

## Persistence of Skewed X-Chromosome Inactivation in Pre-B Acute Lymphoblastic Leukemia of a Female ATRX Mutation Carrier

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### Abstract:

X-linked  $\alpha$ -thalassemia mental retardation syndrome (ATR-X) is a rare familial disorder. Somatic mutations of ATRX are reported as a driver gene in various cancers including acute lymphoblastic leukemia (ALL). Here we present a case of pre-B ALL in a female with a germline ATRX mutation who developed highly aggressive leukemia relapsing after allogeneic stem cell transplantation (allo-SCT) with rapid clonal evolution. We investigated whether germline ATRX mutation was associated with leukemia development through activation of the mutant gene. Leukemic blasts were flow-sorted from pre- and post-transplant bone marrows. T cells were isolated from pre-transplant blood as germline control. Whole genome sequencing (WGS) was performed to cover 100x in tumor cells and 30x in control cells, paired with RNA-seq with 40M reads per sample. WGS confirmed germline mutation of ATRX in both leukemic blasts and germline control, however transcripts of mutant ATRX gene were not detectable in RNA-seq indicating a skewed inactivation of mutant X in both tumor and normal cells. Unsupervised clustering of variant allele frequencies (VAF) of 121 shared somatic mutations in pre- and post-transplant blasts identified founding clones harboring high VAF and three additional sub-clones. RNAseq analysis revealed significant up-regulations of genes enriched in the NOD-like receptor and Jak-STAT signaling pathways. The finding does not support ATRX germline mutation as causative gene in this case, but conversely demonstrated that germline mutation is not always pathologic in tumor cells. Our case study indicates that the biological significance of germline ATRX mutation can vary according to sex and tumor type.

**Conflict of interest:** COI declared - see note

**COI notes:** Dr. Montgomery: Ventana Biosystems (research and travel funding), ArcherDx (research collaboration) Dr. Foster: Shire Plc (honorarium), Bellicum Pharmaceuticals (grant), Novartis Pharmaceuticals (Fees paid to my institution), Amgen Inc. (Fees paid to my institution) Other authors declare no conflict of interest.

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## Introduction

X-linked  $\alpha$ -thalassemia mental retardation syndrome (ATR-X) is a rare familial disorder associated with distinctive craniofacial abnormalities, developmental delay, and  $\alpha$ -thalassemia which predominantly affect males.<sup>1-3</sup> Gibbons et al. first identified the causative gene *ATR*X on chromosome Xq13.3<sup>4</sup>, which encodes for a chromatin remodeling factor of the SW1/SNF family and modulates transcription regulation, DNA repair, and mitotic recombination.<sup>5,6</sup> Recently, somatic mutations of *ATR*X were reported in various human cancers including acute lymphoblastic leukemia (ALL).<sup>7,8</sup> *ATR*X deficiency results in genetic instability through impairment of nonhomologous end joining and increase in sensitivity to DNA-damage in mouse glioma model.<sup>9</sup> However, the exact role of *ATR*X in human carcinogenesis remains unknown. Here we present a female carrier of ATR-X who developed highly aggressive pre-B ALL, relapsing after allogeneic stem cell transplantation (allo-SCT). We performed whole genome sequencing (WGS) and RNA sequencing (RNA-seq) to investigate the role of germline *ATR*X mutation in leukemogenesis of this case.

## Methods

### *Case Report*

A 29-year-old woman was diagnosed with pre-B ALL after presenting with back pain and lymphadenopathy. Her two brothers have X-linked  $\alpha$ -thalassemia mental retardation syndrome with germline mutations in the *ATR*X gene (c.4617\_4622del; p.1540\_1541delDE, NM\_000489.3) while she was found to be a carrier. Her mother, the presumed *ATR*X mutation carrier, was diagnosed with metastatic melanoma and her maternal uncle died from glioma.

Despite of failure to first induction therapy, she achieved a complete remission with clofarabine-based salvage chemotherapy. Eighteen months after the diagnosis, she developed an extramedullary and central nervous system relapse. She was treated with radiation therapy, blinatumomab and intrathecal chemotherapy and referred for allo-SCT using her father as a donor. The pre-transplant evaluation showed a PET-avid lesion in the spine and the bone marrow involvement of 25% B-ALL with a complex

cytogenetic abnormality. She received cranial radiation and clofarabine immediately prior to a myeloablative conditioning regimen using total body irradiation 12Gy, fludarabine, donor lymphocyte infusion (DLI)  $2 \times 10^8/\text{kg}$  CD3<sup>+</sup> cells (day -6), and post DLI-cyclophosphamide. A haplo-identical peripheral blood stem graft of  $8.0 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells was infused followed by sirolimus with ultra-low dose IL-2 for graft versus host disease (GVHD) prophylaxis. Engraftment was rapid with full donor chimerism of CD3 and myeloid cells by day 14. She developed steroid-responsive grade II acute GVHD. On day 74 her leukemia relapsed in bone marrow containing 87% leukemic blasts with increased complexities of cytogenetics consistent with clonal evolution. Despite salvage chemotherapy, she died from septic shock and multi-organ failure.

#### *Biospecimen Collection, Cell and Nucleotide Isolation*

Informed consent was obtained for research use of biospecimen in compliance with the Declaration of Helsinki under the protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (Clinicaltrial.gov: NCT02226861 and NCT00071045). Leukemic blasts were flow-sorted on FACS sorter ARIA II (BD Bioscience, San Jose, CA, USA) and peripheral blood CD3<sup>+</sup> T cells (germline control) and donor B cells were isolated using Robosep (Stemcell Technologies, Vancouver, Canada). Genomic DNA and total RNA were extracted using a column purification method (Allprep DNA/RNA mini kit, Cat No. 80204, Qiagen, Germany).

#### *Whole Genome Sequencing, RNA Sequencing, and Bioinformatic Analysis*

WGS was performed on three samples to cover 100x in two leukemia samples (pre and post-transplant leukemias) and 30x in control cells (germline T cells). RNA-seq with 150-bp and 40 million reads per sample were performed on four samples (germline T cells, two leukemias, and father B cells). Library generation, reverse transcription, and cDNA amplification were performed according to manufacturer's instructions, and both WGS and RNA-seq libraries were sequenced on Illumina HiSeq 3000 (Illumina, San Diego, CA, USA). Somatic mutations were called by Varscan and Mutect2, followed

by annotation using SnpEff. SciClone was used to infer an evolution of sub-clones.<sup>10</sup> Circos was used for graphic demonstration of genomic landscape.<sup>11</sup> Transcriptome was analyzed using STAR alignment and DESeq.<sup>12</sup> Gene Ontology (GO) annotation analysis<sup>13</sup> and Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) were used for gene expression pathway analysis.

## Results

WGS confirmed germline mutation of *ATRX* (c.4617\_4622del; p.1540\_1541delDE, NM\_000489.3) in both leukemic blasts and T cell controls as a high confidence germline mutation. However, transcripts of mutant *ATRX* gene were not detected in RNAseq in leukemic blasts and normal cells, indicating an inactivation of the mutant X. Most female carriers of ATR-X are known to have skewed X-chromosome inactivation (XCI) of the mutant *ATRX* allele. To confirm selective inactivation of maternally inherited X-chromosome, we analyzed heterozygosity of multiple single nucleotide polymorphism (SNP) site on the X chromosome. As shown in **Figure 1**, most SNPs in the X chromosome (from Xp22.11 to Xq28) were selectively activated in the paternal alleles. Transcriptions of the maternal SNP allele were only detectable in pseudoautosomal region (PAR1) (**Supplementary Figure 1**). This highly skewed inactivation of maternal X chromosome persisted in leukemic blasts. Expression levels of the entire *ATRX* transcript by RNAseq also showed no significant changes in expressions between leukemic blasts and controls (**Supplementary Table 1**).

WGS identified various somatic mutations, gene fusions (**Supplementary Table 2**), and copy number variation in pre-transplant leukemia (**Figure 2A**) and post-transplant leukemia (**Figure 2B**). Among somatic mutations, 119 mutations in protein coding genes were persistently detected in both pre- and post-leukemic blasts. Clonal inference and evolution analysis using Sciclone revealed founding clones (cluster 1) with high variant allele frequency (VAF) and three additional sub-clones. One of three sub-clones (cluster 2) showed an increase in VAF following transplant which suggests the clones inherited survival advantages, possibly escaping from graft-versus-leukemia (GVL) effects. (**Supplementary Figure 2, Supplementary Table 3**). RNA-seq analysis revealed upregulations of genes

in the NOD-like receptor and Jak-STAT signaling pathway in post-transplant blasts in comparison to pre-transplant blasts. (**Supplementary Figure 3 and 4**).

## Discussion

Here we report a female carrier of an *ATRX* germline mutation with a strong family history of malignancy who developed a highly aggressive pre-B ALL. Notably, the leukemia could not be attributed to the *ATRX* mutation because the leukemic blasts consistently retained an inactive X-chromosome bearing the mutant *ATRX*. Instead, the leukemia cells demonstrated numerous non-*ATRX* mutations, which may explain pathophysiology of this highly aggressive and refractory leukemia.

Recently two independent groups<sup>14,15</sup> reported osteosarcomas in male ATR-X patients, suggesting genetic predisposition of germline *ATRX* mutations to cancer development. **In contrast, *ATRX* germline mutation was not a provocative event for the pre-B ALL in our heterozygous female carrier.** In a mouse model, skewed XCI in female carriers occurred over time by selection of the cells expressing the wild type *ATRX* allele. The hematopoietic cells underwent selection at the earliest stage of definitive hematopoiesis resulting in highly skewed XCI in adult blood.<sup>16</sup> Therefore, it is likely that XCI in hematopoietic lineage cells is highly conserved, even in leukemic blasts. However, *ATRX* mutations may play a different role in non-hematological cancers. For example, *ATRX* is one of the genes involved in escape from X-inactivation tumor-suppressor (EXITS) contributing to cancer sex bias.<sup>17</sup> *ATRX* mutations have been associated with low grade gliomas in males, whereas escape from X-inactivation of *ATRX* has been described in female brain tissue. Our case study indicates that the biological significance of germline *ATRX* mutation can vary according to sex and tumor type and that germline *ATRX* mutation needs to be interpreted with caution, as it is not always pathologic in tumor cells.

**Disclosures:**

Dr. Montgomery: Ventana Biosystems (research and travel funding), ArcherDx (research collaboration)

Dr. Foster: Shire Plc (honorarium), Bellicum Pharmaceuticals (grant), Novartis Pharmaceuticals (Fees paid to my institution), Amgen Inc. (Fees paid to my institution)

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**Authorship contributions:**

Study concept and design (CB, JB., RG, SI); in vitro experiment and data collection (CB, RP, SI); analysis and interpretation of data (CB, CC, KO, KC, CY, JB, RJ, SI); drafting of the manuscript (CB, CC, KO, NDM, MB, JB, RG, SI); bioinformatic and statistical analysis (CC, CY); clinical data collection (SH, KO, CY, PP, NDM, MCF, MB, JB, RG, SI); and obtained funding and study supervision (JB, SI)



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## Figure legends

Figure 1. Heatmap of single nucleotide polymorphism (SNP) allele expression pattern in X chromosome. Variant allele frequencies (VAF) of SNP genotype from whole genome sequencing (WGS) data were plotted on the first left column. VAF of SNP expressions of four samples from RNAseq data were plotted on the right four columns. Despite whole genome sequencing (WGS) data shows the presence of heterozygosity (i.e. VAF ranges 25- 75%) in each SNP loci, RNAseq data demonstrated the loss of heterozygosity (either 0% or 100%) in majority of leukemia and germline cells, which exclusively express SNP alleles inherited from her father except *FAM104B* and pseudoautosomal region (Supplementary Figure 1). This finding is consistent with persisted X chromosome inactivation in hematopoietic lineage cells in the patient.

Figure 2. Genomic landscape of somatic mutations, gene fusions, and copy number variations in leukemia. Dark blue dots represent somatic mutation with VAF. Light blue lines represent fusion genes. Dark bars represent copy number variations. A) In pre-transplant leukemia, WGS and RNAseq identified 270 somatic mutations (251 mutations in protein-coding genes, 16 in intergenic regions, and 3 in non-coding RNA), 16 fusion genes, and 725 gene loci with copy number variations. B) Post-transplant leukemia harbors 312 somatic mutations (296 in protein coding genes, 19 in intergenic regions, and 6 in non-coding RNA), 13 fusion genes, and 332 gene loci with copy number variations.

# Figure 1

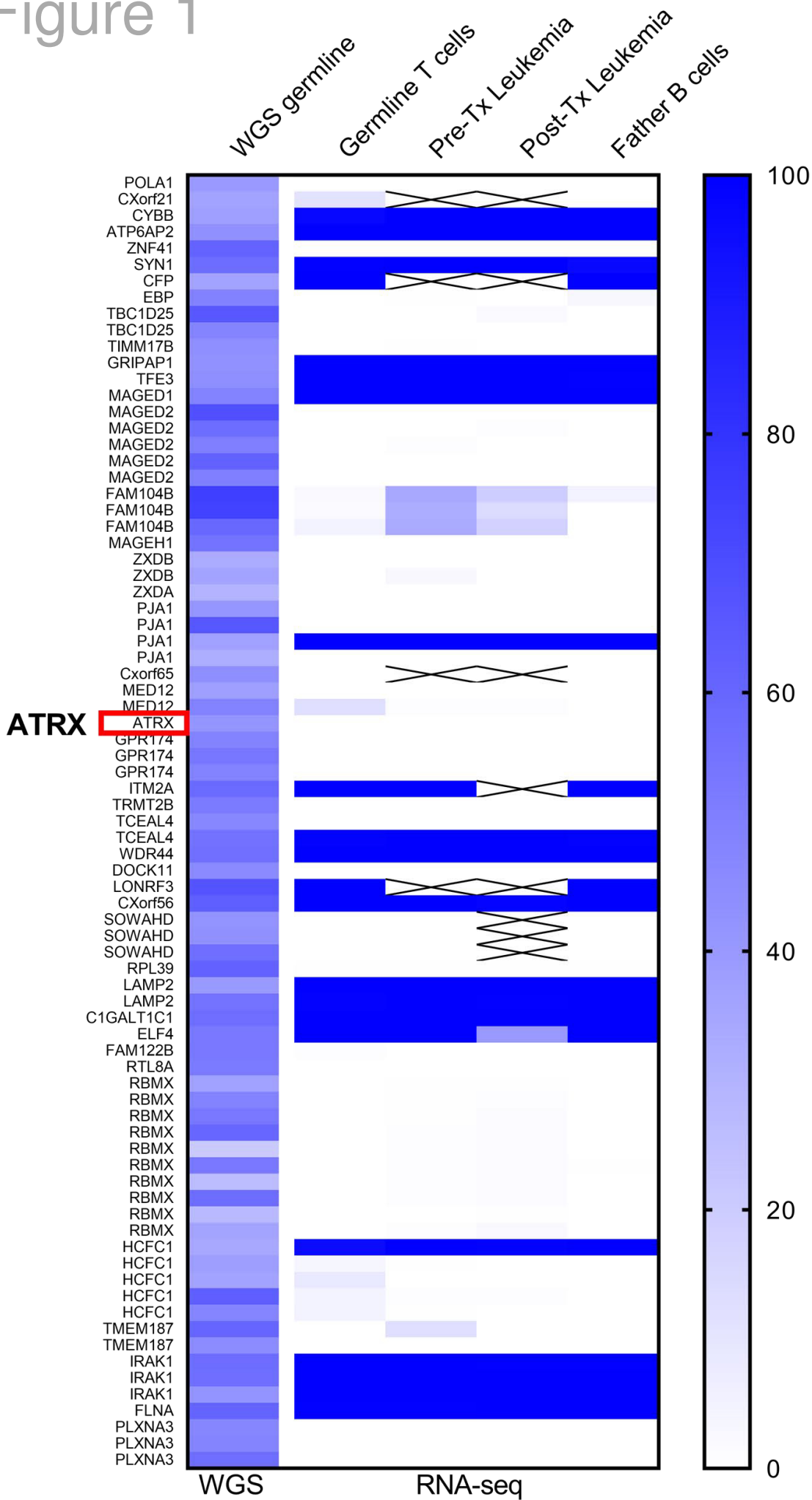
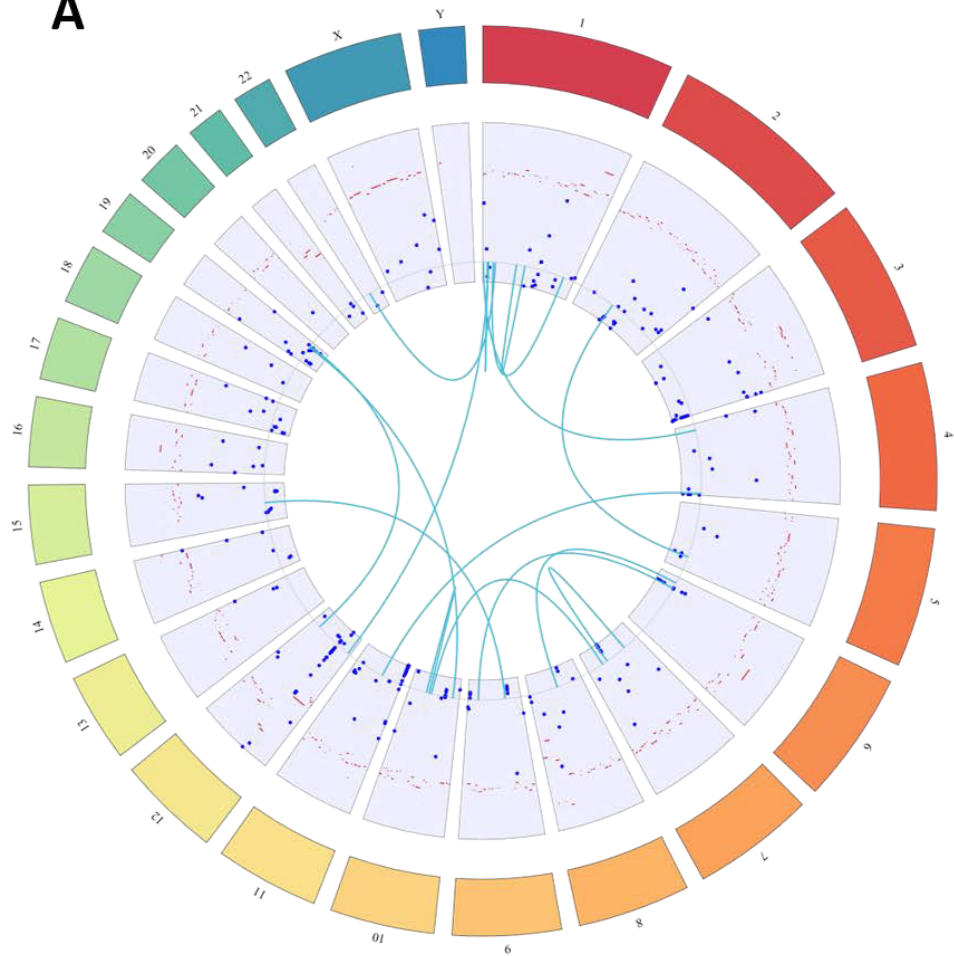


Figure 2

**A**



**B**

