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Evidence of pathogenicity for the leaky splice variant c.1066-6T>G in *ATM*

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Abstract

Mild clinical phenotypes of ataxia-telangiectasia (variant A-T) are associated with biallelic *ATM* variants resulting in residual function of the ATM kinase. At least one regulatory, missense, or leaky splice site mutation resulting in expression of ATM with low level kinase activity was identified in subjects with variant A-T. Studies on the pathogenicity of the germline splicing *ATM* variant c.1066-6T>G have provided conflicting results. Using whole-exome sequencing, we identified two splice site *ATM* variants, c.1066-6T>G; [p.?], and c.2250G>A, [p.Ile709_Lys750del], in a compound heterozygous state in a 27-year-old woman who had been diagnosed as having congenital ocular motor apraxia type Cogan in her childhood. Reappraisal of her clinical phenotype revealed consistency with variant A-T. Functional analyses showed reduced expression of ATM protein and residual activity of the ATM kinase at a level consistent with variant A-T. Our results provide evidence for pathogenicity of the leaky *ATM* splice site variant c.1066-6T>G.

KEYWORDS

ataxia telangiectasia, ataxia-telangiectasia mutated gene, IVS10-6T>G, leaky splice site variant, ocular motor apraxia, variant A-T

1 | INTRODUCTION

Ataxia-telangiectasia (A-T) (MIM 208900) is a rare autosomal recessive disorder clinically characterized by early-onset ataxia, global developmental delay, ocular motor apraxia, oculocutaneous telangiectasia, immunodeficiency, radiation sensitivity, and susceptibility to malignancy, particularly leukemia and lymphoma (Gatti & Perlman, 1999; Rothblum-Oviatt et al., 2016; van Os et al., 2017). A-T is caused by biallelic variants in the *ATM* gene on chromosome 11q22.3. The ataxia

telangiectasia-mutated (ATM) protein, a serine–threonine protein kinase, plays an important role in the cellular response to DNA double strand breaks (DSB) and control of the cell cycle. Cellular processes induced by DSB ATM activation include chromatin modifications via KAP1 phosphorylation, cell-cycle arrest, and apoptosis via CHEK2 phosphorylation (McKinnon, 2012). The classic phenotype of A-T, a severe multisystem condition with a progressive, neurodegenerative course, is due to biallelic *ATM* variants resulting in the loss of ATM kinase activity (Verhagen et al., 2012).

A milder type of A-T designated "variant A-T" is caused by biallelic variants with at least one missense or splice site mutation resulting in expression of ATM with some residual kinase activity (Dörk, Bendix-Waltes, Wegner, & Stumm, 2004; Schon et al., 2019; Sutton et al., 2004; Taylor, Lam, Last, & Byrd, 2015; Verhagen et al., 2012). Previous reports described a wide range of *ATM* missense and leaky splice site mutations, associated with relatively mild phenotypes (Schon et al., 2019). Due to the atypical presentation, these phenotypes often go undiagnosed for years, and many subjects with variant A-T receive the correct diagnosis only in adulthood (van Os et al., 2019).

As part of an ongoing study of clinical, neuroimaging and genetic features in subjects diagnosed as having "congenital ocular motor apraxia" (COMA) (Wente et al., 2016), we used WES and identified two splicing *ATM* variants, c.1066-6T>G and c.2250G>A, in a compound heterozygous state in a 27-year-old woman. This case is of special interest since c.1066-6T>G, also known as IVS10-6T>G, is a relatively common and controversially debated leaky splicing variant (Broeks et al., 2003). We found a marked decrease in the expression level of the ATM protein and its radiation-induced kinase activity in lymphoblastoid cells from this subject, indicating pathogenicity of both variants.

2 | METHODS

2.1 | Editorial policies and ethical considerations

The study was approved by the ethics committee of the Faculty of Medicine, University of Göttingen (file no. 19/5/14).

2.2 | Patient consent

Written informed consent was obtained from the affected subject and her parents.

2.3 | Subject

This 27-year-old woman (#18 in Wente et al., 2016) is the first child of healthy nonconsanguineous parents of German origin. A younger brother is healthy. Ocular motor apraxia (OMA) affecting horizontal saccades was noted at 4 months of age. Her motor development was delayed, she showed early-onset ataxia and did not walk without support until age 24 months. During childhood learning disability, short stature, kyphoscoliosis, and fatigue became apparent. OMA and ataxia persisted, but without any evidence for deterioration. She could never ride a bicycle, and at age 20 years vertical saccades were compromised as well. There were no telangiectasia, choreoathetosis, or signs of immunodeficiency. Presently, she still is fully ambulatory without support and leads an independent life in her own apartment, working part-time as an assistant carer for the elderly. MRI of the brain at age 19 years was normal, notably there was no cerebellar

atrophy. At age 26 years, serum AFP was mildly elevated to 6.6 U/mL [<5.4].

2.4 | Whole-exome sequencing

Whole-exome sequencing (WES) was performed in the affected subject and both parents. Exonic and adjacent intronic sequences were enriched from genomic DNA using the NimbleGen SeqCap EZ Human Exome Library v2.0 Enrichment Kit and were run on an Illumina HiSeq4000 sequencer at the Cologne Center for Genomics, Germany. Data analysis and filtering of mapped target sequences were performed with the "Varbank" exome analysis pipeline. Data were filtered for high-quality variants. The *ATM* variants were confirmed by Sanger sequencing.

2.5 | Functional analysis

We established a lymphoblastoid cell line (LCL) from peripheral white blood cells of the patient using EBV immortalisation. Patient cells were analyzed in comparison with LCLs from a classic A-T patient (negative control) and with LCLs from a healthy donor without *ATM* mutation (positive control). Whole cell extracts were prepared and used for immunoblotting as previously described (Dörk et al., 2004). Immunoreactivity of the ATM protein was tested using rabbit monoclonal antibody 1549-1 (Epitomics) against ATM. We then tested whether the residual ATM protein could be induced to phosphorylate the two targets KAP1 and CHEK2 after irradiation with 6 Gy. The antibodies used were pSer19-CHEK2 (rabbit polyclonal, Cell Signaling) and pSer824-KAP1 (rabbit polyclonal, Bethyl Laboratories). Immunoreactivity toward β-actin as loading control was tested using monoclonal mouse antibody AC15 (Sigma). Anti-mouse and anti-rabbit horseradish peroxidases labeled secondary antibodies were purchased from GE Healthcare. Enhanced chemiluminescence (Dura ECL, Thermo Scientific/Pierce) was used for visualization of immunoreactive bands.

3 | RESULTS

Trio-based whole-exome sequencing of the index patient and her parents excluded de novo and biallelic variants in line with the reported parental nonconsanguinity. Analysis for compound heterozygosity led to the identification of two *ATM* variants in genomic DNA derived from peripheral blood lymphocytes from the affected subject, c.1066-6T>G [p.?], inherited from the mother, and c.2250G>A [p.Ile709_Lys750del], inherited from the father. To assess residual ATM function associated with these variants, we established an EBV-immortalized lymphoblastoid cell line from this patient. We then examined whole cell protein extracts for levels of ATM protein and for radiation-induced phosphorylation of ATM targets.
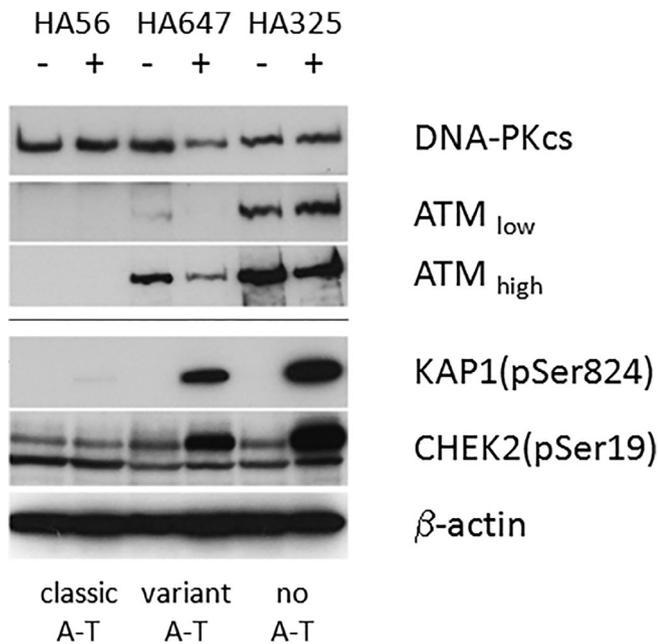
HA56 HA647 HA325
- + - + - +

DNA-PKcs

ATM low

ATM high

KAP1(pSer824)

CHEK2(pSer19)

β-actin

classic variant no
A-T A-T A-T

FIGURE 1 ATM protein levels and kinase activity. Westernblot analysis was performed for total protein extracts from three lymphoblastoid cell lines established from a patient with classic A-T and two frameshift variant in *ATM* (HA56), from a healthy control with no pathogenic *ATM* variant (HA325) and from the patient described in the present study (HA647). Cell lines had been untreated (−) or irradiated with 6 Gy ionizing radiation (+) at 30 min before protein extraction. Immunoreactivity was assessed for ATM, with DNA-PK$_{cs}$ as loading control, and for ATM kinase substrates KAP1 (pSer824) and CHEK2(pSer19), with β-actin as a loading control. ATM results are shown at two different exposure times (ATM$_{low}$, ATM$_{high}$)

Immunoblots revealed a well detectable and variable immunoreactivity of the ATM protein in the LCLs from the patient that was reduced to about 10–30% of the wildtype level from a control cell line, while no ATM was found in the LCLs from a patient with classic A-T (Figure 1). At 30 min after irradiation with 6 Gy, we found phospho-specific immunoreactivity for the two targets KAP1(Ser824) and CHEK2(Ser19) at levels of approximately 30% of wildtype ATM activity (Figure 1) while almost no activity was found in LCLs from classic A-T. This pattern of reduced ATM protein levels and ATM kinase activity strongly indicated a functional deficiency, since A-T heterozygous LCLs show near-normal ATM levels and activity that come close to wildtype homozygotes under these conditions (Fernet, Moullan, Lauge, Stoppa-Lyonnet, & Hall, 2004; Pietrucha et al., 2017).

4 | DISCUSSION

Whole exome sequencing in this 27-year-old subject initially presenting as "congenital ocular motor apraxia" revealed two splicing *ATM* variants, c.1066-6T>G and c.2250G>A, in a compound heterozygous state. Reappraisal of clinical features in this patient showed that early-onset OMA, early-onset ataxia, developmental delay, learning

disability, and short stature are consistent with A-T. However, several other features typically observed in A-T are lacking: conjunctival telangiectasia, dystonia, or choreoathetosis are not apparent, and the subject is not sensitive to infections. There is no evidence for a progressive disorder. Neuroimaging was normal without cerebellar atrophy. As A-T was not considered clinically as a differential diagnosis, serum AFP was only investigated after detection of the *ATM* variants and found to be mildly elevated. Follow-up measurements of AFP in patients with classical A-T showed that AFP levels rise with age (Stray-Pedersen et al., 2007). Therefore, an earlier investigation of the AFP level in this woman may have been deceptively normal.

The splice-donor site variant c.2250G>A is silent at the coding level but alters the final nucleotide of exon 14. It leads to skipping of exon 14 and thus reduces ATM protein expression (Sandoval et al., 1999). This variant was observed in a homozygous or compound heterozygous state in several patients with A-T diagnosed during childhood.

The splice-acceptor site variant c.1066-6T>G leads to incorrect splicing (leaky splicing) of exon 11 and thus to exon skipping, inducing a frameshift and thereby premature protein truncation, though this aberrant splicing is inefficient and leaves some 10–30% of residual full-length ATM RNA (Dörk et al., 2001, our unpublished data). The residual ATM expressed from the c.1066-6T>G leaky splice site mutation is presumed to be normal full length ATM. Whether the c.1066-6T>G variant is disease-causing has been a longstanding matter of debate. Although c.1066-6T>G homozygotes have been described among classic A-T patients (Dörk et al., 2001; Fiévet et al., 2019; R. Gatti pers. comm. 2008), they all harbored second-site *ATM* variants which foiled assessment of the pathogenicity of the c.1066-6T>G variant. For instance, the first A-T patient described with c.1066-6T>G had a full-blown disease which was inconsistent with the leakiness of the splicing variant and with the relatively high frequency of c.1066-6T>G in the general population (Broeks et al., 2003; Dörk et al., 2001). Reevaluation of that patient some years later uncovered a second-site truncating variant that had initially escaped detection by the single-stranded conformation polymorphism analyses at that time and explains the severe A-T phenotype in that patient. Our new case in the present study supports the view that c.1066-6T>G alone does not cause a classic course of A-T. Conflicting data have also been obtained for the estimated breast cancer risk in c.1066-6T>G heterozygotes, and it has been suggested that c.1066-6T>G may be a low-penetrance variant (Broeks et al., 2003).

If so, it would be predicted that compound heterozygosity for c.1066-6T>G and a classic pathogenic *ATM* variant causes a more severe phenotype than the homozygous state of c.1066-6T>G. Indeed, c.1066-6T>G was reported in conjunction with the c.9022C>T (p.Arg3008Cys) variant in two siblings with variant A-T at ages 22 and 20, diagnosed with gait ataxia or dystonia, respectively, but without immunodeficiency and with normal serum AFP (Albertyn et al., 2010). Furthermore, c.1066-6T>G in conjunction with c.9022C>T was reported in a 48-year-old patient with a milder phenotype of A-T and multiple myeloma (Austen et al., 2008). The vector-expressed Arg3008Cys mutant ATM protein showed absence of ATM kinase

activity, whereas residual ATM kinase activity was detected in cells of this patient, possibly due to leaky expression from the c.1066-6T>G allele. The authors concluded that the partial ATM function is consistent with leaky expression of a low level of normal ATM from this allele, resulting in a milder A-T phenotype and relative longevity in this patient (Austen et al., 2008).

In the case presented here, c.1066-6T>G is located *in trans* with a known disease-causing allele, c.2250G>A, in a patient with variant A-T and no other pathogenic *ATM* allele detected after exome sequencing. The results of our functional analysis are consistent with observations reported previously in patients with variant forms of A-T (Dörk et al., 2004; Taylor et al., 2015; van Os et al., 2019) and with previously described patients who were reported with the c.1066-6T>G variant (Albertyn et al., 2010; Austen et al., 2008). This provides strong evidence for the pathogenicity of the c.1066-6T>G allele, although the attenuated and partial expression of clinical symptoms is indicative of a reduced penetrance and reduced expressivity of this variant. Given that c.1066-6T>G (rs201686625) occurs at a carrier frequency of 1/200 in some European populations (Broeks et al., 2003), compound heterozygosity for c.1066-6T>G with a severe A-T causing variant could be as common as classic A-T in those countries, but an accurate diagnosis may be hampered when some of the lead symptoms are missing.

Early-onset—but not *infantile-onset*—OMA is well known to occur in A-T, hence, this differential diagnosis was not considered in this patient. Ataxia is generally regarded as the presenting sign in A-T, and OMA usually emerges only at the age of 2–4 years (Lewis, Lederman, & Crawford, 1999; Taylor et al., 2015). Onset of OMA in infantile age, as observed in this patient at age 4 months, is unusual. Variant A-T should be considered as differential diagnosis in every child with early-onset OMA, even if cerebellar atrophy is lacking and serum AFP is normal or only mildly elevated, as an early diagnosis of A-T entails ample consequences for the medical management of patients and their families. Many *ATM* mutated heterozygotes also have an increased cancer risk and should be advised to participate in intensified tumor surveillance programs (van Os et al., 2016).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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