Case #4 had a TTE done one month prior to anagrelide therapy that showed an EF of 73%. She took anagrelide for just over one year before developing peripheral edema and dyspnea on exertion. TTE showed an EF of 30%. Anagrelide was discontinued leading to improvement in symptoms and a repeat TTE 3 months later showed an EF of 55%.

Case #5 was treated with anagrelide for 10 months before developing chest pain. Coronary angiogram was normal, but TTE showed an EF of 35%. Anagrelide was stopped with improvement in symptoms. Follow-up TTE one month later showed an EF of 44%.

Case #6 had a TTE done 9 months into anagrelide treatment (EF = 58%). Two months later, still on anagrelide therapy, she developed symptoms of heart failure. TTE showed an EF of only 10%. Anagrelide was stopped leading to immediate clinical improvement and a repeat TTE, performed within a week, showed a substantial improvement in EF (34%).

Treatment in the 5 patients with ICM that was not associated with anagrelide therapy consisted of either phlebotomy alone (3 patients) or other cytoreductive agents (2 patients). The clinical course of ICM in these 5 cases was stable in terms of both symptoms and EF.

The observations from the current study strongly suggest a potentially reversible drug-induced cardiomyopathy in anagrelide-treated patients with either PV or ET. The mechanism of action is currently unknown but may involve the drug's known cardiovascular effects including positive inotropism, vasodilatation, and tachyarrhythmia.[5] In regards to this last effect, the reversible nature of anagrelide-associated ICM is reminiscent of tachycardia-induced cardiomyopathy.[6]

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Key words: anagrelide, cardiomyopathy, congestive heart failure, drug toxicity.

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Acute Myeloid Leukemia

ABC transporter ABCA3 is expressed in acute myeloid leukemia blast cells and participates in vesicular transport

Drug resistance is a major issue in the treatment of acute myeloid leukemia (AML), and drug efflux by ATP-binding-cassette (ABC) transporters is one of the main mechanism involved in this resistance. We determined the prevalence of the intracellular transporter ABCA3 in specimens from patients with AML, and addressed its biology with attention to intracellular compartmentalization.

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We recently described a subpopulation of leukemic progenitor cells with high intrinsic drug efflux capacity, and detected the expression of ABCA3 in the cells of the AML-SP1 leukemia model.[1-3] Whereas expression of ABCA3 was absent or low in normal hematopoietic tissues, reverse transcription polymerase chain reaction (RT-PCR) revealed expression of the transporter in 81% and 88% of mononuclear cells from adult and pediatric patients (n=33), respectively (Figure 1A). The median blast count of the samples was 80% (range 5% to 98%), and the blast percentages did not correlate with ABCA3 levels (correlation coefficient -0.05). The expression varied broadly with a trend towards higher levels in specimens from patients with relapsed or resistant disease (median 28.4%, n=6) than in specimens from those with primary disease (median 12.5%, n=27; p=0.16 two-tailed t-test for two samples with unequal variances). With the mean ABCA3 expression levels of FAB classes being 18.5% in MI (n=4), 13.0% in M2 (n=7), 13.5% in M4 (n=8) and 13.3% in M5 (n= 4), there was no segregation with FAB subtypes, nor with the prognostic implications of the karyotype. Immunocytology with a polyclonal antibody against ABCA3 was performed on seven cases and showed high staining intensities in two, intermediate/low in three, and no staining in 2 cases. The corresponding RT/PCR were values 150 and 32% in the two cases with high staining intensity, 27%, 2.1% and 11.9% in the cases with intermediate/low staining and 0% in the two with no staining. The immunostains showed a vesicular pattern of ABCA3 in the cytoplasm of the leukemic blasts (Figure 1B). Overexpression of ABCA3 in 293A cells augmented the number and size of acidic vesicles, as visualized by the fluorescent dye *lysotracker red*® specific for acidophilic organelles. Exploiting the fluorescent characteristic of daunorubicin, which is quenched when distributed into a non-nuclear compartment of cytoplasmic organelles,[4] we observed quenching of daunorubicin fluorescence in ABCA3 expressing cells which suggested that daunorubicin translocates from the nucleus into an extranuclear cellular compartment (Figure 2A). It is not yet clear whether the decrease in nuclear daunorubicin is due to an increased extranuclear compartment of the cells, transport the daunorubicin by ABCA3, or both mechanisms. To adress the volume of the extranuclear space we established a flow cytometric assay with *lysotracker red*® fluorescence as an indicator of lysosomal mass per cell. In this assay, a staining ratio (LSR) was defined by the mean fluorescence intensity of a given blast cell population divided by the fluorescence intensity of normal lymphocytes stained and measured on the flow cytometer in strict parallel:

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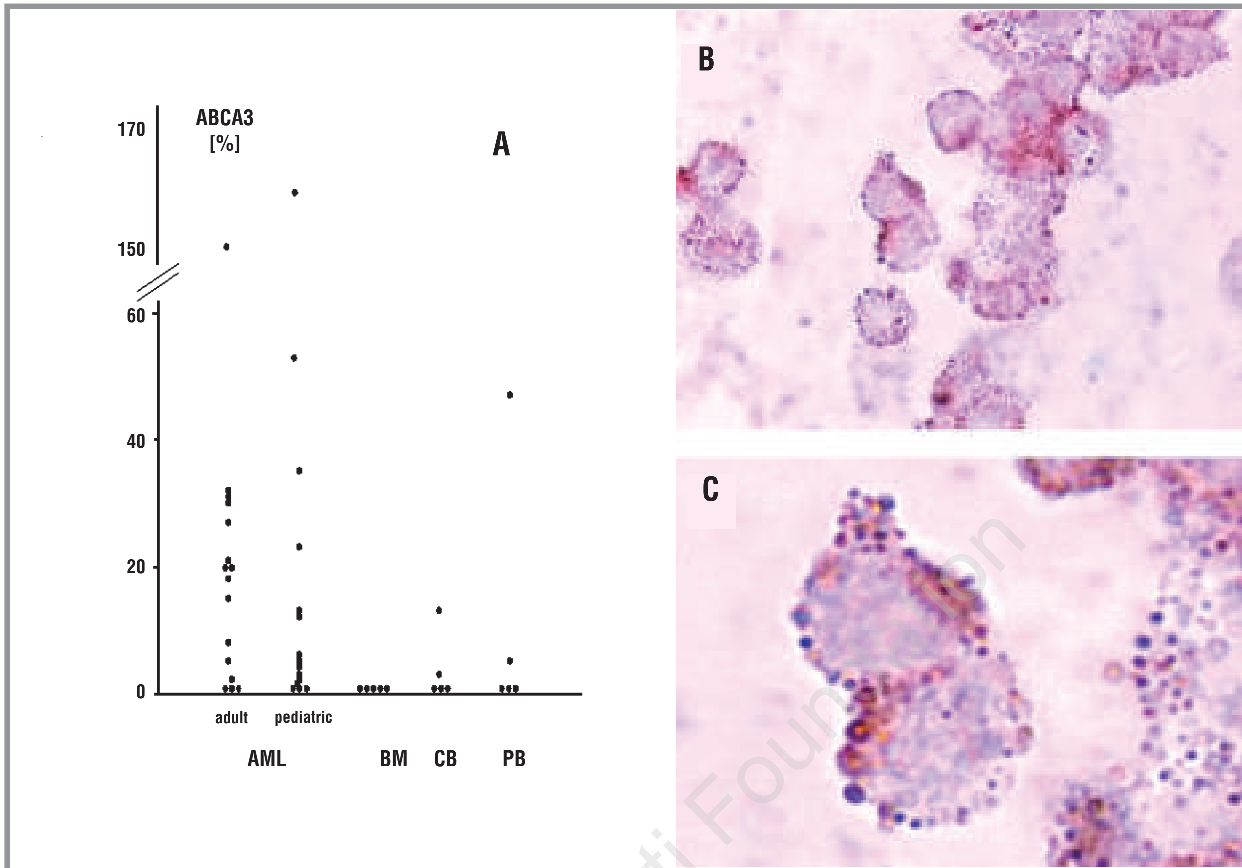

Figure 1. Expression of ABCA3 in bone marrow specimens of patients with AML, in normal hematopoietic cell populations and cytoplasmic localization of ABCA3 in AML blast cells. The prevalence of ABCA3 is reported as PCR product ratios of ABCA3/ABL or ABCA3/GAPDH, expressed in percentages (1 A). The AML samples were grouped into those from children (age 1 to 18 years) and those from adult patients (age >18 years). Specimens of normal peripheral blood (PB) and bone marrow (BM) were from adult volunteers, cord blood (CB) samples were taken from the umbilical vein. Indirect immunocytology with a polyclonal antibody against ABCA3 showed a vesicular cytoplasmic staining pattern in AML blast cells (1 B, C). 40x magnification in panel B, digital enlargement from panel B to C.

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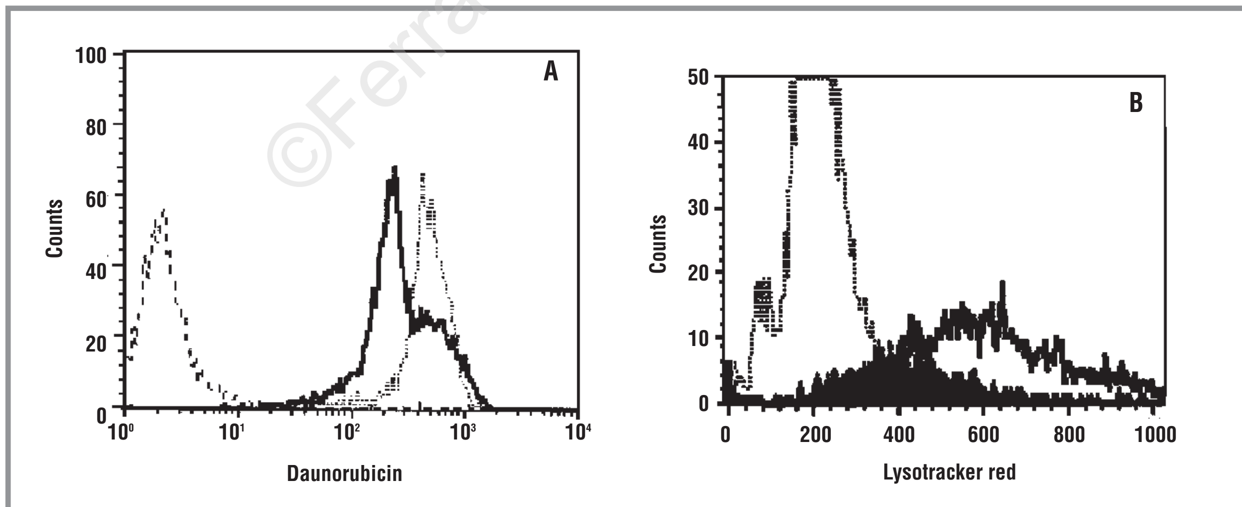

Figure 2. Augmentation of acidophilic vesicles by forced ABCA3 overexpression and *lysotracker red®* staining profile of normal and leukemic cells. ABCA3 encoding plasmid (70% transfection efficiency) and staining with the fluorescent drug daunorubicin (5 μg/mL, 90 min) revealed a shift to lower fluorescence in the ABCA3 transfected cells (2A, bold line), compared to in the mock transfected cells (dotted line) and the unstained control (dashed line). In a typical example of a *lysotracker red®* stain the leukemic blast population (2B, full line) is shown overlaid on normal lymphoid cells (dotted line) and myeloid/monocytic cells (shaded). The mean fluorescence of this AML specimen was 622, the LSR 2.9.

(LSR sample=mean $\text{fluorescence}_{\text{blast cells}}$/mean $\text{fluorescence}_{\text{lymphocytes}}$). While the LSR for normal myeloid/monocytic cells was 1.82 + 0.19 (n=6), we observed LSR of 3.2, 2.7 and 2.6 from the leukemic cell lines K562, HL60 and ML2, respectively, and a LSR of 2.35±0.35 (n=7) in clinical AML blast cell samples. Thus leukemic cell lines as well as primary AML blasts have a high volume of lysosomal space per cell. In addition to drug extrusion by ABC transporters across the outer plasma membrane, subcellular drug sequestration in acidic organelles has repeatedly been described to occur in resistant cells.[5,6] In acidic vesicles weakly basic drugs are protonated, lose membrane permeability and are secreted by fusion of the vesicles with the plasma membrane (the so-called protonation, sequestration and secretion (PSS) model.[7] The microscopic data available for ABCA3 are consistent with it having a role in sequestration and secretion.[8,9] It remains to analyze whether ABCA3-involving secretory mechanisms are similar in different cell populations such as pneumocytes, hematopoietic cells and malignant leukemic cells. In this regard, with AML as a malignant disease of progenitor cells, it will be particularly important to elucidate whether the expression of ABCA3 in leukemic cells is a reflection of its expression in precursor cell subsets.

In summary, ABCA3 is overexpressed in adult and pediatric AML, may have a role in the turnover of acidic vesicles, and may participate in subcellular drug sequestration. Understanding ABCA3 biology may give novel insight into mechanisms of drug resistance and leukemic blast cell physiology.

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Acute Myeloid Leukemia

Prognostic significance of *N-RAS* and *K-RAS* mutations in 232 patients with acute myeloid leukemia

Reports on the prognostic impact of mutations in the *RAS* proto-oncogenes in patients with acute myeloid leukemia (AML) are conflicting. A peptide nucleic acid (PNA)-based technique was used on 232 AML samples to detect point mutations of the hotspots in *N-RAS* and *K-RAS*. No significant correlations between *RAS* mutations and clinical features, karyotype or FLT3 were found.

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The presence of defined karyotypes are among the most important prognostic factors in acute myeloid leukemia (AML). However, even within defined cytogenetic groups stability of remission and long-term survival may vary significantly. Therefore, additional recurrent aberrations may have a prognostic impact. The role of Ras proteins in human leukemias has been analyzed extensively,[1] but their prognostic role, if any, is still disputed. The uncontrolled activation of Ras proteins, which can be found in a variety of malignancies, contributes to several aspects of the malignant phenotype including the deregulation of tumor-cell growth and programmed cell death.[1] In this correlation study, we analyzed *RAS* mutations using a PNA-based detection method in 232 patients with centrally confirmed and classified AML. All patients were treated within a large prospective multicenter study exploring a risk-adapted treatment strategy (SHG AML 96). The study design has been published elsewhere.[2,3] Patients with acute promyelocytic leukemia (FAB M3) were excluded. Morphologic analysis and immunophenotyping were done centrally in designated reference laboratories in Dresden and Erlangen, respectively. The cytogenetic risk assessment was done as described previously.[2,3] Mutations of codons 12, 13, 61 of *N-RAS* and codons 12, 13 of *K-RAS* were detected using a polymerase chain reaction (PCR)-based technique with PNA-clamping as described.[2,4] The PNA molecules were designed to hybridize to the wild type sequences, thus inhibiting primer binding to wild type DNA. In the case of a mutated allele the PNA molecule does not bind thus allowing preferential amplification of the mutated DNA. Additionally, in 197 out of 232 samples FLT3-activating mutations either through internal

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