Real-time quantification of multidrug resistance–1 gene expression in relapsed acute promyelocytic leukemia treated with arsenic trioxide

The multidrug resistance-1 (MDR-1) gene expression in 16 patients with relapsed acute promyelocytic leukemia (APL) treated with arsenic trioxide (AS_2O_3) and consolidated with idarubicin was quantified by quantitative polymerase chain reaction. MDR-1 expression was increased in nine patients (56%) at relapse (10-3,000 times the presentation level), and further increased in about half who relapsed post-AS_2O_3 treatment. MDR-1 expression had no effect on the response to AS_2O_3, and did not increase relapses after AS_2O_3 and chemotherapy. We conclude that the increased MDR-1 expression in relapsed APL might be overcome by AS_2O_3. Factors other than MDR-1 expression might be involved in relapses post-AS_2O_3 treatment.

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Arsenic trioxide (As_2O_3) induces remissions in most patients with relapsed acute promyelocytic leukemia (APL),[1,2] but optimal post-As_2O_3) consolidation is undefined. Only 1/3 to 1/2 of patients treated with As_2O_3) alone remain in remission,[3,4] suggesting that additional post-As_2O_3) chemotherapy is necessary.

The P-glycoprotein (P-gp) encoded by the multidrug resistance (MDR)-1 gene is a transmembrane efflux pump for chemotherapeutic drugs. MDR-1 expression in acute promyelocytic leukemia (APL) at presentation is low, but may be increased at relapse.[5] In this study, we quantified MDR-1 expression in relapsed APL with real time polymlerase chain reaction (Q-PCR), and examined whether MDR-1 expression was related to the response to As_2O_3 and chemotherapy. Sixteen consecutive patients with relapsed APL gave informed consent and were studied (Table 1). Treatment comprised As_2O_3 (10 mg/day) until complete remission (CR), and idarubicin for consolidation (54 mg/m^2 in 9 divided doses).[6] All patients achieved CR with As_2O_3. At a median follow-up of 18 months, seven patients had relapsed again. Two died before treatment, and five received As_2O_3 (10 mg/m^2) and ATRA (45 mg/m^2), and have remained in remission.[7]

MDR-1 expression was quantified by Q-PCR (ABI Prism 7700, PE Biosystems, Foster City, CA, USA). Briefly, total mRNA was extracted from diagnostic marrow after Ficoll-density centrifugation (blast percentage >90%). Q-PCR primers and the TaqMan probe for target genes are shown in Figure 1. The MDR-1 transcript copy number was normalized to an internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Threshold cycles (CT) at which a significant increase in fluorescence signal first occurred were determined. Serial dilutions of RNA from the MDR-1 expressing leukemic line CEM 1.0 (V. Ling, British Columbia Cancer Research Center)[8] were used to construct a standard curve of CT versus log RNA (Figure 1A). Patients' samples were tested in triplicates, and the starting mRNA copy numbers were determined from the CTs (Figure 1B).

Presentation samples were available in eleven cases. The median MDR-1 expression was 0.018 (0.004–0.282) (× dilution of CEM 1.0) at presentation and 0.193 (0.014–223.4) at relapse. For paired comparison, there was an overall significantly increased MDR-1 expression (p=0.026, Wilcoxon sign rank test). In seven cases, MDR-1 expression was significantly (> 10 times) increased at relapse, sometimes up to 40-3,000 times that of presentation, and 223 times the expression level CEM1.0 (Table 1) (Figure 1C and D). There was no significant change in three cases, and in one case (patient 7), MDR-1 expression was significantly decreased (> 10 times). In five cases without presentation samples, two cases (patients 11 and14) showed very high levels of MDR-1 expression (28.1 and 12.6×CEM 1.0) at relapse. Thus, evidence of increased MDR-1 expression was found in 9/16

Table 1. Clinicopathologic features and results of *MDR-1* gene quantification in 16 relapsed APL patients treated with As_2O_3.

Cases	Sex/Age	Status at As_2O_3 therapy	Time from last CR to As_2O_3	DFS	Outcome	MDR-1 expression (×10^{-3} dilution of CEM) P	R1	R2	R3
1	M/25	R1	9 m	10 m	CR2 = 10 m, R2 as EMD, died in R2	4	571	–	–
2	M/24	R1	11 m	34 m +	CR2	10	22	–	–
3	M/46	R1	7 m	33 m +	CR2	–	167	–	–
4	F/47	R1	14 m	36 m +	CR2	5	75	–	–
5	M/50	R1	24 m	18 m +	CR2	66	223,376	–	–
6	M/33	R1,R2	12 m	4 m +	CR2 = 12 m, R2 treated with As_2O_3 and ATRA, CR 3 = 4 m	–	367	–	
7	F/26	R1	21 m	38 m +	CR2	282	23	–	–
8	F/26	R1, R2	16 m	14 m +	CR2 = 21 m, R2 treated with As_2O_3 and ATRA, 19 CR3 = 14 m	193	258,975	–	–
9	F/47	R1, R2	17 m	3 m +	CR2 = 17 m, R2 treated with As_2O_3 + ATRA, CR3 = 3m	21	4,082	124	–
10	M/37	R1	6 m	18 m +	CR2	22	8,822	–	–
11	M/37	R1, R2	14 m	9 m +	CR2 = 19 m, R2 treated with As_2O_3 and ATRA, CR3 = 9 m	–	28,116	8	–
12	F/20	R1	11 m	12 m +	CR2	11	171	–	–
13	F/46	R2, R3	19 m	20 m +	CR3 = 19 m, R3 treated with As_2O_3 and ATRA, CR4 = 20 m	18	34	1,104	2,028
14	F/39	R2	15 m	7 m	CR3 = 7 m, died in R3 before treatment could be given	–	–	20	7
15	M/34	R1	10 m	5 m +	CR 2	18	14	–	–
16	M 60	R1	84 m	3 m +	CR 2	–	12,641	–	–

CR: complete remission; DFS: disease-free survival from latest As_2O_3 treatment; EMD: extramedullary disease; R: relapse; m = month; P: presentation; –: not done because of lack of sample.

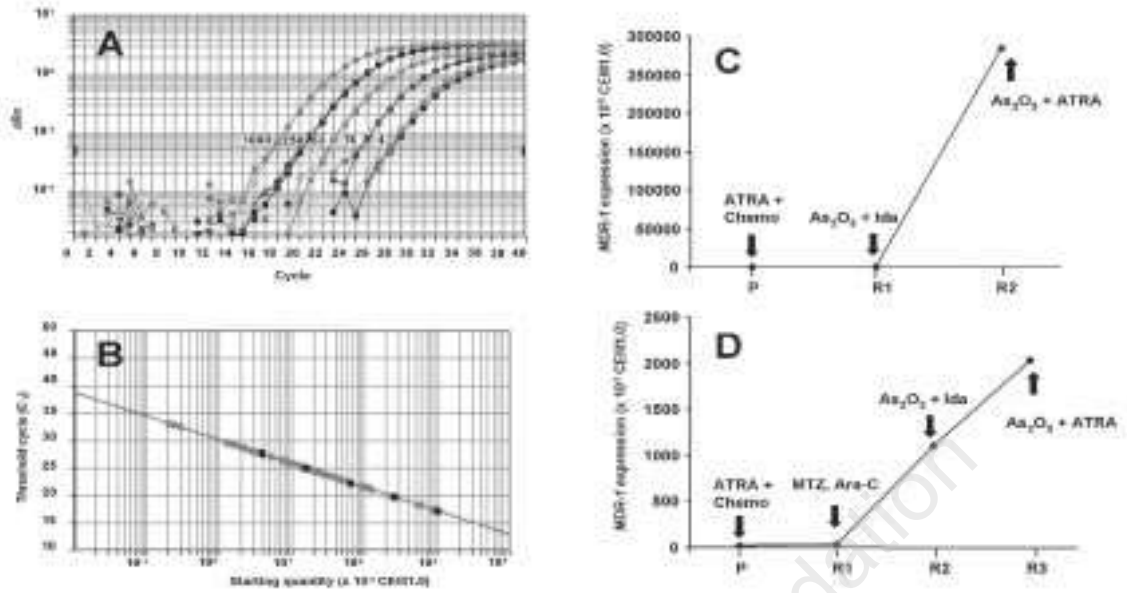

Figure 1A. Q-PCR of *MDR-1*. Primers (forward primer : 5'– CCC AGG AGC CCA TCC TGT – 3', backward primer : 5'– CCC GGC TGT TGT CTC CAT A – 3') and the dual-labeled TaqMan probe (5' – [FAM] TGA CTG CAG CAT TGC TGA GAA CAT TGC [TAMRA] – 3') were designed with the Primer Express software (PE Biosystems). Primers were used at a final concerntration of 300 nM, and the probe at 200 nM. The Taqman EZ RT-PCR kit (PE Biosystems) was used for Q-PCR, according to the instructions of the manufacturer, with the reverse primer of *MDR-1* used for first strand c-DNA synthesis. Amplification plot of different dilutions of 1,000 ng of CEM 1.0 mRNA (4 – 1000×10^{-3}, denoted by boxes next to the corresponding curves). Cycle number is plotted against change in normalized reporter signal ΔRn). For each reaction, the fluorescence signal of the reporter dye (FAM for *MDR-1* and JOE for *GAPDH*) is divided by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalized reporter signal (Rn). *GAPDH* was similarly amplified with forward (5' – GAA GGT GAA GGT CGG AGT C – 3') and backward (5' – GAA GAT GGT GAT GGG ATT TC – 3') primers and detected by the TaqMan probe (5' – [JOE] CAA GCT TCC CGT TCT CAG CC [TAMRA] – 3'), and used an internal control for the amount and quality of input mRNA. Figure 1B. Plot of standard curve of starting CEM1.0 mRNA amount against CT. Black circles represent CEM1.0 standards as shown in Figure 1A. Grey circles represent some of the patients' samples. Figure 1C. Case #8, showing a dramatic increase in *MDR-1* gene expression with second relapse. P: presentation; R: relapse; ATRA: all trans retinoic acid; Chemo: chemotherapy; As_2O_3: arsenic trioxide; Ida: idarubicin. Figure 1D. Case #13, showing a progressive but moderate increase in *MDR-1* gene expression with consecutive relapses. MTZ: mitoxantrone, Ara-C : cytosine arabinoside.

(56%) cases of relapsed APL. Eight patients remained in remission, including four cases with high MDR-1 expression at first relapse (R1). Of seven cases with post-As_2O_3 relapses, five had increased MDR-1 expression at their initial relapse. Hence, the incidence of post-arsenic relapse was apparently unrelated to MDR-1 up-regulation at R1 ($p=0.608$, χ^2). There was also no relation between the magnitude of increased MDR-1 expression at R1 and post- As_2O_3 relapse ($p=0.388$, Mann Whitney test). At further relapses, MDR-1 expression was increased in two cases (patients 8 and13) (Figure 1 C and D), but significantly decreased in two other cases (patients 9 and 11). MDR-1 expression had no effect on the response to As_2O_3 and ATRA, as all treated cases achieved CR (Table 1).

There are a number of interesting observations in this study. Our results show, for the first time, that in some cases of APL, MDR-1 expression might be dramatically increased at relapse, reaching up to 4 to 223 times that of a cell line selected for the MDR phenotype in vitro. The impact of MDR-1 expression on treatment outcome in relapsed APL has not been fully examined in the literature, especially with the recent advent of arsenic therapy, which is presently the treatment of choice. MDR-1 expression in MDR and P-gp-transfected cell lines was found not to affect the in vitro sensitivity to As_2O_3.[9] This was shown in our study, as all patients treated with As_2O_3 achieved a remission irrespective of MDR-1 expression, whether initially or at post-As_2O_3 relapse. However, as As_2O_3 alone is not curative in at least half of the patients, additional chemotherapy may be needed. Therefore, MDR-1 expression might be reasonably expected to influence relapses after As_2O_3 treatment. Interestingly, in this study, the presence or magnitude of MDR-1 overexpression at R1 was not shown to be related to further relapses post-As_2O_3/idarubicin treatment. Hence, it is possible that arsenic may play a more important role than idarubicin in eradicating chemotherapy-resistant APL clones at relapse. Moreover, although further MDR-1 upregulation was seen in two cases (1.1 and 258 times that of CEM 1.0) after As_2O_3/idarubicin, MDR-1 expression was in fact decreased to low levels (0.007 – 0.12 times CEM 1.0) in three cases. To explain these observations, one might postulate that at initial relapse, different APL clones with or without increased MDR-1 expression existed. As As_2O_3 treatment was unaffected by MDR-1, the survival of APL clones with different MDR-1 expression might either be probabilistic, or determined by factors related to As_2O_3 resistance rather than P-gp.

Finally, high levels of cellular glutathione and the multidrug-resistance-associated protein 1 have been observed in As_2O_3-

resistant MDR cells.[10] The contribution of these proteins to As_2O_3 resistance in APL patients will need to be further examined.

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Identification and functional characterization of a new hemoglobin variant in Sardinia: Hb Muravera [β47 GAT→ GTT, (CD6) Asp→Val]

Hemoglobin Muravera [β47 Asp→Val] is a new, slightly unstable hemoglobin variant found in Sardinia during a screening for β-thalassemia. Purified Hb Muravera displays an oxygen affinity higher than that of HbA in the absence of 2,3 DPG, and a faster than normal rate of auto-oxidation. The functional alterations of Hb Muravera could be due to the structural modification induced by the type and position of the substituted amino acid.

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The β47 (CD6) residue of human hemoglobin (Hb)A is normally an aspartic acid that forms intrachain contacts with β53 Ala, β54 Val and β57 Asn.[1] The β47 (CD6) residue is in an external non-helical segment that, although not directly involved in the heme contacts, might contribute significantly to maintaining the structure of the heme pocket, therefore establishing the oxygen binding properties of the molecule.[2] We report here a new hemoglobin variant, resulting from a GAT→GTT mutation at codon 47 of the β globin gene, which predicts an Asp→Val amino acid substitution. This variant was detected during screening to identify β-thalassemia carriers in Southern Sardinia. Hemoglobin analysis of the proband performed by high performance liquid chromatography (Variant I, Bio-Rad, Milan, Italy) showed an abnormal peak eluting with HbA_2, in the amount of 33.3%. On cellulose acetate electrophoresis at alkaline pH, the abnormal band was in the HbS-like position, but the sickling test was negative. The proband had mild microcytosis (MCV = 78.1 fL) and hypochromia (MCH = 26.2 pg), but a normal hemoglobin level (Hb = 15.6 g/dL). HbA_2 determined by DE-52 microchromatography was 3.3% (Figure 1). The isopropanol test was weakly positive. Globin chain synthesis analysis showed an α/non-α globin chain synthesis ratio of 0.75, compatible with an α-thalassemia carrier state confirmed by α-globin gene analysis (genotype –3.7 α/αα). The proband's sister had mild reticulocytosis, a weakly positive isopropanol test, and some red blood cells containing inclusion bodies, after 1 h incubation at

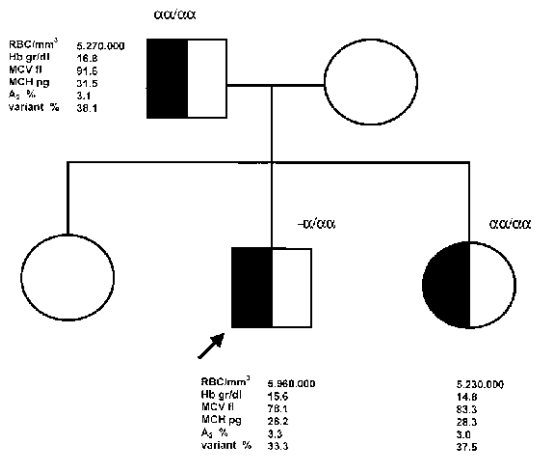

Figure 1. Pedigree of the family. The proband is indicated by the arrow.