A novel 5′ ATRX mutation with splicing consequences in acquired α thalassemia-myelodysplastic syndrome

Background and Objectives. Acquired α thalassemia (hemoglobin H (HbH) disease) is a rare complication of neoplastic chronic myeloid disorders, especially myelodysplastic syndrome. Acquired HbH has recently been associated with mutations in an X-linked gene, ATRX, previously linked to inherited ATR-X syndrome (α thalassemia-retardation-X linked).

Design and Methods. A Swiss man with chronic myelomonocytic leukemia complicated by various autoimmune disorders and by strikingly microcytic, hypochromic anemia was analyzed for the presence of acquired HbH. After HbH detection, we sought an underlying genetic cause. We used denaturing high-performance liquid chromatography to screen for an ATRX mutation, and measured ATRX expression by reverse transcriptase polymerase chain reaction.

Results. The patient had 50% HbH-containing cells on supravital staining. Marrow karyotype and the α globin cluster were normal. A clonally-restricted ATRX point mutation was detected in the conserved splice donor motif in intron 4 (IVS 4 +2 T→C). Plasmid vector cloning of patient ATRX cDNA demonstrated both exon 4 skipping and partial intron retention with activation of a cryptic splice site, both outcomes resulting in frameshifts with premature stop codon generation in exon 5 and near-decimation of ATRX expression in myeloid cells. Normal exon 6 alternative splicing was retained.

Interpretation and Conclusions. Intronic ATRX mutations with splicing consequences, uncommon in inherited ATR-X syndrome because of their devastating effect on expression of functional protein, should be routinely sought when undertaking molecular analysis of acquired HbH disease. Detection of an acquired ATRX mutation can help support clonality in karyotypically normal ambiguous myeloid disorders with HbH.

Key words: α thalassemia, myelodysplastic syndrome, splicing, acquired hemoglobinopathy.

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The myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, a variable risk of evolution to acute leukemia, and characteristic dysplastic morphologic abnormalities in blood and marrow. Chronic myelomonocytic leukemia (CMML), defined by peripheral blood monocytosis and once considered a form of MDS, often has features of both MDS (dysplasia and anemia) and myeloproliferation (e.g., splenomegaly and leukocytosis), and is now considered in a separate MDS-myeloproliferative overlap category in the World Health Organization’s (WHO) current classification of hematopoietic neoplasia. Both MDS and CMML are associated with diverse clinical presentations and hematologic phenotypes, including acquisition of both functional and structural erythrocyte, leukocyte, and platelet defects.

Occasionally, patients with chronic myeloid disorders such as MDS or CMML develop disorders of hemoglobin synthesis, especially α thalassemia. Acquired α thalassemia can be demonstrated by in vitro detection of hemoglobin H (HbH), usually by supravital staining or chromatographic and electrophoretic techniques. Such patients are most often recognized when anemia associated with a myeloid disorder is microcytic and hypochromic, instead of exhibiting more typical macrocytosis or normocytic indices. While inherited α thalassemia is extraordinarily common globally, affecting hundreds of millions of persons in tropical and subtropical regions, the acquired forms are currently believed to be very rare – only about 70 cases have been reported since the first description in 1960.
(registry is available at URL http://www.imm.ox.ac.uk/groups/mrc_molhaem/ home_pages/Higgs/index.html, accessibility verified 30 August 2005), mostly in Northern Europeans. Because acquired HbH almost always arises in the context of MDS, α thalassemia complicating hematopoietic neoplasia is termed α thalassemia – myelodysplastic syndrome (ATMDS, Online Mendelian Inheritance in Man (OMIM) entry #580448). Recently, acquired somatic point mutations were found in the ATRX gene (MIM *300032) on chromosome Xq13 in 15 of 19 ATMDS cases analyzed. In one remarkable case, an acquired macrodeletion involving the globin cluster on chromosome 16p was causative, and five cases were unexplained. The ATRX (α thalassemia-mental retardation-X linked) gene is named after the inherited ATR-X syndrome of profound mental retardation, developmental abnormalities, and mild thalassemia afflicting boys with germline ATRX mutations; it encodes a nuclear chromatin remodeling protein with profound, diverse effects on DNA methylation and gene expression. These effects are not completely understood, but include down-regulation of α globin expression, despite the fact that the ATRX protein has not yet been demonstrated at the globin cluster on itself. Homology to known SWI2/SNF2 family members, and the presence of seven highly conserved co-linear domains similar to those possessed by proteins with helicase-ATPase function, suggest that ATRX influences gene expression via an effect on chromatin, probably as part of a multi-protein complex, but its precise biochemical function is not understood.

This report describes a novel ATRX gene mutation in a conserved 5′ intronic splice donor motif predicted to affect mRNA splicing, in an individual with CMMML and multiple autoimmune complications who developed microcytic anemia and acquired HbH (ATMDS). In contrast to the three previously reported ATMDS patients with ATRX genomic mutations with a probable splicing effect for whom RNA was not available (two cases) or in whom mature processed ATRX mRNA was not expressed due to nonsense-mediated decay (one case), and three other patients in whom aberrant splicing abnormalities were found but no corresponding genomic DNA mutation could be located, in this case both the genomic mutation and its splicing consequences were directly demonstrable. This success resulted from several factors: fresh myeloid-derived mRNA was available from the patient, some expression of the mutant gene was retained despite probable nonsense-mediated decay (which must have been only partially efficient), and the proportional clonality of the mutation was high. Therefore, we were able to demonstrate splicing consequences of the mutation with certainty.

**Design and Methods**

**Clinical information**

A 59-year-old previously healthy Swiss-French businessman developed a chronic multisystemic disorder characterized by autoimmune complications, anemia, and eventually, monocytosis. Since the onset of bilateral relapsing anterior granulomatous uveitis 8 years previously (successfully treated with corticosteroid and diclofenac sodium eye drops) and steroid-responsive bilateral Achilles' tendinitis 3 years later, he had endured pleuritis and pericarditis requiring pericardectomy, diabetes insipidus, recurrent pulmonary embolism, and multiple episodes of neutrophilic dermatitis. There was no evidence of underlying infection and no serological evidence of a classic connective tissue disorder, and his family history was unremarkable.

Microcytic anemia (hemoglobin 9-10 g/dL) supervened when the patient was 56 years old. Iron studies and initial bone marrow examination were unremarkable, and chronic erythropoietin supplementation resulted in moderate improvement in hemoglobin and transfusion avoidance. The patient was also treated with chronic oral prednisone. Eventually, persistent monocytosis developed, and an unstable hemoglobin consistent with HbH was detected. Bone marrow examination was repeated, and was suggestive of a chronic myeloid disorder. Multi-specialty consultation at the Mayo Clinic in Rochester, Minnesota was requested.

The patient's hematologic data are presented in Table 1. Bone marrow examination showed a hypercellular marrow (95%) with left-shifted granulopoiesis, erythroid hyperplasia with normoblastic maturation and an inverted myeloid:erythroid ratio, monocytosis (confirmed by esterase staining, immunochemistry with CD68 and PGM-1, and flow cytometry), scattered atypical megakaryocytes, and no excess in undifferentiated myeloblasts. Ringed sideroblasts were not observed. A focal increase in fibrosis (1′) was demonstrated by reticulin staining. Marrow karyotype was normal (46,XY) and fluorescent in situ hybridization analysis of 500 interphase cells for BCR/ABL was negative. The underlying hematologic diagnosis was felt to be most consistent with WHO-defined CMMML, subtype I (<5% blasts).

Sedimentation rate (4 mm/hr), urinalysis and renal function tests, and multiple autoimmune serology investigations including complement titers, antinuclear antibodies, rheumatoid factor, extractable nuclear antigen antibody panels, anti-neutrophilic cytoplasmic antibodies (p- and c-ANCA), antibodies to double-stranded DNA, anti-phospholipid antibod-
ies, and cryoglobulins, were all normal or negative. The C-reactive protein was slightly elevated at 1.7 mg/dL (normal <0.8 mg/dL). Lymphocytosis showed only a slight increase in CD16+, CD56+ natural killer cells (842/µL; normal range 80-597/µL); protein electrophoresis demonstrated polyclonal hypergammaglobulinemia, with negative immunofixation.

**DNA/RNA preparation and polymerase chain reaction (PCR)**

The patient gave written consent for analysis of his blood and marrow cells and genetic material, and the study was approved by the Institutional Review Board of the Mayo Clinic. Compliance with the Federal Health Insurance Portability and Accountability Act and Minnesota Statute On Access To Health Records (144.335) was assured.

Peripheral blood granulocytes and mononuclear cells and marrow mononuclear cells were isolated from EDTA-anticoagulated specimens using dual density Ficoll-Hypaque centrifugation (Sigma Chemicals, St. Louis, MO, USA). Genomic DNA was obtained using a resin-based DNA extraction kit (HighPure DNA Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) and RNA extract-
ed with RNEasy Mini Kit (QIAGen, Venlo, The Netherlands). RNA quality was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and cDNA generated using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). DNA amplification was performed by PCR: reagents included GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA, USA), MgCl₂ (final concentration 1.5 - 3 mM – see Table 2; Applied Biosystems), dNTPs (200 µM; Roche), forward and reverse primers (40 µM; Table 2; Integrated DNA Technologies, Coralville, IA, USA), 100 ng template DNA, and a 5:1 ratio of AmpliTaq Gold DNA polymerase (total, 1 U; Applied Biosystems) to PfX DNA polymerase (total, 0.2 U; Roche). PfX polymerase was included to provide 3'-5' exonuclease activity and improve the performance characteristics of the denaturing high performance liquid chromatography (DHPLC). Amplicons were designed to cover the entire 5' untranslated region, protein coding region, canonical splice donor and splice acceptor sites, and the 3' polyadenylation consensus signal of ATRX (Table 2). The total reaction volume was 50 µL. Reactions were carried out on a Dyad DNA Engine Peltier Thermocycler (MJ Research, Waltham, MA, USA). Amplification conditions were as follows: 95°C for 10 minutes, followed by 35 cycles of: 95°C for 30 seconds, the optimal annealing temperature (Table 2) for 30 seconds, and 72°C for 40 seconds.

**DHPLC**

PCR-amplified samples were warmed to 95°C, then slowly cooled to room temperature to encourage heteroduplex formation. A wild-type DNA control was included for each amplicon. In view of the tissue DNA admixture (normal/clonal) in unfractionated blood and marrow that is characteristic of chronic myeloid disorders, the patient’s DNA was not mixed with wild type before heteroduplexing. For DHPLC analysis, we used the WAVE 3500HT DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA) with Navigator Software, as previously described.¹⁰

**Subcloning and sequencing**

Subcloning was performed using the pGEM-T Easy Vector System (Promega; Madison, WI, USA) and DH5α competent cells (Invitrogen) as previously described.¹⁰ Sequencing of subclones bearing inserts was performed by the Mayo Clinic Molecular Biology Core Facility using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The generated sequence data were analyzed using the Sequencher
globin analysis

5'-TGAAAGGCTAATTAAGGGAATTCTT-3' 1.5 57 54.6, 56.6

PCR annealing DHPLC

5'-CTTGGTCGAAAGGAGTTGTCCAC-3' 1.5 60 55, 57

5'-AATTGGTGCTGCCAGAACCAC-3' 1.5 60 55.6, 57.6

5'-TGTACCAGCAATGTTGGCTT-3' 1.5 57 55.6, 56.6

5'-AAATCCTGCTGGGATTTTTG-3'

5'-AAAGAAGGCAGTCATTGTCAAGG-3' 1.5 60 56.2, 57.2

5'-GCAGCAGTGGAACTGAACAA-3' 1.5 60 55.4, 57.4

5'-TGGGTATTGTTCCAGTTGTCAG-3' 1.5 60 54.1, 55.1

GAPDH

5'-TGAGCATTTCATTGGGGAAT-3' 1.5 60 54.1, 55.1

5'-GCACTGAAAAGTTACCTGAGCGAG-3' 1.5 60 54.5, 55.5

5'-CCCCTACTTGCTGATTGACC-3' 1.5 60 56.2, 57.2

5'-TCAGTCCTTCCTCAGCTCGT-3' 1.5 60 54.9, 55.9

5'-GGAATGCATACAGAGGTAGAACA-3' 1.5 58 55.9, 56.9

5'-CCTCTTTTGAGACAGGTCATGC-3' 1.5 55 57.5, 58.5

5'-ACTGTCCAGAGGGGAAAAAT-3'

5'-GCTTCTCTACACTGCCAAAAGTG-3' 1.5 60 55.2, 57.2

globin gene cluster was per-

5'-TTCCTGGATCTGAGAATGTGG-3'

FAM multiplex

5'-TGATGCATATTTCAGTGGGAAT-3' 1.5 60 55.4, 57.4

5'-GTTGGCAAATGGAAGGATTC-3' 1.5 58 56.4, 58.4

5'-TGAGCAAGGTGGAAAATCTG-3'

5'-CAGTTTCCTGAAAGAAGGGAA-3' 1.5 57 58, 59

5'-CGAGGCATTTTAAAGGCTGA-3' 1.5 55 55.5, 56.5

5'-CAATGACTATCCATCCCTCCATAG-3' 3 62 55.3, 57.3

2

5'-TCCTGGAGATTTTCCCCAG-3' 1.5 57 53.4, 55.4

5'-AAGTTGTGGTCTGAACCCC-3' 1.5 60 56.2, 57.2

WBC cells (Figure 1) which, in the setting of CMML and

some amplicons (e.g., exon 8) normally include a small heteroduplex peak on DHPLC analysis, perhaps because of low sequence complexity and poor polymerase fidelity, or

either mis-primer. Comparisons with simultaneously analyzed wild-type DNA samples can be helpful, and sequencing with or without cloning may sort ambiguous cases. 

Eluate time shifts when using high-throughput DNAsep column may be necessary to visualize peaks, depending on local conditions (buffer lot, etc.). These DHPLC tempera-
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A34

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are shown in Table 1. The presence of HbH was confirmed by hemoglobin electrophoresis and isoelectric focusing. Analysis of the \( a \) globin loci by Southern blotting and sequencing was normal. Analysis of the entire coding region of the \( ATRX \) gene by DHPLC using DNA from blood mononuclear cells (enriched for monocytes, presumed to be clonal in CMML) revealed a heteroduplex peak in exon 4; all other amplicons were wild-type. Sequencing demonstrated a thymidine to cytidine point mutation of mixed clonality in the second base of the canonical GT splice donor motif just 3' to \( ATRX \) exon 4 (IVS 4+2 \( T \rightarrow C \), Figure 2A) not present in non-hematopoietic cells. The same mutation was detectable in the patient’s granulocytes but not in DNA from 24 healthy controls (7 British, 8 Icelandic, 8 Jamaican, 1 Frisian). The 5' end of \( ATRX \) cDNA made from the patient’s monocyte RNA was then amplified and sequenced; electrophoresis of raw PCR product demonstrated multiple abnormal bands, and the multiple overlapping chromatograms at the exon 3-4 junction suggested the presence of several novel spliceoforms (Figure 2B).

Subcloning of amplified cDNA demonstrated two consequences of this mutation on \( ATRX \) splicing (Figures 2B and 3); neither aberrant isoform was present in cDNA from 9 healthy European-American individuals. In one of the resulting aberrant spliceoform groups in the patient, exon 4 was skipped; exon 3 was spliced directly to exon 5. Since exon 4 is 53

![Supravital staining of a peripheral blood smear from the patient with chronic myelomonocytic leukemia (CMML) and microcytic, hypochromic anemia. Multiple hemoglobin H-containing golf ball cells (arrows) are visible. The stain also highlights reticuloocytes (arrowhead). (Brilliant cresyl blue, 100X.)](image1)

![Fluorescent dye chemistry sequencing chromatograms of genomic DNA amplicon, including exon 4. This demonstrates IVS 4+2 \( T \rightarrow C \) point mutation (arrow) of mixed clonality (predominantly mutant) in the CMML patient (Pt, top); the wild type (WT) chromatogram is at the bottom. Mixed clonality \( ATRX \) mutations such as this are consistent with either somatic, acquired mutation or mosaicism, since \( ATRX \) is X-linked and males are hemizygous. B. Sequencing chromatograms of cDNA from the patient reveals a confused sequence at the the exon 3-exon 4 junction (middle). Subcloning demonstrates exclusion of exon 4 (bottom – arrow indicates start of exon 5) and intron inclusion (not shown – the appearance is the same at the exon 3-4 junction as the wild type chromatogram at top.](image2)
base pairs in length, this results in a frameshift and generation of a premature stop codon in the 5' end of exon 5. Exon 6 is normally alternatively spliced and this accounts for the fact that there were two different variants of this spliceform, since the primers used for amplification and sequencing the cDNA (5'-GCCCATGAGTGAAAGCAAGT-3' and 5'-AGCTCACAATCCCATGAAGC-3') spanned exons 1-7. The other spliceform pair included intact exons 3, 4 and 5, but retained 43 base pairs of intron 4 (including the TÆC point mutation in the splice donor site). The sequence at the 3' end of this retained intron is a characteristic splice donor motif not normally used by the cell, and represents activation of a cryptic splice site. The intron retention also resulted in a frameshift with generation of a premature stop codon in exon 5. Rare normally spliced transcripts were also observed during cloning, presumably synthesized by cells from residual normal hematopoietic clones. Expression of ATRX mRNA in the patient's monocytes, measured by RT-PCR using the probe to the helicase domain, was 11.1% of control mRNA, and in the patient's granulocytes, expression was 15.3% of normal. In view of the 5' position of the splicing mutation and frameshift, but recovery of multiple clones, this likely represents nonsense-mediated decay of the transcript.

Discussion

Pre-mRNA splicing abnormalities are increasingly recognized in association with human disease, including neoplastic conditions, and may represent potential therapeutic targets. There are multiple ways in which dysfunctional mRNA splicing isoforms, such as those observed here, can be generated, including point mutations in conserved splice donor, recipient, and branch site motifs, or mutations in less well-understood cis-acting exonic and intronic splicing enhancers and silencers. In addition, trans-acting alterations in components of the extraordinarily complex spliceosome or, perhaps more commonly, in non-spicosomal RNA binding protein regulators, can cause varied and unpredictable splicing abnormalities. This class of mutation may account for aberrant or alternative spliceforms where a cis-acting cause is sought and not found, as often happens in cancer. For instance, a splicing regulator is suspected to underlie the recently recognized helicase-domain exon skipping in ATRX and parallel changes in the structurally similar PASC/HELLS/SMARCA6 gene in leukemia. Global genome-wide screening of human alternative splicing patterns using array-based methods indi-
cates that at least 74% of human multi-exon genes are normally alternatively spliced. Because normal alternative splicing is so common, when transcript isoforms not previously recognized are discovered, as in this report, it may be challenging to decide whether they are actually pathogenic, or are simply rare alternative variants dependent on special developmental or environmental circumstances for expression. Dramatic changes in gene expression – and especially generation of premature stop codons, which are known to cause nonsense-mediated decay when located in the 5’ region of a gene, as here – support pathogenicity, because no functional protein results.

In this case, the pathogenic nature of the splicing mutation seems clear. First, the patient had clear evidence for the phenotype of acquired α thalassemia, recently associated with coding mutations in the ATRX gene, and the α globin cluster itself was normal. Second, the mutation detected is in a highly conserved splice donor motif in a conserved region of ATRX. Third, while human ATRX has several known alternative mRNA isoforms in healthy individuals, including a conserved truncated isoform, alternative splicing of exon 6 and intron 5 retention, we sought but did not detect the specific aberrant isoforms seen in this study in any healthy individuals, and to our knowledge they have not been described by others. Finally, this mutation was associated with striking down-regulation of ATRX expression, although not complete abrogation of expression as was described for the first acquired ATRX mutant discovered.

Germline ATRX splicing mutations do occur, but are an uncommon cause of inherited ATR-X syndrome. Point mutations or small deletions/insertions are more typical, and some expression of full-length protein is usually retained. It is likely that this class of mutation, unless rescued by normal alternative splicing or insertion of heterologous DNA that keeps the transcript in frame and allows acceptable protein folding, is particularly detrimental to the developing male fetus and usually not compatible with life. Alternative splicing may also modulate the ATR-X phenotype when detrimental point mutations are present.

The observation of multiple apparent autoimmune complications in this patient is worthy of comment. Various immune disorders such as acute and chronic vasculitides, classical connective tissue disorders (especially relapsing polychondritis), and atypical autoimmune phenomena have been reported in a minority of patients with myeloid neoplasia, and these appear to be especially common in association with CMML. This observation has motivated clinical trials of immunosuppressive therapies, including anti-thymocyte globulin and cyclosporine in MDS, which have met with some success. To our knowledge, autoimmunity has not been reported with acquired or inherited ATRX mutations.

There are occasions in clinical practice when a bone marrow examination, undertaken for evaluation of peripheral blood cytopenias, is non-diagnostic. In this setting, the presence of a karyotypic abnormality typical for MDS can help secure the diagnosis and overcome ambiguity. The present case is an example of how discovery of a clonally restricted point mutation might also serve such an end, although in this case the marrow morphology was unambiguous. Other point mutations could serve similar ends; ATRX is convenient because mutations have such an easily detectable erythrocyte phenotype, although it is not clear how common acquired HbH actually is in myeloid disorders. The search for other point mutations with greater frequency and pathophysiological significance should be a high priority for MDS researchers.

In conclusion, acquired cis-acting point mutations in conserved splicing motifs of ATRX leading to aberrant splicing can be associated with the ATMD syndrome, and can provide proof of clonality in diagnostically challenging cases. In future analyses seeking ATRX mutations, splicing abnormalities should be routinely considered.

Appendix

Alternative splicing can lead to discrepancies in the exon numbering systems in published reports, as with what has sometimes been described as ATRX exon 7, an incorrectly spliced human-specific transcript without murine or other equivalent, which is not routinely detectable in human samples in health or disease. In this report we have followed the lead of others and GenBank Accession NM_000489.2, the longest isoform in the NCBI PubMed database, and have chosen to ignore exon 7 in our numbering system, and renumber the previous exons 8 and 9 as exon 7.

MEN performed the PCR, DHPLC, and cloning under direct supervision by DHPLC, and drafted the manuscript. PJT evaluated the patient clinically, recognized the possibility of acquired HbH, and obtained the patient’s consent and research samples. JDH performed the metabolic HB analysis. DPS did the CBCh stain and α globin sequencing, edited, submitted, and revised the manuscript, co-ordinated and supervised the entire project, and is ultimately responsible for the figures and contents.

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