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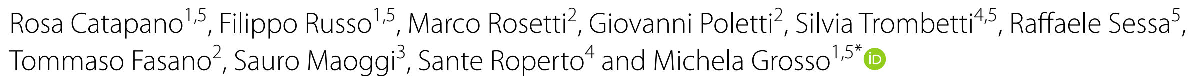

Identification of a novel ATR-X mutation causative of acquired α-thalassemia in a myelofibrosis patient

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Dear Editor,

Acquired alpha-thalassemia mental retardation X-linked (ATRX) mutations are associated with the onset of α-thalassemia in several hematological malignancies including myelodysplasia, acute lymphoblastic leukemia, myelofibrosis, essential thrombocythemia, and acute myeloid leukemia (acquired α-thalassemia myelodisplastic syndrome, ATMDS) [1]. The ATRX gene (NM_000489.6) is located at Xq21.1 and encodes a chromatin remodeling protein which contributes to regulate the structure and function of chromatin in centromeric heterochromatin and telomeric domains to control different cellular pathways including DNA damage response and senescence mechanisms [2, 3]. ATRX is also involved in the epigenetic regulation of α-globin genes: loss-of-function mutations in the ATRX gene cause the transcriptional repression of the α-globin gene (HBA), thus resulting in a decreasing production of α-globin chains [4]. In this regard, mutations of the ATRX gene have been reported in association with a rare inherited pathology called X-linked α-thalassemia and mental retardation syndrome (or ATR-X syndrome) characterized by mental retardation, facial and urogenital abnormalities along with an α-thalassemia trait with elevated levels of β-globin or γ-globin tetramers (HbH or Barts' hemoglobin), the amount of which is directly related to the severity of the α-globin chain deficiency [5].

Here we report a novel single-nucleotide variant (SNV) in the *ATRX* gene, found by Next-Generation Sequencing (NGS) analysis in a 77-year-old Italian man previously healthy who had been hospitalized for myelofibrosis and was referred to our Centre to investigate the possible genetic cause of an acquired form of α-thalassemia with elevated levels of HbH. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Naples Federico II (project approval number 443/21). Genomic DNA was extracted using the Nucleon BACC3 kit (GE Healthcare, Life Sciences, Chicago, IL, USA) and analyzed by a customized NGS gene panel recently developed by our group to identify acquired or inherited mutations associated with thalassemic disorders. The DNA libraries were prepared with the SureSelect$^{XT\ HS}$ Target Enrichment System kit (Agilent Technologies, Santa Clara, CA, USA) after enzymatic fragmentation and according to the manufacturer's protocol. Library quality and quantity were checked with the TapeStation system

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(Agilent Technologies) and Qubit dsDNA High Sensitivity assay kit on Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Libraries were sequenced with MiSeq Reagent Kit v2 (300-cycles) by loading a concentrated pool (9 pM) and 1% Phix on a MiSeq Illumina® instrument (Illumina; San Diego, CA, USA). To exclude any kind of contamination, a blank negative control was included, and it followed all procedure's steps, from DNA extraction to sequencing. Data analysis was performed using Alissa Report v1.1.6–2023-03 and Alissa Interpret v5.4.2 software (Agilent Technologies) and revealed the presence of a T > G transition at codon 520 in exon 7 of the *ATRX* gene (c.520T > G) with a variant allele frequency of 89.9% (179/199 variant coverage) which deviates from the expected values for germline mutations, thereby in agreement with the acquired origin of the variant. This SNV leads to a missense p.Cys174Gly mutation in the PHD-like domain, a hot-spot region for ATMDS defects [1, 6]. The mutation was confirmed by Sanger sequencing (Fig. 1A). NGS and MLPA analysis also excluded the presence of point mutations or large deletions in the α-globin gene cluster that are responsible of inherited α-thalassemia (Fig. 1B) [7].

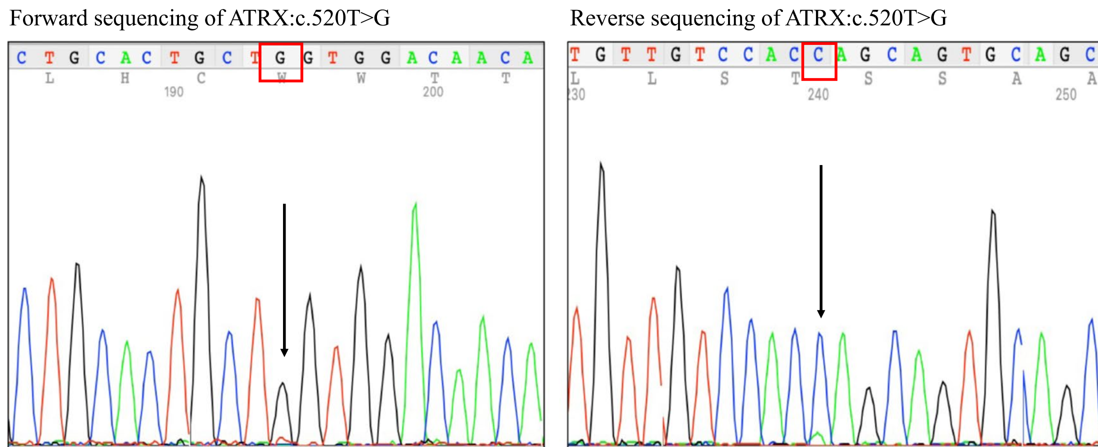
To our best knowledge and according to GnomAD exome, GnomAD genome, and ClinVar databases, this SNV is an unreported variant in the *ATRX* gene. Thirteen out of 18 *in-silico* prediction tools (CADD, Polyphen2 HVAR, Polyphen2 HDIV, FATHMM, M-CAP, MutPred, MVP, FATHMM-MKL, LRT, PrimateAI, PROVEAN, SIFT, SIFT4G) supported the possible pathogenicity of this SNV, whereas other five tools (BLOSUM, DANN, DEOGEN2, LIST-S2, MutationTaster) classified it as of uncertain significance (Table 1). In addition, six different meta-scores for *in-silico* pathogenicity assessment determined a very strong, strong, or moderate pathogenic prediction, basing on multiple tools as reported in Table 1. Furthermore, an analysis of base conservation scores on 99 vertebrate genome sequences aligned to the human genome (represented by PhyloP100way scores provided by the VarSome platform, https://varsome.com/about/resources/acmg-implementation) revealed that c.520T is a highly conserved nucleotide in the human genome, as represented in Fig. 1C. Indeed, this mutation falls in the PHD-like region of the protein, a functional domain where several other ATMDS mutations have been identified so far [8]. Based on this information, we classified this mutation as potentially pathogenic. In fact, according to the criteria of the American College of Medical Genetics and Genomics (ACMG), the detected SNV met three criteria which allow to establish its pathogenicity [9]: first, there are several computational systems supporting a possible deleterious effect of this mutation (PP1 rule); secondly, this mutation is located in a mutational hotspot genomic area (PM1 rule); finally, no frequency data for this sequence variation are reported in the main genetic databases, such as the Exome Sequencing Project, 1000 Genome Project, or the Exome Aggregation Consortium (PM2 rule).

In conclusion, here we report a novel ATRX mutation in a patient with myelofibrosis in which the onset of HbH disease can be explained by impaired ATRX functions leading to altered expression of the α-globin genes. This report contributes to better define the ATRX gene mutational spectrum, with the purpose of improving genetic screening and diagnosis of rare diseases.

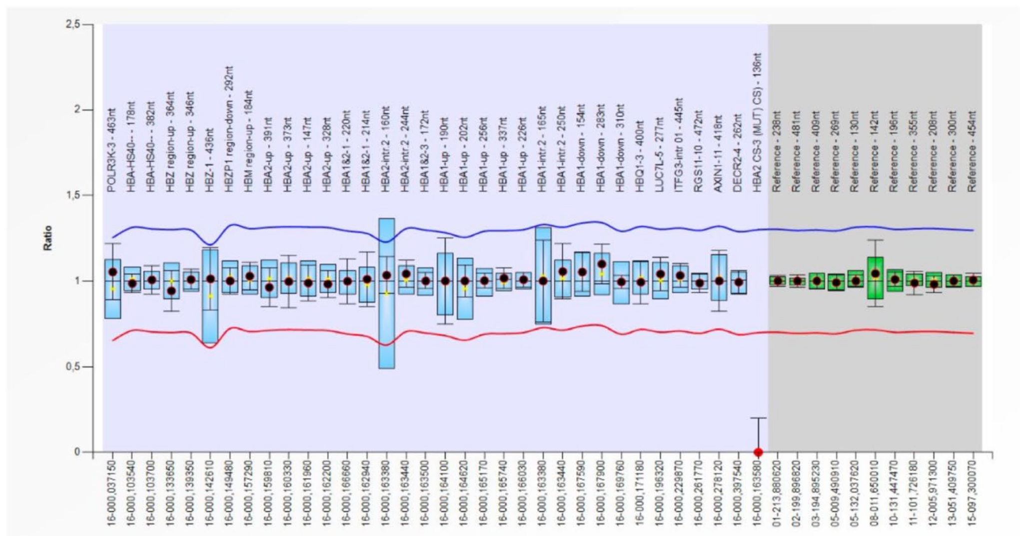
(See figure on next page.)

Fig. 1 Analysis of the ATRX:c.520T > G variant. **A** Sanger sequencing with forward and reverse primers to confirm the presence of the novel mutation previously identified in the proband by NGS. The arrow indicates the mutated base; **B** MLPA analysis showing the absence of α-thalassemia deletions in the α-globin cluster, as previously described [7]; **C** Base conservation scores of 18 bases on the X chromosome's negative strand of exon 7 of ATRX (and the respective amino acid encoded). Below each base, the PhyloP100way score from the VarSome database is presented in diagram form and color-coded. The PhyloP100way score calculation is based on multiple alignments of 99 sequences of genomes from different vertebrates compared to the human genome. It represents the conservation level of a specific nucleotide in the human genome: the higher the score, the more that nucleotide is conserved (red = highly conservated; yellow = moderately conservated; light green = mildly conservated; dark green = very mildly conservated). The asterisk indicates the position of the ATRX:c.520T > G variant (p.Cys174Gly) colored with red diagonal stripes

(A)



(B)



(C)

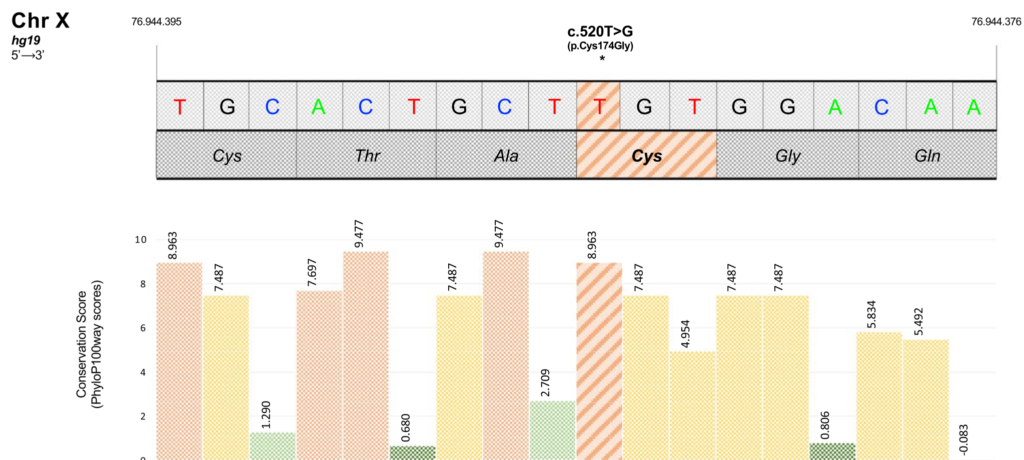


Fig. 1 (See legend on previous page.)

Table 1 Pathogenicity prediction meta-score

Meta-score		Integrated tools	Score calculated	Pathogenic prediction
BayesDel addAF	Deleteriousness meta-score: the higher the score means the variant is more likely pathogenic [10] *Score range:* − 1.29334 to 0.75731	Deleteriousness predictors combined in the Bayesian approach using ClinVar variants as a standard to determine the cutoff value, which includes maximum activation flow rate (MaxAF)	0.7156	Very Strong
BayesDel noAF	Deleteriousness meta-score: the higher the score means the variant is more likely pathogenic [10] *Score range:* − 1.31914 to 0.84087	Deleteriousness predictors combined in the Bayesian approach using ClinVar variants as a standard to determine the cutoff value, which not includes maximum activation flow rate (MaxAF)	0.7901	Strong
MetaLR	Logistic Regression (LR) ensemble score: larger value means the SNV is more likely to be damaging [11] *Score range:* 0 to 1	9 prediction scores: SIFT, PolyPhen-2, GERP + +, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy, Phylo allele frequencies database: 1KG	0.975	Moderate
MetaRNN	Recurrent neural network (RNN) ensemble score: larger value means the SNV is more likely to be damaging [11] *Score range:* 0 to 1	16 prediction scores: SIFT, Polyphen2_HDIV, Polyphen2_HVAR, MutationAssessor, PROVEAN, VEST4, M-CAP, REVEL, MutPred, MVP, PrimateAI, DEOGEN2, CADD, fathmm-XF, Eigen and GenoCanyon 8 conservation scores: GERP, phyloP100way vertebrate, phyloP-30way mammalian, phyloP17way primate, phastCons100way vertebrate, phastCons30way mammalian, phastCons17way primate and SiPhy) allele frequency databases: 1000 Genomes Project (1000GP), ExAC, and gnomAD	0.9787	Strong
MetaSVM	Support Vector Machine (SVM) ensemble score: larger value means the SNV is more likely to be damaging [11] *Score range*: − 2.0058 to + 3.0399	9 prediction scores: SIFT, PolyPhen-2, GERP + +, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy, PhyloP allele frequencies database: 1KG	1.0847	Moderate
REVEL	Pathogenicity predicting ensemble score of missense variants: higher scores reflect a greater likelihood of disease-causing variant [12] *Score range:* 0 to 1	13 prediction scores: MutPred, FATHMM v2.3, VEST 3.0, PolyPhen-2, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP + +, SiPhy, phyloP, and phastCons	0.977	Strong

Each meta-score was calculated by using different integrated tools for *in silico* evaluation of the mutation pathogenicity.

Abbreviations

ATRX Alpha-thalassemia mental retardation X-linked
ATMDS Acquired α-thalassemia myelodisplastic syndrome
HBA α-globin gene
SNV Novel single-nucleotide variant
NGS Next-generation sequencing
MLPA Multiplex ligation-dependent probe amplification
ACMG American College of Medical Genetics and Genomics

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Author contributions
Conceptualization was performed by MG; methodology by RC and RS; software by FR; validation by RC, RS, and ST; investigation by RC, RS, and ST; data curation by RC and FR; writing—original draft preparation by RC and FR; writing—review and editing by MG, MR, GP, TF, and SM; visualization by SM and SR; supervision by MG. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The data supporting the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of University of Naples Federico II (protocol code 443/21; date of approval: 24/02/2022).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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