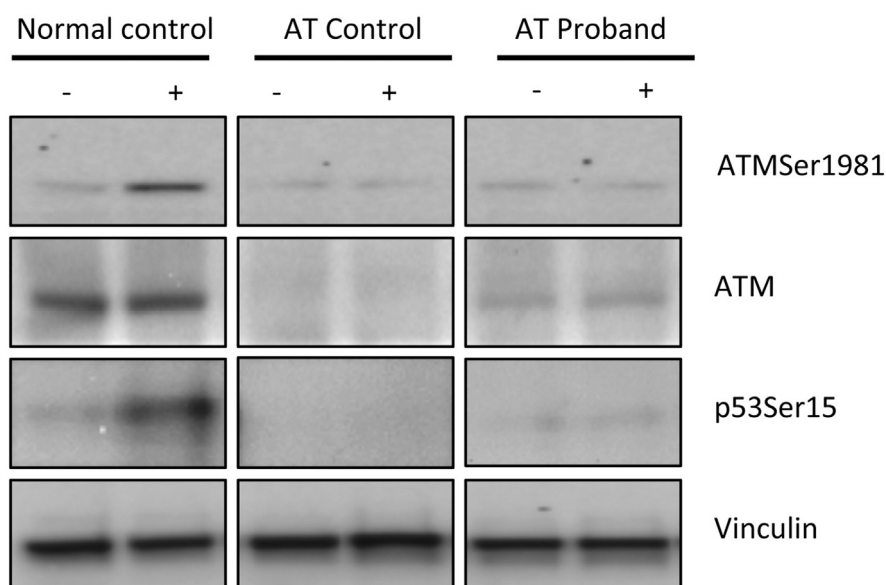
**Fig. 2.** Pedigree of the proband's family. The filled symbol denotes the affected patient who is compound heterozygous c.[3291delC];[8198A>C] in *ATM* gene, each of them inherited from one of the parents. The parents are heterozygous carriers (half-filled symbol): I:2 heterozygous for c.3291delC mutation and I:3 heterozygous for c.8198A>C. Square symbols denote males, the circles denote females. NA = not analyzed. c.[3291delC];[8198A>C] referred to II:1 and II:3, genotyped for mutations, denote normal coding DNA reference sequence on both chromosomes.



**Fig. 3.** Molecular study. (A) Western Blot analysis of ATM protein in LCLs lysates from: normal control, lanes 5 and 7; mother (I:3), lane 1; AT proband (II:2), lane 2; father (I:2), lane 3; brother (II:3) lane 4; AT control, lane 6. MagicMark (Invitrogen) lane 8. (B) Sequencing analysis. The mutations on electropherograms are indicated with black arrows.

foetal calf serum (Gibco BRL), 2 mM L-glutamine, 100 U/ml Hepes buffer (Fluka) and 100 U/ml streptomycin and maintained in a 37 °C incubator (Forma Scientific) in a 5% CO<sub>2</sub> atmosphere and 100% nominal humidity. To test whether expressed ATM protein was functional, the autophosphorylation of ATM-Ser1981 and phosphorylation p53Ser15 were determined by WB and phosphorylation of γ-H2AX by Irradiation-induced Foci (IRIF) assay. Cells were X irradiated in a Gilardoni RadGil instrument at a dose rate of 5 Gy and harvested after 1 h. Untreated and treated cells were washed with PBS plus 1 mM Na<sub>3</sub>VO<sub>4</sub>, pelleted

and lysed with RIPA buffer supplemented with Complete Mini EDTA-free protease Cocktail and PhosStop phosphatase inhibitor tablet cocktail (Roche Diagnostics, Burgess Hill, UK). Aliquots containing proteins (10 to 50 µg/ml) were size fractionated by Tris Acetate 3–8% gel electrophoresis (Invitrogen). After blocking with 5% BSA in phosphate-buffered saline plus 0.1% Tween (Invitrogen), the membranes were incubated with polyclonal antibody to ATM (a gift of D. Delia), ATM (phospho S1981) (AbCam 2888), Phospho-p53 (Cell Signaling 9284), anti-β-actin (A2066 SIGMA) and anti-vinculin (V9131 SIGMA) for



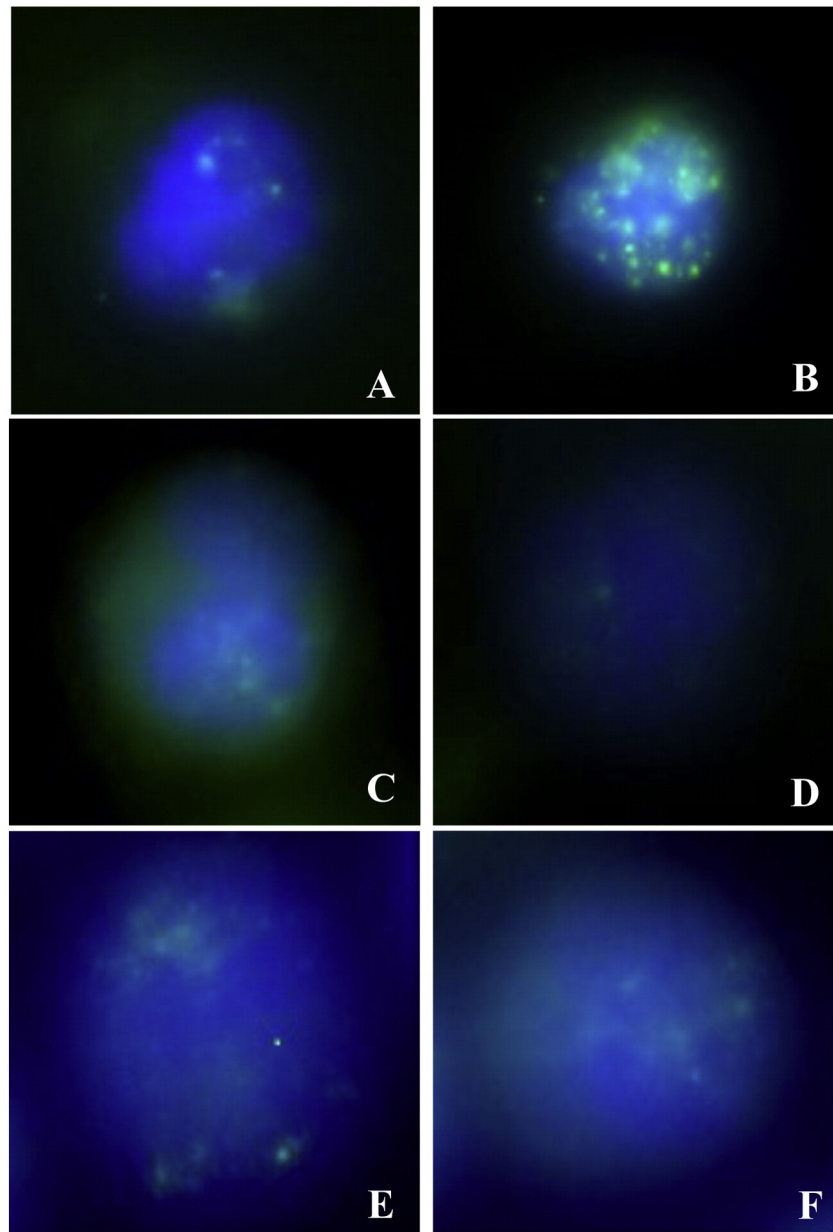
**Fig. 4.** Western Blot showing levels of ATM kinase activity in lysates from AT proband compared with AT and normal control. There are two lanes for each cell line, dependent on the absence (–) or presence (+) of ATM kinase activation by exposure of cells to 5 Gy. Normal cells show activation of ATM kinase by autophosphorylation of ATM Ser1981, and phosphorylation of p53Ser15. AT patient's cells show neither autophosphorylation of ATM Ser1981 nor ATM kinase activity for failure to phosphorylation of p53.

normalisation. After incubation with a peroxidase conjugated secondary antibody, the immunoreactive bands were visualised by ECL Supersignal on autoradiographic films. To examine by immunofluorescence the IRIF of  $\gamma$ -H2AX 1 h after irradiation (5 Gy) aliquot of the II:2, AT and normal control LCLs unirradiated and irradiated were re-suspended in 70% ethanol and maintained at 20 °C until the time of  $\gamma$ -H2AX immunostaining and analysis. After fixation the cells were washed with TST (Tris-HCl pH 7.5 plus 4% foetal bovine serum and 0.1% Triton X-100) and incubated for 1 h at 37 °C with antiphospho-H2AX monoclonal antibody (Millipore) diluted 1:500 in TST. After, cells were rinsed twice in TBS (Tris-HCl pH 7.5 plus 2% foetal bovine serum) and incubated for 1 h at 37 °C with goat anti-mouse IgG conjugated with FITC (Molecular Probes, Eugene OR, USA) diluted 1:500. Finally, after two washing in TBS, cells were counterstained with Vectashield with 0.2  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc. Burlingame, CA, USA). Slides were analyzed using an Olympus fluorescence microscope at 1000 magnification. Nuclei were classified as  $\gamma$ -H2AX positive or negative, positive nuclei being those with at least one large focus or a granular pattern of green

fluorescence. The analysis of the significance for  $\gamma$ -H2AX foci and of the effect of the X-rays versus the control samples was performed with the Student's *t*-test (ts) for paired samples ( $p < 0.01$ ).

### 3. Results

At sequencing the patient was found to be compound heterozygote c.[3291delC];[8198A>C] for two mutations in *ATM* gene (Fig. 3B), never described previously in AT patients (<http://chromium.liacs.nl/LOVD2/home.php>, <http://www.hgmd.cf.ac.uk/ac/index.php>). The c.3291delC mutation located in exon 25 of *ATM* gene is predicted to lead to a frame-shift with a premature termination codon (p.Phe1097fs), resulting in a truncated protein. The missense mutation c.8198A>C in exon 58 leads to a Gln to Pro non-synonymous substitution at position 2733 (p.Gln2733Pro) in the phosphatidylinositol-3 kinase domain of ATM protein. Scores from SIFT, Polyphen and Mutation Taster (0.00, 0.903 and 76 respectively) were indicative of a deleterious variant. In contrast, Align-GVGD predicts this missense mutation as neutral (Class C0).



**Fig. 5.** Irradiation-induced Foci (IRIF) within 1 h after 5 Gy irradiated cell.  $\gamma$ -H2AX foci in LCLs from normal control (A, B), AT control (C, D), AT proband II:2 (E, F).

WB for ATM protein expression showed a band of 360 kDa corresponding to the size of ATM protein in normal control cell line. The cells from the AT proband (II:2) when compared with the normal control show an amount of 50% of ATM protein. The analysis was performed also in the cells from the patient's father (I:2), heterozygous for the c.3291delC null mutation, and from the mother (I:3), heterozygous for the c.8198A>C missense mutation; they showed respectively 50% and normal amount of ATM protein (Fig. 3A). The kinase assay performed by WB on LCLs from the proband and controls at 1 h after a dose of 5 Gy X-rays showed no detectable ATM pSer1981 autophosphorylation and absence of phosphorylation in p53 Ser15 in the proband (Fig. 4). The phosphorylation of H2AX was performed in normal control, AT control and AT proband (II:2) LCLs at 1 h after a dose of 5 Gy X-rays. Respect to their relative un-irradiated controls, the irradiated samples (Fig. 5) show a statistically significant ( $p < 0.01$ ) induction of  $\gamma$ -H2AX foci/cell only in the wild type cell line, while in both the AT control and in the AT proband cell lines no increase was observed (Fig. 6).

#### 4. Discussion

The increased risk of developing cancer in AT patients has triggered several investigations on the role of *ATM* mutations in the pathogenesis of neoplastic lesions both in patients and in sporadic tumours. It is well known that ATM protein plays a key role in regulating the cell cycle and repairing DNA double-strand breaks (DSBs) [10,11,12]. Errors in the repair of DSBs, the major form of DNA damage, give rise to small deletions or insertions at the lesion site that may result in chromosomal translocations and genomic instability and finally in cancer [12].

In our patient the sequencing of *ATM* gene showed a compound heterozygous genotype c.[3291delC];[8198A>C] for a null and a missense mutation (Fig. 3B). At our knowledge, none of these mutations have been previously reported in AT patients. The c.3291delC (Fig. 2C) is a frameshift mutation in the exon 25 of *ATM* gene predicted to lead to the premature termination of the translation (p.Phe1097fs), resulting in the absence of expression of ATM protein. Cells from the proband's father, heterozygous for this mutation, showed only 50% of the normal amount of ATM protein, consistent with the presence of a null allele (Fig. 3A).

The second mutation was an A to C transition at nucleotide 8198 in exon 58 (Fig. 3B) that leads to a glutamine to proline substitution

(p.Gln2733Pro) in the PI3-kinase domain of ATM protein. This mutation is not present in 200 control chromosomes that we have screened by DHPLC analysis. Using Align-GVGD algorithm, which predicts substitution effects based on assessment of chemical dissimilarity between residues and phylogenetic conservation, this variant is considered unlikely to be pathogenic (Class C0), but in silico tools SIFT, Polyphen and Mutation Taster classify this mutation as probably damaging and not tolerated. LCLs derived from our proband show the presence of mutant ATM protein (Fig. 3A) without detectable kinase activity. Fig. 4 shows the absence of autophosphorylation at ATM Ser1981 and the absence of phosphorylation in p53Ser15 in the proband cell line when compared to AT and normal control. The results of X-ray-induction of  $\gamma$ -H2AX detected by immunocytochemistry and fluorescence microscopy in the LCLs clearly show an enhancement of  $\gamma$ -H2AX foci in presence of a functional ATM protein and no induction when ATM is absent, as well as in the cell line with the missense/truncation mutations. The phenotypic manifestations in Ataxia Telangiectasia show a continuous spectrum from severe classical AT to a relatively mild disorder depending on the presence of ATM protein and kinase activity. The allelic heterogeneity in *ATM* gene results in clinical heterogeneity: age of onset, clinical presentation, occurrence and type of tumours in AT patients depends on the type of mutations and retention or absence of kinase activity of ATM protein [13,14]. The development of tumours in childhood (lymphoid and brain) is associated almost exclusively with absence of ATM kinase activity [15].

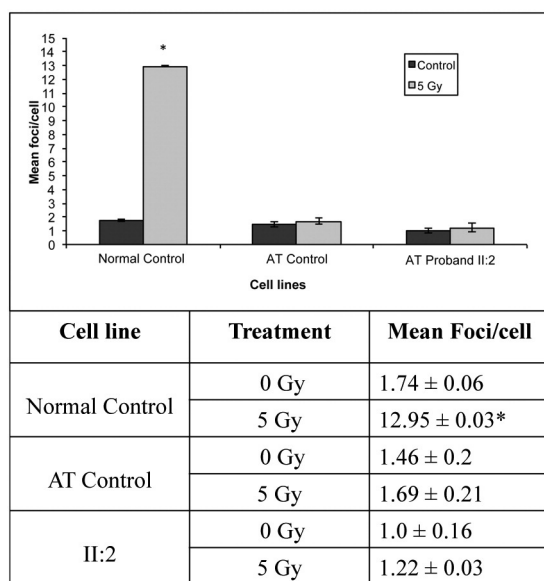
The cancer predisposition particularly to lymphoid tumours in AT has been known since the time of the full description of the disorder. In a retrospective analysis of 279 AT patients were reported 69 patients (24.5%) with cancer, the majority hematologic cancers [5]. Reiman et al. [15] presented similar observations, with cancer occurring in 25% of 296 AT patients analyzed; primarily lymphoma or leukemia, but also breast, gastric and other solid tumours. The reported incidence of various cancer types in AT is 13% for non-Hodgkin's lymphoma, 2–8% for leukemia, 4% for Hodgkin's lymphoma, 2–3% for breast cancer, <1% for pancreatic carcinoma, <1% for thyroid carcinoma. Single case of adenocarcinoma of stomach, dysgerminoma, gonadoblastoma, medulloblastoma and colon cancer were described [5,15,16,17].

In our study we present the case of a young AT patient with a left cerebellar astrocytoma and a right cerebellar infarction. Approximately one-third of AT patients develop a malignancy during their lifetimes, usually with a characteristic trend: hematopoietic and lymphoid neoplasms account for the majority of neoplastic lesions in younger patients, while a wide range of solid tumours have been reported after the first 15 years of life (dysgerminomas, breast, gastric, pancreatic and hepatic carcinomas) [7,8]. Our AT patient developed a primary CNS tumour earlier than the expected age, since it was already detected by the first MRI when she was 1y7m old. Moreover, though low-grade cerebellar astrocytomas represent 12–18% of all paediatric brain tumours [18], they are an uncommon condition in patients with AT [15]. Concerning the CNS, at our knowledge only few cases of medulloblastoma, craniopharyngioma and glioma have been reported in the literature as associated with AT [6,7,8]. After 5 months from the complete surgical resection and recovery, our patient developed an ischemic infarction in the contralateral cerebellar hemisphere, considered as an independent event, as the post-operative brain CT performed the same day of surgery did not show any hematoma or ischemia in the right cerebellar hemisphere.

This study reports for the first time a case of AT young patient with cerebellar astrocytoma complicated by contralateral cerebellar infarction and expands the phenotypic and mutational spectrum of Ataxia Telangiectasia.

#### Funding

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**Fig. 6.** Induction of  $\gamma$ -H2AX foci in LCLs from AT proband (II:2), AT and normal control. Histogram showing the level of induction of  $\gamma$ -H2AX foci; the columns represent the mean value of positive cells from un-irradiated and irradiated control cells and AT proband at 1 h after 5Gy irradiation. \*Student's *t*-test, X-rays versus control,  $p < 0.01$ .

## Conflict of interest

The authors report no conflicts of interest.

## Acknowledgments

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