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Missense mutations in the PML/RARα ligand binding domain in ATRA-resistant As_2O_3 sensitive relapsed acute promyelocytic leukemia

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Abstract

Background and Objectives. **Acute promyelocytic leukemia is characterized by the chromosomal translocation t(15;17) which yields the fusion product PML/RARα. All-trans retinoic acid probably induces differentiation of atypical promyelocytes and clinical remission in APL patients by binding to the ligand binding domain (LBD) of the RARα portion of the PML-RARα chimeric protein. Structural alterations of the LBD of the PML/RARα have been revealed in ATRA resistant APL cell lines and in a few APL patients with acquired clinical resistance to ATRA therapy. Two APL relapsed patients with clinical resistance to ATRA therapy were evaluated for the presence of nucleotide mutations in the LBD of PML/RARα gene and then treated with arsenic trioxide (As_2O_3).**

Design and Methods. **DNA fragments from the LBD of the PML/RARα chimeric transcript were obtained by reverse-transcribed polymerase chain reaction. Direct sequencing was performed by an unambiguous bidirectional automatic analysis. Samples representative of APL onset and relapse were analyzed from both patients.**

Results. **In both patients, at the ATRA resistant relapse, a missense point mutation in the LBD of the PML/RARα gene was found. The mutations, absent at APL onset, led to an Arg272Gln and to an Arg276Trp amino acid substitution, according to the sequence of the RARα protein. Both patients had complete clinical and hematologic remission after treatment with As_2O_3.**

Interpretation and Conclusions. **LBD missense mutations appear to be a significant mechanism of acquired ATRA-resistance *in vivo*, closely related to clinical APL relapse. The two cases reported here provide the first *in vivo* evidence of APL relapsed patients, who have become ATRA resistant for molecular reasons, being sensitive to arsenic trioxide.**

Key words: acute promyelocytic leukemia, PML/RARα, ATRA-resistance, missense point mutations, arsenic trioxide

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The chromosomal translocation t(15;17), characteristic of acute promyelocytic leukemia (APL), involves the retinoic acid receptor-α (RARα) gene on chromosome 17 and the PML gene, a putative transcription factor, on chromosome 15, generating the PML/RARα chimeric gene.[1-3] The resulting PML/RARα chimeric protein is crucial to the pathogenesis of APL.[4]

Moreover, APL has the unique characteristic of responding *in vitro* and *in vivo* to differentiating therapy with all-trans retinoic acid (ATRA).[5,6] The PML/RARα protein retains the majority of the functional domains of the RARα gene, in particular the DNA- and the ligand-binding domain (LBD), maintaining the ability to bind ATRA and activate transcription.[7-9] ATRA used as single agent in APL therapy induces clinical remission in the great majority of patients. Nevertheless, acquired clinical ATRA resistance appears within a few months of treatment and relapse occurs in almost all.[10] For this reason, modern treatment of APL combines ATRA with cytotoxic chemotherapy, obtaining long remission in the majority of patients.[11,12] However, relapse still occurs in about 20-30% of patients. In these patients clinical ATRA resistance is often present. Several mechanisms have been postulated to explain this clinical ATRA resistance, including pharmacokinetic reasons[13] and an increase in the cellular retinoic acid binding protein (CRABP) or multidrug-resistance (MDR) gene products.[14,15] Moreover, APL cells from clinically ATRA-resistant patients often exhibit ATRA-resistance or decreased sensitivity *in vitro*, suggesting cellular mechanisms leading to the selection of ATRA resistant APL subclones, in the presence of pharmacological concentrations of ATRA.[14] A point mutation in the LBD sequence, abolishing the ATRA binding activity of the PML/RARα, has been detected in an ATRA-resistant NB4 subclone,[16] suggesting that similar alterations might be the cause of ATRA resistance *in vivo*. Recently, two reports[17,18] demonstrated the presence of such mutations in 2 and 3 APL relapsed ATRA-resistant patients.

Here we report the presence of mutations in the LBD of PML/RARα gene in another two APL relapsed ATRA-resistant patients, who were subsequently shown to be clearly sensitive to As_2O_3 treatment.

Design and Methods

Bone marrow aspirates were obtained from two patients with APL. Patient #1 was referred to the Hematology Division, Oncology Department, University of Pisa and patient #2 to the Hematology Division, Department of Medical Sciences of the University of Modena and Reggio Emilia, Italy. Total RNA was extracted from Lymphoprep (Nycomed Pharma AS, Majorstu, Norway) isolated-mononuclear cells by a guanidinium thiocyanate-phenol-chloroform method.[19,20]

cDNA was synthesized using 1.5 µg of total RNA in a 30 µL reaction mixture using 400 U of M-MLV reverse transcriptase (GibcoBRL, Gaithersburg, MD, USA), 20 U of RNAsin (Roche Diagnostics, Mannheim, Germany), 1 µg of oligo-dT10 primer (Roche Diagnostics, Mannheim, Germany) in 50 mmol/L Tris-HCl, pH 8.3, 60 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, 0.5 mmol/L dNTPs at 42°C for 1 hour followed by 3 minutes at 95°C. Five microliters of cDNA products were used in the further PCR reactions. The presence of the chimeric PML/RARα mRNA was detected by nested RT-PCR in accordance with the primer sequences and PCR conditions described by Borrow *et al.*[21] In order to define the type of chimeric PML/RARα (bcr1, bcr2 or bcr3) detected, the M2 primer, as reported by Lo Coco *et al.*, was also used.[22] In order to screen the RARα or PML/RARα LBD mutations a 728 nt PCR product was obtained using oligonucleotide E2 as the sense primer (5'-GCA TCA TTA AGA CTG TGG AG-3') and EF1a as the antisense primer (5'-GTA GAA GGC AGA GAA AAG C-3') in a 50 µL reaction mixture containing 0.225 mmol/L dNTPs, 1 µmol/L of each primer, 2.5 U of Taq polymerase (Roche Diagnostics, Mannheim, Germany), 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 5% dimethyl sulfoxide. The conditions of amplification were: 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C for 40 cycles with 2 minutes of an initial denaturation step at 94°C and 10 min of a final extension step at 72°C.

A sense primer corresponding to exon 1 of RARα (RAR-1A primer: 5'-ATG GCC AGC AAC AGC AGC TCC TGC CCG AC-3') and to exon 3 of PML (PML primer: 5'-TGT GCT GCA GCG CAT CCG CA-3') together with E4 antisense primer (5'-GCG AAG GCA AAG ACC AGG-3') were used in different PCR reactions performed under the same conditions in order to obtain differential amplification of the LBD of the RARα and of the PML/RARα genes. Using the PML oligonucleotide as the sense primer a fragment of 991 nt was obtained from patient #1; this represented the bcr3 type of the PML/RARα mRNA. Two fragments of 1,465 and 1,321 nt, representative of the PML/RARα bcr1 type, were obtained from patient #2. A fragment of 937 nt representative of the RARα mRNA was obtained from the patients when primers RAR-1A and E4 were used. Under the same condition, 2.5 µL of the PML-E4 and of the RAR-1A-E4 PCR reactions were re-amplified using E2 sense and E4 antisense primers, obtaining a fragment of 234 nt that was used for further sequencing analysis.

RT-PCR fragments excised from 1% low-melting agarose and further purified by Wizard PCR preps DNA purification kit (Promega, Madison, WI, USA) were directly sequenced using a BigDye terminator cycle sequencing kit and a DNA sequencer (ABI Prism 310 - PE Applied Biosystem, Foster City, CA, USA). Sequencing reactions were performed with the same oligonucleotides as those used in PCR amplifications by either the sense or antisense primers.

Clinical history

Patient #1

BP, a 30-year old man was diagnosed in December 1996 as having APL with the presence of a typical PML-RARα chimeric mRNA of the short type (bcr3). The patient was treated with idarubicin plus ATRA (45 mg/m^2/d) and with Ara-C plus idarubicin or mitoxantrone for three consolidation courses, according to the Italian GIMEMA AIDA protocol.[12] The patient achieved complete clinical and molecular remission and was treated with ATRA 45 mg/m^2/d for 15 days every three months as consolidation therapy; he received three courses. Nine months after the end of the consolidation therapy the patient clinically relapsed and RT-PCR also revealed the presence of PML/RARα chimeric mRNA. The patient was again treated with ATRA and idarubicin according to the AIDA protocol induction regimen, but without success. Therefore, arsenic trioxide (As_2O_3) associated with ATRA (45 mg/m^2/d) was started at the dosage of 10 mg/d by i.v. infusion over 2 hours for 20 consecutive days. The patient achieved complete clinical and molecular remission. Further details about the clinical course of this patient have already been published.[23]

Patient #2

AF, a 56 year-old man was diagnosed as having APL in November 1996 by PB and BM morphologic examination. Nested RT-PCR revealed the presence of the long form (bcr1) PML/RARα chimeric mRNA. The patient had thrombosis of the common iliac artery as well a slowly-resolving staphylococcal pneumonia, so treatment with ATRA (45/m^2/d) as a single agent was started. The patient obtained a complete clinical and hematologic, but not molecular remission. ATRA administration was maintained until the disease relapsed after 8 months. Induction chemotherapy was started with idarubicin followed by three consolidation courses with Ara-C plus idarubicin or mitoxantrone in accordance with the AIDA protocol,[12] producing a second good clinical and hematologic, but not molecular, remission. Two months after the end of the consolidation therapy (June 1998), a second relapse was evident and ATRA therapy at the same standard dosage was started again and maintained for a month without any sign of clinical or hematologic response. The ATRA ther-

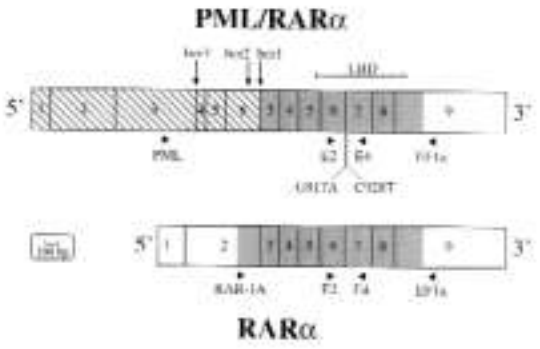

Figure 1: Schematic representation of the PML/RARα (upper) and RARα (lower) cDNAs. The PML portion is hatched, the RARα coding regions are grey and the RARα untranslated regions are clear. Numbered boxes are PML and RARα exons. The upper vertical arrows indicate the three breakpoint sites (bcr1, bcr2, bcr3) of the PML gene involved in the PML/RARα translocation. Arrowheads indicate the site of the primers used for PCR and sequencing analysis. Position and the type of the missense mutations in the PML/RARα ligand binding domain (LBD) found in the patients examined are also indicated.

apy was, therefore, stopped and the patient treated with As_2O_3 10 mg/d by i.v. infusion over two hours for 42 days, achieving a third complete, but not molecular remission.

Results

We screened for mutations of LBD PCR products using different oligonucleotides as primers in the RT-PCR experiments as shown in Figure 1. The different amplicons obtained were analyzed by an unambiguous bi-directional automatic direct sequencing analysis. The E2-EF1a fragment of 728 nucleotides was initially obtained from BM specimens taken during patient #1's APL first relapse and patient #2's second relapse. This PCR fragment is representative of the complete LBD of both PML/RARα and normal RARα genes. Patient #1's specimen revealed the presence of a G to A missense mutation at position 917, according to the nucleotide sequence of the RARα gene (reference sequence, *Giguere et al.*[24]). This mutation leads to the replacement of an arginine (Arg) amino acid by glutamine (Gln), corresponding to RARα codon 272. The same mutation has already been reported by Imaizumi *et al.* in an APL patient.[17] Patient #2's specimen revealed an as yet unreported mutation in which a T replaces a C at position 928, leading to the substitution of an arginine (Arg) by tryptophan (Trp) at codon 276. The same analysis was performed on specimens from both patients at the onset of APL, when no nucleotide substitutions were found. In order to confirm the presence of the mutations and to distinguish in which gene, either RARα or PML/RARα, the

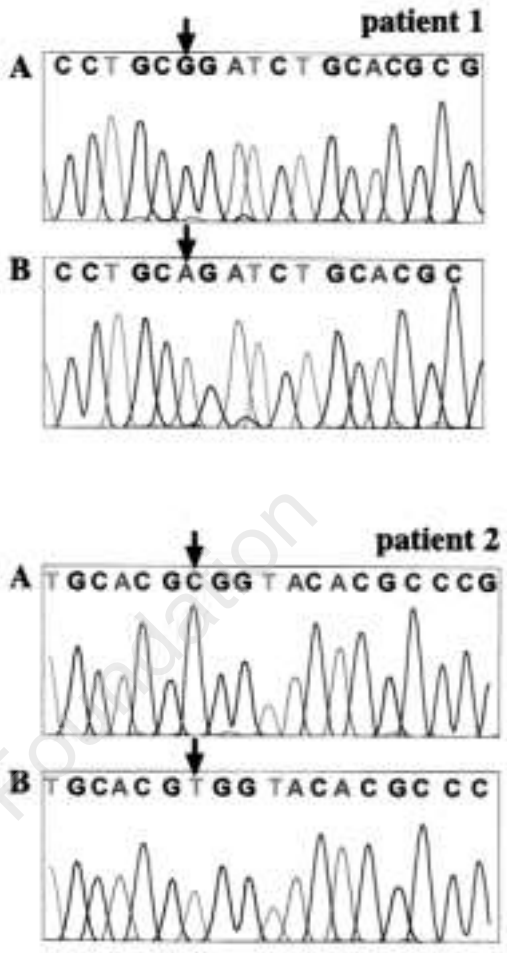

Figure 2. Automated sequencing analysis of the PCR products derived from the ligand binding domain of the RARα (A) and of the PML/RARα (B) of the patients with APL examined during ATRA-resistant relapse. Vertical arrows indicate the normal RARα nucleotides and the correspondent mutated PML/RARα nucleotides.

point mutations were present, we performed differential amplifications of the PML/RARα and RARα genes, using as sense primers, the RAR-1A oligonucleotide specific to the RARα gene and the PML oligonucleotide specific to the PML/RARα gene in two different PCR reactions, using as antisense primer the E4 oligonucleotide. Both reactions were re-amplified using E2 and E4 primers. Sequence analysis revealed the presence of the missense mutations in the PML/RARα derived amplicons from both patients. The normal RARα derived amplicons were unmutated (Figure 2). Other BM specimens taken from patient #2 throughout the course of the disease were available, allowing the timing of the appearance of the missense mutations to be studied. The C928T mutation was absent in the BM specimens collected 2 and 5 months after the onset of APL and first appeared in

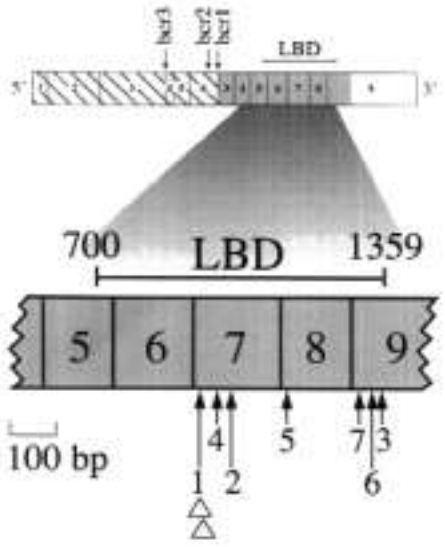

Figure 3. Summary of the LBD PML/RARα mutations so far associated with ATRA-resistance. The ligand binding domain (LBD) comprised between nucleotide #700 and #1359, according to the nucleotide number assignment by Giguere *et al.*[24] is represented. Arrows with numbers indicate the site of point mutations in APL patients (1-5) and cell lines (6,7). 1: G917A (Arg272Gln) and 2: A991T (Met297Leu) reported by Imaizumi *et al.*[17] 3: T1340C (Met413Thr), 4: C970G (Leu290Val) and 5: C1282T (Arg392Trp) reported by Ding *et al.*[18] 6: C1322T (Pro407Leu) reported by Kitamura *et al.*[34]). 7: T1295C (Leu398Pro) reported by Shao *et al.*[32] White arrowheads indicate the sites of the two point mutations reported in this paper.

the BM specimen collected 8 months after the onset of APL. This was the time of the patient's first relapse. The mutation was present in all subsequent BM samples.

Discussion

ATRA treatment of APL induces terminal cell differentiation and complete clinical remission. Nevertheless, these remissions are transient and ATRA-resistance often appears at relapse. There are different mechanisms underlying this acquired resistance: during continuous administration, ATRA plasma concentrations decrease as a result of enhanced activity of P450 as well as up-regulation of the CRABP protein.[25,26] Nevertheless, ATRA-resistance is also present at a cellular level. Several laboratories have identified ATRA-resistant sub-clones of the NB4 cell lines. Of interest, in the retinoic-resistant NB4-R4 subclone, Shao *et al.* identified a missense mutation in the LBD of the RARα portion of the PML/RARα but not of the RARα gene.[16] This mutation abrogates the ability of the PML/RARα protein to bind its ligand and inhibiting, in a retinoic-independent dominant-negative manner, the co-expressed wild-type RARα. Very recently, Imaizumi *et al.* and Ding *et al.* independently reported the presence of point missense mutations in the LBD of the PML/RARα gene in 5 ATRA-resistant relapsed patients, in particular in those patients who had received prolonged or intermittent administration of ATRA before relapse.[17,18]

In this study we identified two new ATRA-resistant APL patients with a mutated PML/RARα. The mutations were present only at relapse, and were not detected at the onset of APL in either patient examined. Moreover, analysis of different BM specimens from patient #2 indicates that the appearance of the mutation is strictly related to APL relapse, since early BM samples collected during remission did not contain mutations, but were still positive for PML/RARα chimeric mRNA by RT-PCR. These data strongly suggest that APL LBD-mutated cells may have a significant proliferative and survival advantage over APL LBD-wild-type cells during the course of ATRA therapy, so that their clonal expansion may give rise to clinical relapse.

The missense mutations found in the two patients examined are both in the 5' portion of the LBD. In particular, Arg272 of RARα appears to be very important in making van der Waal contact with the acyl-chain of ATRA.[27] In fact, site direct mutagenesis at Arg272 is able to impair the ATRA binding capacity of RARα.[28] Therefore, the Arg272Gln mutation found in patient #1, already reported by Imaizumi *et al.*,[17] seems to play a crucial role in the ligand function of RARα. This alteration probably causes the clinical ATRA-resistance shown in this patient. In patient #2 an as yet unreported Arg276Trp substitution, closely related to Arg272, was observed. In this case too the involved amino acid residue seems to play a crucial role in ligand binding capacity of RARα, making a weak salt bridge with the retinoic acid carboxylate. It also contributes to the inter-aminoacidic hydrogen-bound network of the LBD RARα region.[26]

In 1997 investigators from China reported that As_2O_3 was able to induce complete remission in APL relapsed patients.[29] These data have been very recently confirmed by Soignet *et al*.[30] Both patients reported here clearly demonstrated clinical resistance to ATRA and cytotoxic chemotherapy. We therefore decided to treat them with As_2O_3, which induced complete remission in both the patients without significant toxic side effects. A recent report by Nason-Burchenal *et al.* revealed that reduction of PML/RARα expression in the NB4-R1 ATRA resistant APL cell line, bearing a mutation in the LBD of the PML/RARα, is incompatible with leukemic cell growth.[31] Therefore targeting PML/RARα could have therapeutic potential in retinoic-resistant APL cells. This might be the advantage of arsenic trioxide which is able to induce partial differentiation and apoptosis of APL cell lines with wild-type or LBD mutated PML/RARα, inducing rapid degradation of the chimeric protein.[32,33] Further *in vivo* and *in vitro* molecular studies are necessary in order to

define whether the retinoic acid binding capacity is in fact affected in all the LBD mutated cases and whether degradation of the PML/RARα protein still occurs during As_2O_3 treatment. The two cases reported here provide the first *in vivo* evidence that APL cells bearing LBD point mutations are sensitive to As_2O_3, indicating that this apoptotic agent may have a use in APL patients who become ATRA resistant for molecular reason.

Contributions and Acknowledgments

MR was responsible for data collection, literature revision and writing of the manuscript. PZ, PV, SG carried out PCRs and sequencing analysis. GL, AD, MP were the clinicians responsible for the patient's clinical management. ML contributed to data analysis and revision of the paper. GT supervised the entire study and revised the final version of the paper.

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Disclosures

Conflict of interest: none.

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