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

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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.[1,2] Chronic myelomonocytic leukemia (CMML), defined by peripheral blood monocytosis and once considered a form of MDS,[3] often has features of both MDS (dysplasia and anemia) and myeloproliferation (e.g., splenomegaly and leukocytosis),[4] and is now considered in a separate MDS-myeloproliferative overlap category in the World Health Organization's (WHO) current classification of hematopoietic neoplasia.[5] 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.[6,7] Acquired α thalassemia can be demonstrated by *in vitro* detection of hemoglobin H (HbH), usually by supravital staining or chromatographic and electrophoretic techniques.[7] 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,[8] 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.[7] 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 #300448). Recently, acquired somatic point mutations were found in the *ATRX* gene (MIM *300032) on chromosome Xq13 in 13 of 19 ATMDS cases analyzed.[9,10] In one remarkable case, an acquired macrodeletion involving the globin cluster on chromosome 16p was causative, and five cases were unexplained.[11] 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.[12-16] 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 itself.[17] 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.[18]

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 CMML 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,[9,10,19] 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 pericardiectomy, 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),[20] 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 CMML, subtype I (<5% blasts).[20]

Sedimentation rate (4 mm/hr), urinalysis and renal function tests, and multiple autoimmune serology investigations including complement titers, anticentromere antibodies, rheumatoid factor, extractable nuclear antigen antibody panels, anti-neutrophilic cytoplasmic antibodies (p- and c-ANCA), antibodies to double-stranded DNA, anti-phospholipid antibod-

Table 1. Patient's hematologic data.

Complete blood count		
Hemoglobin	10.0 g/dL	(normal*, 13.5-17.5 g/dL)
Red blood count	6.00×10^{12}/L	(normal, $4.32\text{-}5.72\times10^{12}$/L)
Mean corpuscular hemoglobin	16.7 pg	(normal, 26-33 pg)
Mean corpuscular volume	54 fL	(normal, 85.2-95.1 fL)
Platelet count	432×10^{9}/L	(normal, $150\text{-}450\times10^{9}$/L)
White blood count	30.3×10^{9}/L	(normal, $3.5\text{-}10.5\times10^{9}$/L)
Differential		
Neutrophilic segs	59%	(normal, 42-75%)
Monocytes	13%	(normal, 1-11%)
Lymphocytes	11	(normal, 16-52%)
Basophils	2%	(normal, 0-4%)
Eosinophils	0%	(normal, 0-7%)
Metamyelocytes	9%	(normal, 0-1%)
Myelocytes	4%	(normal, 0-0.5%)
Undifferentiated blasts	2%	(normal, none)
Nucleated red blood cells	1/100 WBC	(normal, none)
Peripheral smear		
Dimorphic red cell population with marked anisopoikilocytosis, polychromasia, and poorly hemoglobinized erythrocyte *ghosts*		
Reticulocyte count	3.89%	(normal, 0.60-1.83%)
Absolute reticulocytes	238.8×10^{9}/L	(normal, $29.5\text{-}87.3\times10^{9}$/L)
Coombs' test:	1+ positive	
Markers of hemolysis (lactatedehydrogenase, bilirubin, haptoglobin,urine hemosiderin):	normal	
Hemoglobin electrophoresis		
Hemoglobin A	89.9%	(normal 95.0-98.0%)
Hemoglobin A2	2.3%	(normal 2.0-3.3%),
Hemoglobin F	0.3%	(normal <2.0%)
Hemoglobin H	7.5%	(normal, none)

**Normal ranges effective in the Mayo Clinic Clinical Hematology Laboratory in July 2005.*

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). Lymphocytoflow showed only a slight increase in $CD16^{+}$, $CD56^{+}$ natural killer cells (842/uL; normal range 80-597/uL); 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 extracted 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 RTS 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), $MgCl_2$ (final concentration 1.5 - 3 mM – see Table 2; Applied Biosystems), dNTPs (200 μM; Roche), forward and reverse primers (40 pM; 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

Table 2. PCR and DHPLC conditions for ATRX coding region mutation screening.

EXON	Primers	PCR $MgCl_2$ concentration (mM)	PCR annealing temperature (°C)	DHPLC temperatures (°C)
1	5'-CTAACCCACCAAGCGAAC-3' 5'-GGCCCCCAAAGAAGGTG-3'	1.5	60	64, 65
2	5'-TGAAAGGCTAATTAAGGGAATTCTT-3' 5'-GAAGGTATAGCACATTCTTTTTCAA-3'	1.5	57	54.6, 56.6
3	5'-TGTGAGAATGGGTTTGTGGA-3' 5'-TTCTCAAACTGGAAAACAACGA-3'	1.5	60	55.8, 56.8
4	5'-TTCTAGGCACATAAATAGCATCTCA-3' 5'-TGTCCCTTCAACATCAACCA-3'	3	57	56.1, 57.1
5	5'-TGGGTATTGTTCCAGTTGTCAG-3' 5'-GCCATGTTTGGTCGTTTGTA-3'	1.5	60	54.1, 55.1
6	5'-TGTACCAGCAATGTTGGCTT-3' 5'-AAGCACATCCGATTTTCCAA-3'	1.5	57	55.6, 56.6
7	5'-GGAATTTGCCAAGGTTGTC-3' 5'-TCTTCAAGACTGTGCCCTCA-3'	1.5	58	55.6, 56.6
8	5'-TCCTGGAGATTTTCCCCAG-3' 5'-AATGACGATACTATGAAAGAC-3'	1.5	57	53.4, 55.4
9A	5'-CAGTTTCCTGAAAGAAGGGAA-3' 5'-TTCTTATTTTGCTGCAACAACTG-3'	1.5	57	58, 59
9B	5'-GCAGCAGTGGAACTGAACAA-3' 5'-TCATTTTCAACCAAATGCTCA-3'	1.5	60	55.4, 57.4
9C	5'-CTTGGTCGAAAGGAGTTGTCCAC-3' 5'-ATCCATCGCTCGAAACTCGG-3'	1.5	60	55, 57
9D	5'-AAATTCCGAGTTTCGAGCGATG-3' 5'-GACAATCAGCACCTTTAATTGGGG-3'	1.5	60	55, 57
9E	5'-AAGTTGTGGTCTGAACCCC-3' 5'-TGACTCATCCTGCTCACCTCTTTG-3'	1.5	60	56.2, 57.2
9F	5'-AATTGGTGCTGCCAGAACCAC-3' 5'-GCTCAGGTAACTTTTCAGTGCC-3'	1.5	60	55.6, 57.6
9G	5'-GGATAAGCGTAATTCTTCTGACAG-3' 5'-CACTTGCTTGCTGCTTCTTAGGAAG-3'	1.5	60	55.5, 56.5
9H	5'-GCAGAGAAATTCCTAAAGAAAGACC-3' 5'-GAACTCTTTCCAAGCAACTTGCAC-3'	1.5	60	55.2, 56.2
9J	5'-GCACTGAAAAGTTACCTGAGCGAG-3' 5'-TCTCCTTGACAATGACTGCCTTC-3'	1.5	60	54.5, 55.5
9K	5'-AAAGAAGGCAGTCATTGTCAAGG-3' 5'-CAATGTAGTAACTCAAGAGGGG-3'	1.5	60	56.2, 57.2
10	5'-GCTGGCACTTCACAAATAACC-3' 5'-CCCATGCCTAGGGTGTGTTA-3'	1.5	58	54.6, 55.6
11	5'-TGGTGTATTTCACCCCCAGA-3' 5'-AAGTTTTAAACATTTCCATGAACTC-3'	1.5	58	55.1, 56.1
12	5'-CGAGGCATTTTAAAGGCTGA-3' 5'-GGCATAAGCCACTGTCTGGT-3'	1.5	55	55.5, 56.5
13	5'-TGCTCTGTTTTAATGTCGAGTCA-3' 5'-TGAAGGCATGGTCATTCAGA-3'	3	55	54.1, 57.1
14	5'-GGAATGCATACAGAGGTAGAACA-3' 5'-TTCAAGCATGTGGTAAATGTCA-3'	1.5	58	55.9, 56.9
15	5'-CCACCTTTTCCTGCTGTGTT-3' 5'-CACTTGAGCCCAAGAGTTCG-3'	1.5	62	57.1, 58.1
16	5'-TTTTGGAAAATCTCCCTTGG-3' 5'-AGCTGCCTCACACCCAAATA-3'	1.5	58	55.6, 56.6
17	5'-TGCTGTTTCTTAGAAGTTTTGGTT-3' 5'-TCTTCAGCCCCTACGACTGT-3'	1.5	60	55.4, 57.4
18	5'-AACGAAGGTGACAATCTGTTTTC-3' 5'-TTCCCACTGAAATATGCATCAC-3'	1.5	58	53.8, 55.8
19	5'-TGATGCATATTTCAGTGGGAAT-3' 5'-AAAAGGCAAAAACCTGAAGGA-3'	1.5	60	55.4, 57.4
20	5'-AATGGATCACATTTTCAGTTTTATT-3' 5'-GGAAATACCAATATTCTACTGCATAA-3'	1.5	58	53.5, 55.5
21	5'-TGAGCATTTCATTGGGGAAT-3' 5'-GCGGGAAAGAAAACACAAAA-3'	1.5	60	54.1, 55.1
22	5'-TGAGAACTTGTAGTTCATTCCTGTT-3' 5'-TCAACAAGGTGTATTGTTTACCTG-3'	1.5	58	54.1, 56.1
23	5'-GCTTCTCTACACTGCCAAAAGTG-3' 5'-TTCTGCTTCCAATAGATGCTTT-3'	1.5	60	55.2, 57.2
24	5'-TCAGTCCTTCCTCAGCTCGT-3' 5'-AGAGGTCAATCAGCAAGTAGGG-3'	1.5	60	54.9, 55.9
25	5'-CCCCTACTTGCTGATTGACC-3' 5'-TCCATGATAAAGGCAACATTCA-3'	1.5	60	56.2, 57.2
26	5'-CCCCATGGGTAGGTCTTTTT-3' 5'-GGAAGGAAGGAAAAGCAACA-3'	3	58	55.6, 56.6
27	5'-AAATCCTGCTGGGATTTTTG-3' 5'-GGGTAGTTTTGTTTCTTTTGTTGC-3'	1.5	60	55.1, 56.1
28	5'-TGAGCAAGGTGGAAAATCTG-3' 5'-TGACTGTACCTTGCATTTGCT-3'			
29	5'-AAGAAATGAATTCTCTGAACTCTTGA-3' 5'-TCACATAAACTTTCAATATGAAAAAG-3'	3 3	62 58	55.5, 56.5 53.2, 55.2
30	5'-TCCTTGATTAAGTTTCATGCTTACA-3' 5'-TCCTTAGTCCACGTTGGTTTG-3'	1.5	55	56.5, 57.5
31	5'-ACTGTCCAGAGGGGAAAAAT-3' 5'-GCATCAACTACCTTCTTCTCTGC-3'	1.5	55	57.6, 58.6
32	5'-TTCCTGGATCTGAGAATGTGG-3' 5'-CACAACTCACCTCCAGCTGTT-3'	1.5	55	55.9, 58.4
33	5'-GTTGGCAAATGGAAGGATTC-3' 5'-AGTAGGGGGTGGAGGGTACA-3'	1.5	58	56.4, 58.4
34	5'-CAATGACTATCCATCCCTCCATAG-3' 5'-AGATAGCCAGCCCAGGTACA-3'	3	62	55.3, 57.3
35	5'-CCTCTTTTGAGACAGGTCATGC-3' 5'-GGCATTTAAGGGGACCAAAC-3'	1.5	55	57.5, 58.5

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 else mis-priming. 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 temperatures represent a minimum for screening of the coding region; some mutations may not appear at these temperatures, and broader ranges must be used if screening of the entire ATRX gene is unrevealing and there remains a high index of suspicion.

v4.2 software package (Gene Codes, Ann Arbor, MI, USA).

Hemoglobin and α globin analysis

Fresh peripheral blood was incubated for 4 and 24 hours with 1% brilliant cresyl blue (Sigma) in 0.9% NaCl, smeared on a glass slide and examined for intraerythrocytic HbH inclusions. Hemoglobin stability testing was performed on washed, freshly hemolyzed erythrocytes incubated in isopropanol (Sigma) at 37°C for 20 minutes, and the solution examined for turbidity (normally there is none.) Hemoglobin electrophoresis was performed in alkaline (pH 8.6) and acid (pH 6.2) conditions using the Spife 3000 electrophoresis system (Helena Laboratories, Beaumont, TX, USA). For isoelectric focusing, an agarose gel slab with a pH gradient of 6 to 8 was used (Isolab, Inc, Akron, OH, USA). Hb A_2 and Hb F were measured by high performance liquid chromatography using a weak cation exchange column (Bio-Rad Variant, Bio-Rad Laboratories, Hercules, CA, USA).

Southern blot analysis for typical deletion-type mutations of the α globin gene cluster was performed in the Mayo Molecular Genetics Clinical Laboratory using two DNA probes, one directed to α globin genes and the other to ζ globin gene (pseudo-ζ), using three restriction endonucleases: *Eco RI*, *Bgl II*, and *Bam HI*. The α globin loci were also directly sequenced to rule out non-deletional forms of thalassemia.

ATRX expression analysis

Real-time PCR (RT-PCR) was performed using TaqMan Universal Master Mix, an ABI 7900 RT-PCR system, the Hs00230877_m1 *ATRX* FAM multiplex primer-probe set (interrogates exon 21/22 in the helicase domain of *ATRX* NM_138270 GenBank RefSeq) and results normalized with TaqMan glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control reagent with JOE probe (all Applied Biosystems). The patient's granulocyte and mononuclear cell RNA were compared with RNA from the same cell subpopulations from healthy male donors. Assays were performed in triplicate. Expression ratios were calculated using the $2^{-\Delta\Delta C_T}$ method.[21]

Results

Supravital staining of the patient's peripheral blood demonstrated approximately 50% HbH-containing *golf ball* cells (Figure 1) which, in the setting of CMML and known previously normal hematology, is consistent with ATMDS. Hemoglobin stability testing demonstrated abnormal turbidity and electrophoresis results

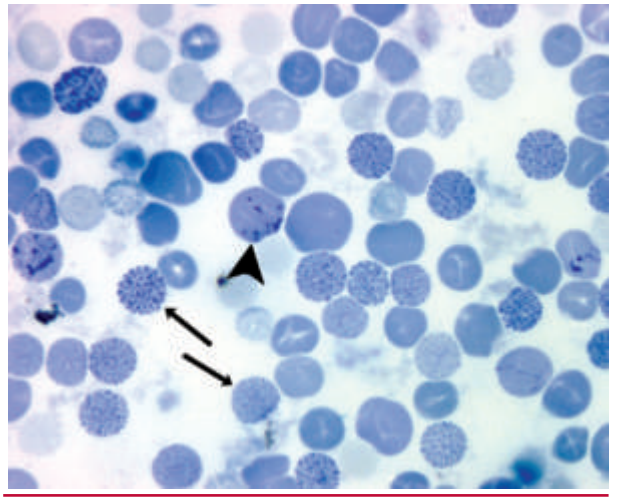

Figure 1. Supravital staining of a peripheral blood smear from the patient with chronic myelomonocytic leukemia (CMML) and microcytic, hypochromic anemia. Multiple hemoglobin H-containing *golf ball cells* (arrows) are visible. The stain also highlights reticulocytes (arrowhead). (Brilliant cresyl blue, 100×).

are shown in Table 1. The presence of HbH was confirmed by hemoglobin electrophoresis and isoelectric focusing. Analysis of the α 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→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

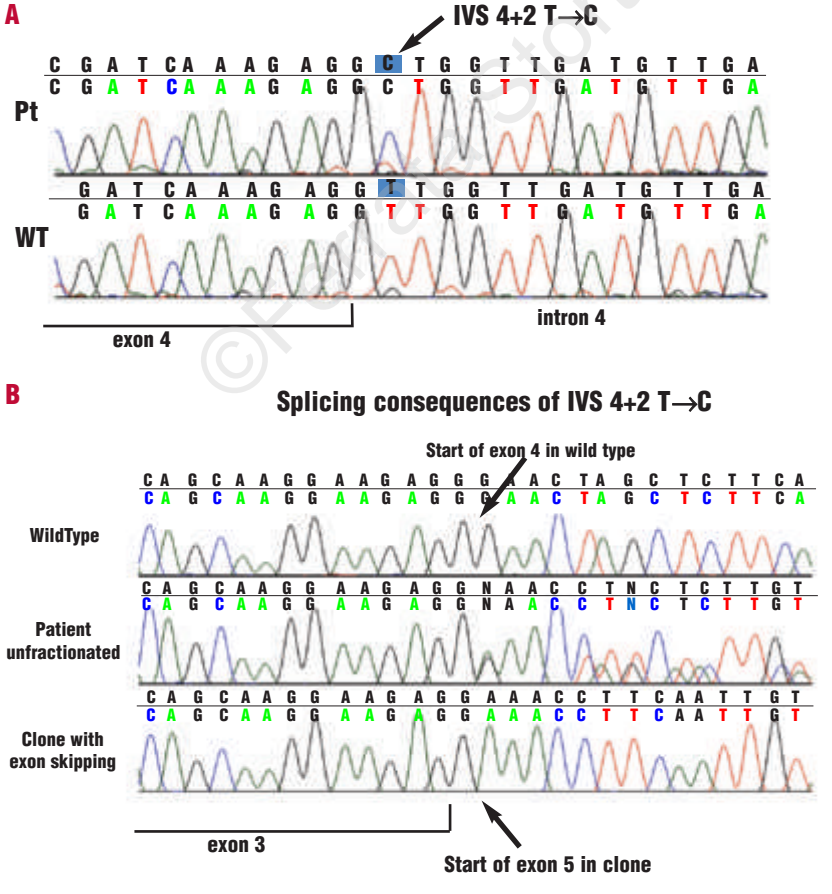

Figure 2. A. Fluorescent dye chemistry sequencing chromatograms of genomic DNA amplicon, including exon 4. This demonstrates IVS 4+2 T→C point mutation (arrow) of mixed clonality (predominantly mutant) in the CMML patient (Pt, top); the wild type (WT) chromatogram is at the bottom. Mixed clonality *ATRX* 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 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).

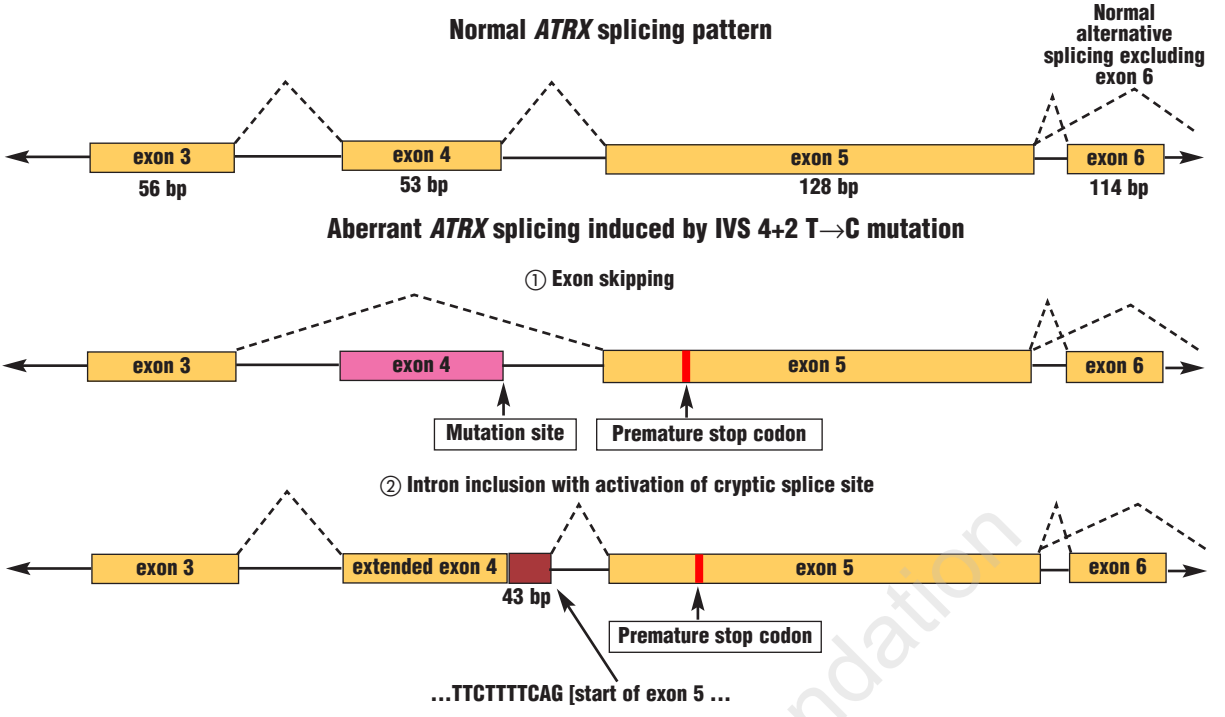

Figure 3. Ideogram of detected splicing consequences of the IVS 4+2 T→C mutation. Introns and exon 6 are not drawn to scale. The 5' end of *ATRX* normally exhibits alternative splicing of exon 6 (top.) The novel point mutation 3' to exon 4 results in exon 4 skipping (middle) or intron 4 retention with recruitment of a cryptic splice site (bottom). Both shift the open reading frame, with premature stop codon generation.

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 spliceoform, since the primers used for amplification and sequencing the cDNA (5'-GCCCATGAGTGAAAGCAAGT-3' and 5'-AGCT-CACAATCCCATGAAGC-3') spanned exons 1-7. The other spliceoform pair included intact exons 3, 4 and 5, but retained 43 base pairs of intron 4 (including the T→C 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,[22-25] and may represent potential therapeutic targets.[26-29] 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.[24] In addition, *trans*-acting alterations in components of the extraordinarily complex spliceosome[30,31] or, perhaps more commonly, in non-spliceosomal RNA binding protein regulators,[32] can cause varied and unpredictable splicing abnormalities. This class of mutation may account for aberrant or alternative spliceoforms where a *cis*-acting cause is sought and not found, as often happens in cancer.[24] 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 *PASG/HELLS/SMARCA6* gene in leukemia.[19,33,34]

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.[35-38] 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[39] 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* .[18] Third, while human *ATRX* has several known alternative mRNA isoforms in healthy individuals, including a conserved truncated isoform,[40] alternative splicing of exon 6,[41,42] and intron 33* retention,[18] 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.[9] Germline *ATRX* splicing mutations do occur, but are an uncommon cause of inherited ATR-X syndrome.[43-45] 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,[46] 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.[47]

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,[48] and these appear to be especially common in association with CMML.[49] This observation has motivated clinical trials of immunosuppressive therapies, including anti-thymocyte globulin and cyclosporine in MDS, which have met with some success.[50] 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.[51] 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;[52] *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 ATMDS phenotype, 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,[18] which is not routinely detectable in human samples in health or disease. In this report we have followed the lead of others[42,53] 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[9,10,14] exon 8 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 BCB 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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