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Association of *ABCB1* polymorphisms with survival and *in vitro* cytotoxicity in *de novo* acute myeloid leukemia with normal karyotype

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Running title: *ABCB1* genotypes in AML

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Abstract

Over expression of the multi-drug transporter P-glycoprotein, encoded by the *ABCB1* gene, is a clinically relevant problem in acute myeloid leukemia (AML). Polymorphisms in *ABCB1* might contribute to cancer risk and therapeutic response. We therefore investigated the influence of polymorphisms G1199A, C1236T, G2677T/A and C3435T on cancer susceptibility, *in vitro* cytotoxicity and overall survival in 100 *de novo* AML patients with normal karyotype. Patients with 1236C/C or 2677G/G genotypes showed poorer survival than patients with other genotypes (P=0.03 and P=0.02, respectively). Both these genotypes were significant factors for survival in multivariate analysis, along with age, *NPM1* and *FLT3* mutation status. *In vitro* cytotoxicity studies demonstrated that leukemic cells from 1236T/T and 2677T/T patients were significantly more susceptible to mitoxantrone (P=0.02), and tended to be more susceptible to etoposide and daunorubicin (P=0.07-0.09), but not to cytarabine. No significant difference in allele frequencies were found between patients and healthy volunteers (n=400).

Keywords: *ABCB1*, acute myeloid leukemia, single nucleotide polymorphisms, anthracyclines

Introduction

Cytogenetic aberrations are important prognostic factors in acute myeloid leukemia (AML). Based on cytogenetic abnormalities, cases of AML are usually classified into three groups, with favorable, intermediate and adverse prognosis.¹ The largest of these is the “intermediate” group, within which patients with normal karyotype constitute about 45% of cases with *de novo* AML.²⁻³ AML with normal karyotype is a heterogeneous group where some patients rapidly relapse while others go into complete remission and currently attempts are being made to distinguish different prognostic subgroups. During the last few years, mutations in the *FLT3* (fms-related tyrosine kinase 3) and *NPM1* (nucleophosmin) genes have been described and they have become established markers of clinical outcome and survival in cases of *de novo* AML with normal karyotype.⁴⁻⁵ Internal tandem duplication (ITD) within *FLT3* occurs in approximately 30% of all cases of AML with normal karyotype and it correlates with poor outcome, whereas absence of this mutation in the presence of *NPM1* mutation is associated with favorable prognosis.⁴⁻⁷ However, there is still a large group of patients with normal karyotype with intermediate risk who lack reliable prognostic markers and it is obvious from the clinical setting that there is a need for further markers to guide treatment decisions.

The development of multidrug resistance during cancer chemotherapy is a clinically relevant obstacle to successful treatment of AML. Increased expression of P-glycoprotein encoded by the *ABCB1* gene is a well-characterized mechanism by which cancer cells in culture avoid the action of chemotherapeutic agents. P-glycoprotein is capable of extruding cytotoxic drugs with different chemical structures and mechanisms of action, such as anthracyclines, vinca alkaloids and epipodophyllotoxins. All of these are used in the treatment of AML and cross-resistance occurs.⁸⁻⁹ The activity of P-glycoprotein has also been shown to affect the absorption and the elimination of several drugs.¹⁰⁻¹¹ Interestingly, several single nucleotide

polymorphisms (SNPs) in the *ABCB1* gene have been identified, of which C1236T (silent), G2677T/A (Ala893Ser/Thr) and C3435T (silent) have been associated with altered P-glycoprotein expression and phenotype.¹²⁻¹⁴ Altered transport activity due to the different genetic variants might lead to reduced capacity for the cells to avoid potential harmful xenobiotics including the cytotoxicity of chemotherapeutic drugs. The SNPs in *ABCB1* have also been associated with susceptibility to cancer, altered pharmacokinetics and treatment response to several drugs, including anticancer agents.¹⁵⁻¹⁷

Both *FLT3* and *ABCB1* are important for chemoresistance in AML¹⁸, and *NPM1* and *FLT3* status have recently become useful prognostic markers. Several studies have addressed the importance of genetic variants of *ABCB1* in the treatment of AML, with varying results.¹⁹⁻²² However, no study has yet attempted to take both these resistance mechanisms into account, especially not in patients with normal karyotype. We therefore investigated the importance of five different *ABCB1* SNPs (i.e. G1199A, C1236T, A1308T, G2677T/A and C3435T), *FLT3*-ITD and *NPM1* mutation status for the response and overall survival in 100 *de novo* AML patients with normal karyotype. We also determined the relevance of the *ABCB1* SNPs on AML susceptibility and on the *in vitro* drug cytotoxicity on isolated leukemic cells from the AML patients.

Design and methods

Patients

Peripheral blood samples were collected at diagnosis from 100 adult patients (mean age 63, range 20-85) with *de novo* AML and normal karyotype. No cases of relapsing or secondary leukemia were included. The patients were treated according to national guidelines (Swedish Haematology Association 2007) at Linköping University Hospital and Karolinska University Hospital in Huddinge, Sweden. All but four patients were treated with regimes that included anthracyclines or mitoxantrone in combination with cytarabine and the patients were evaluated up until 4 years. Patient characteristics and details of the induction treatment regime are presented in Table 1. The response after chemotherapy was evaluated as non-complete remission (no CR) or morphologic complete remission (CR).²³ To get a more specific effect of the chemotherapy on the survival patients treated by means of allogeneic bone marrow transplantation ($n=21$) were censored at time of transplantation in the survival analysis.

A Swedish reference material of 400 healthy volunteers of comparable age (median 60, range 22-77) and sex distribution (51% male and 49% female) was also included for evaluation of *ABCB1* genotype susceptibility to develop AML. DNA samples were obtained from a regional DNA bank consisting of genomic DNA isolated from selected individuals representing the population in the southeastern part of Sweden after obtaining their informed consent.

The study was approved by the local ethical committee and all patients included gave their written informed consent for genetic analysis for evaluation of therapeutic efficacy.

ABCB1, NPM1 and FLT3-ITD genotyping

The *ABCB1* G1199A, C1236T, A1308T, G2677T/A and C3435T genotypes were determined using pyrosequencing as previously described.²⁴⁻²⁵ In short, genomic DNA was isolated using QIAamp® DNA mini-kits (Qiagen, Sweden) according to the manufacturer's protocol. HotStarTaq master mixture (VWR International, Sweden) was used for PCR amplification and all reactions were carried out on a Mastercycler gradient instrument (Eppendorf, Germany) in a total volume of 25 µl. The SNPs were analyzed by a Pyrosequencing PSQ96MA instrument (Qiagen, Sweden) according to the manufacturer's protocol and as previously described.²⁴⁻²⁵ Insertion mutations in exon 12 of the *NPM1* (Gene ID: 4869) gene were detected by fragment analysis of PCR products as described previously.²⁶ For analysis of *FLT3-ITD* (*FLT3* Gene ID: 2322), PCR and fragment analysis were performed as detailed earlier.²⁷

In vitro cytotoxicity assay

Leukemic cells were isolated by centrifugation on metrizoate-dextran (Lymphoprep, Axis-Shield PoC, Oslo, Norway) from patients being treated at the Karolinska University Hospital (n=56). The cells, >90% pure as assessed by light microscopy, were incubated and cultured for 4 days with a panel of cytotoxic drugs, as previously described.²⁸ The drug concentrations were as follows: Ara-C 0.5 µM, daunorubicin 0.2 µM, etoposide 20 µM, mitoxantrone 0.1 µM. All incubations were performed in duplicate and with a drug-free control. Incubations with conventional chemotherapeutic drugs were designed to mimic the *in vivo* situation.²⁹ After incubation, cytotoxicity was assessed by means of a bioluminescence method, measuring the intracellular ATP concentration as a marker of cell viability after drug exposure.²⁸ The cell survival was expressed as percentage of viable cells compared to the control.

Statistical analysis

For comparison of genotype and allele distribution between patients and the reference material as well as between CR and no CR the generalized Fisher's exact test was used. Kaplan–Meier analysis was applied to estimate overall survival and the log-rank test to determine significance. Multivariate analysis was performed using the Cox regression model. Survival was evaluated as number of days after the date of diagnosis until death, the latest follow-up date or allogeneic bone marrow transplantation. When comparing groups in terms of differences in sensitivity in the *in vitro* drug panel, two-sided non-equal variance Student's *t*-test was used. P-values of <5% ($P < 0.05$) were taken as statistically significant. Results are presented as means and 95% confidence intervals (CI).

Results

No difference in ABCB1 genotypes between AML patients and controls

All 100 patients with *de novo* AML and the 400 healthy controls were successfully genotyped for the five *ABCB1* SNPs. No significant difference in genotype frequencies was found between patients and the reference population (Table 2), and the distribution of four of the SNPs (G1199A, C1236T, G2677T/A and C3435T) were in Hardy-Weinberg equilibrium. However, for A1308T, only the A/A genotype was found among these 500 individuals. In addition, all patients but one were successfully analyzed for *FLT3*-ITD and *NPM1* mutations (Table 1). No significant difference in distribution of *ABCB1* genotypes were found depending on *FLT3*-ITD or *NPM1* mutation status (data not shown).

NPM1 mutation, but not *FLT3*-ITD or *ABCB1* genotype, correlates with higher rate of complete remission

Of the 100 AML patients included, 72 patients achieved CR, 24 did not reach CR and 3 could not be evaluated. We could not find any significant correlation between the different *ABCB1* SNPs the response rate in the AML-patients. Neither were there any significant differences in *FLT3* status between patients that achieved CR and those that did not. However, *NPM1* mutations correlated significantly with CR rates ($P=0.04$). Of the 46 patients that carried mutated *NPM1* 39 (85%) achieved CR, while only 33 of the 50 patients (66%) with wild-type reached CR.

The *ABCB1* SNPs C1236T and G2677T as well as *FLT3*-ITD/*NPM1* influence the long-term survival of AML patients

In accordance with previous studies,³⁰ our data indicated that *NPM1*-positive and *FLT3*-ITD-negative patients showed better overall survival than the other patients (Figure 1A, P=0.06). Since only one patient had the 1199A/A genotype, we compared the overall survival of patients with the 1199 G/G genotype versus all patients with G/A and A/A genotypes; this comparison showed borderline significance (Figure 1B, P=0.06). The estimated mean survival for patients with the 1199G/G and G/A genotypes was 1.2 and 0.9 years, respectively, whereas the patient carrying the A/A genotype survived for 16 days. For C1236T, patients carrying the C/C genotype had a significantly shorter survival than those with other genotypes (Figure 1C, P=0.03). The mean survival was 0.7, 1.3 and 1.8 years for patients with the C/C, C/T and T/T genotypes, respectively. The 2677A allele was only present in four patients: two were heterozygous for G/A and two heterozygous for T/A, and they were excluded from analysis due to the low frequency. Patients carrying the wild-type 2677G/G had a significantly shorter survival than those with other genotypes (Figure 1D, P=0.02). The mean survival time of patients with G/G, G/T and T/T genotype of SNP G2677T/A was 0.7, 1.2 and 1.7 years, respectively. The C3435T genotype did not significantly correlate with survival (Figure 1E, P>0.05) and excluding patients that were transplanted from the analysis had minor impact on the statistics (data not shown).

In a multivariate Cox regression model the influence of age, *NPM1* mutation, *FLT3*-ITD, and the SNPs in the *ABCB1* gene on overall survival was investigated. However, the SNPs C1236T and G2677T are present in linkage disequilibrium^{16, 31} i.e. if the patient has the wild-type allele of one of the SNPs there is a high probability that the patient also has the wild-type variant of the other, and the effect of these SNPs could not be distinguished from each other. We therefore conducted two separate Cox regression analyses, one for each of these SNPs, to

determine the influence of the separate genotypes on survival (Table 3). Both the *ABCB1* SNPs C1236T and G2677T had a significant impact on survival in these models: the hazard ratios for patients carrying the 1236T/T and 2677T/T genotypes were 0.24 and 0.22 as compared to patients having the 1236C/C and 2677G/G genotypes, respectively. *NPM1* and *FLT3*-ITD were independent factors for survival in the C1236T model and there was also an indication in the G2677T model. The hazard ratios for *NPM1* and *FLT3*-ITD were 0.6 and 1.7-1.9, respectively (Table 3). Age was a significant variable in both models. G1199A lost its significance in the multivariate models probably due to multiple testing and the low allele frequency.

The *ABCB1* SNPs influence the cytotoxicity of leukemic cells in vitro

We tested the *in vitro* sensitivity of leukemic cells from 56 patients to chemotherapeutic drugs commonly used in the treatment of AML. Since it has previously been shown that *FLT3*-ITD has an impact on the cells' susceptibility to cytotoxic stimuli³², the effect of the *ABCB1* SNPs were investigated in cells from patients with wild-type *FLT3*, in which the resistance is not affected by the *FLT3*-ITD. The susceptibility of leukemic cells from patients with the 1199G/A, 1236T/T, 2677T/T and 3435T/T genotypes of the *ABCB1* SNPs was compared to the susceptibility of cells from all the other patients (Figure 2). We choose to compare the genotype which in the survival analysis gave the best survival to the rest, since they might show the lowest cell survival and therefore lowest variability in a viability assay. For the SNPs C1236T and G2677T there was a significant difference in the *in vitro* survival of cells exposed to mitoxantrone (P=0.02), and borderline significance for cells exposed to etoposide and daunorubicin (P=0.07-0.09). Cells of different genotypes did not differ in their sensitivity to the cytotoxic effects of cytarabine. The SNPs G1199A and C3435T did not affect the *in*

vitro cytotoxicity; however, since only three of the patients from which leukemic cells were isolated were heterozygous for G1199A, cautious interpretation is warranted.

Discussion

In this study we show that normal karyotype AML patients with the C/C and G/G genotype of *ABCB1* SNPs C1236T and G2677T had a significantly shorter overall survival than other patients with normal karyotype AML. We also found an indication that the genetic variant G1199A influenced the survival. In a multivariate analysis the SNPs 1236C/C and 2677G/G were shown to be independent prognostic factors, along with *FLT3*-ITD, *NPM1* and age. The SNPs G2677T and C1236T were also shown to influence the *in vitro* resistance of isolated leukemic cells from patients against several P-glycoprotein substrates.

Our results indicate that *ABCB1* genotype has potential as a prognostic marker for predicting survival in AML patients with normal karyotype. Hence, the group of patients with intermediate risk, and in whom treatment response is uncertain, might be diminished further by *ABCB1* genotyping. A previous publication by Illmer *et al.* that investigated the *ABCB1* SNPs C1236T, G2677T/A and C3435T in unselected Caucasian AML patients (n=405) showed that patients with the wild-type variants of C1236T, G2677T/A and C3435T (1236C/C, 2677G/G and 3435C/C) had shorter overall survival and a greater risk of relapse than patients with other genotypes.¹⁹ Our results support these findings in normal karyotype *de novo* AML, since patients carrying the C/C or G/G genotypes of C1236T and G2677T have shorter overall survival in our study. In contrast, a study by van der Holt *et al.* (n=150) showed no difference in complete remission, relapse-free survival or disease-free survival for the same *ABCB1* SNPs.²² The population studied by van der Holt *et al.* was older than 60 years and came from a clinical trial investigating the P-glycoprotein inhibitor PSC-833; which might explain the discrepancy between their results and those reported by us and Illmer *et al.*¹⁹ In younger patients with relapsed AML (n=30), van den Heuvel-Eibrink *et al.* have shown that homozygosity for G2677T (G/G and T/T vs G/T) was associated with shorter relapse-free

periods and shorter overall survival.²¹ Moreover, two studies on Korean populations with AML have been published, where Hur *et al.* could not demonstrate any significant difference in terms of survival between *ABCB1* C3435T genotypes²⁰ whereas Kim *et al.* reported that patients carrying 2677G/G and 3435C/C had a greater chance of CR and longer event-free survival, but not longer overall survival.³³ However, the impact of the *ABCB1* SNPs might differ in the different ethnic populations. The SNP G1199A was not investigated in any of the other studies, but we found an indication that this SNP might have an impact on survival, although this has to be studied further. Our results agree with those of a small study on ovarian cancer, where heterozygous patients (1199G/A) showed clinical resistance to paclitaxel-carboplatin treatment and a shorter progression-free survival.²⁴ Our results also concur with *in vitro* data on this SNP showing that cells expressing the 1199A variant have increased resistance to other chemotherapeutic agents such as doxorubicin, vincristine and vinblastine.³⁴⁻³⁵

The effect of C1236T and G2677T/A on survival remained significant in multivariate analysis and – together with age, *NPM1* and *FLT3* status – these SNPs seem to be important variables for therapeutic response in AML. Noteworthy is that the hazard ratios of these *ABCB1* SNPs had higher values than *NPM1* and *FLT3* in this population. This would indicate that the *ABCB1* genotype has relatively strong impact on survival. We also found that the *ABCB1* SNPs were not a major factor in genetic susceptibility to AML, which is in accordance with previous findings.³⁶ It is noteworthy that neither our study nor any of the other studies in Caucasians showed a correlation between the *ABCB1* genotypes and CR. However, we showed that *NPM1* mutations but not *FLT3*-ITD is a positive prognostic marker for CR. This is in accordance with recent data showing that *NPM1* mutations predict early response parameters such as CR and early blast cell clearance.³⁷

Our clinical observation of the effect of the *ABCB1* SNPs C1236T and G2677T is also supported by the *in vitro* cytotoxicity data: leukemic cells from patients with the best prognosis (i.e. those with 1236T/T and 2677T/T genotypes), had a significantly lower *in vitro* survival. Daunorubicin, etoposide and mitoxantrone are known *ABCB1* substrates *in vitro*³⁸⁻³⁹, while the pyridine analogue cytarabine are believed not to be transported by P-glycoprotein⁴⁰. Our *in vitro* data suggest that only the drugs that are known as P-glycoprotein substrates are affected by the *ABCB1* genotypes. The functional consequences of these *ABCB1* SNPs have not been extensively studied *in vitro* for these substrates. In contrast to our study, Schaefer *et al.* showed in membrane vesicle preparations that, as compared to the wild-type variant (2677G), the maximum transport velocities of vincristine were significantly increased by 1.5- and three-fold for the 2677T and the 2677A variants, respectively.⁴¹ This would indicate a higher resistance for the 2677T variant than for 2677G, which is in contradiction to the lower survival we observed in isolated leukemic cells from patients homozygous for T in this position. In another study using HeLa cells the wild-type showed a slightly higher efflux of paclitaxel than the Ser893 variant (2677T)⁴², in agreement with our results. In contrast, transport of other substrates such as verapamil, vinblastine, calcein-AM, prazosin, bisantrene, forskolin, digoxin and cyclosporin A was not affected by G2677T/A or C3435T variants of P-glycoprotein; however, for each substrate only one concentration was tested.^{31, 42-43}

In conclusion our findings suggest that certain *ABCB1* SNPs (e.g. C1236T and G2677T) affect the survival of AML patients with normal karyotype after chemotherapy and might provide useful information for treatment strategies and individualized chemotherapy. Our data show that patients carrying the 1236T/T or 2677T/T genotypes benefit from standard AML

treatment with anthracyclines and cytarabine, and might be considered low-risk patients. Conversely, patients carrying the 1236C/C and 2677G/G genotypes have a poorer prognosis when treated according to standard regimes and should be considered for allogeneic bone marrow transplantation or chemotherapy containing fludarabine and cytarabine or second generation nucleoside analogues. If *ABCBI* genotype proves to be a reliable prognostic marker, the number of intermediate risk patients might be decreased even further than today.

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Authorship

H.G. was the principal investigator and takes primary responsibility for the paper. HG was also involved in conception and design, collection and compilation of data, data analysis, and writing the manuscript; I.J.F.: collection and compilation of data, data analysis, and helping write the manuscript; K.L.: conception, data analysis and patient recruitment; M.H.: *FLT3* and *NPM1* analysis; R.R.: data analysis, strategic suggestions, writing the manuscript, *FLT3* and *NPM1* analysis; E.P. and C.P.: collection of data and patient recruitment; H.N.: conception, data analysis and patient recruitment.

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Table 1. Patient characteristics

Patient characteristics	n
Sex	
Male	52
Female	48
<i>NPM1</i>	
Wild-type	51
Mutated	48
Missing data	1
<i>FLT3</i>	
<i>FLT3</i> neg	68
<i>FLT3</i> -ITD	31
Missing data	1
Induction treatment	
Daunorubicin and cytarabine	48
Daunorubicin, cytarabine and thioguanine	9
Idarubicine and cytarabine	14
Idarubicine, cytarabine, etoposide and/or cladribine	16
Mitoxantrone and cytarabine	2
Mitoxantrone, cytarabine and etoposide	7
Fludarabine, cytarabine, hydrea and/or G-CSF	4

Table 2. Genotype frequencies in the Swedish reference population and AML patients.

All genotypes are in Hardy-Weinberg equilibrium and there were no significant differences in genotype frequencies between the two groups ($P>0.05$).

SNP	Genotype	Ref. pop. n=400	AML-pat. n=100	P=
G1199A	G/G	362 (90.5%)	92 (92%)	0.45
	G/A	37 (9.25%)	7 (7%)	
	A/A	1 (0.25%)	1 (1%)	
C1236T	C/C	133 (33.25%)	34 (34%)	0.86
	C/T	187 (46.75%)	44 (44%)	
	T/T	80 (20.0%)	22 (22%)	
G2677T/A	G/G	124 (31%)	31 (31%)	0.96
	G/T	184 (46%)	43 (43%)	
	T/T	75 (18.75%)	22 (22%)	
	G/A	10 (2.5%)	2 (2%)	
	T/A	6 (1.50%)	2 (2%)	
	A/A	1 (0.25%)	0 (0%)	
C3435T	C/C	87 (21.75%)	19 (19%)	0.79
	C/T	175 (43.75%)	47 (47%)	
	T/T	138 (34.5%)	34 (34%)	

Table 3. Cox regression analysis of age, *FLT3*, *NPM1*, *ABCB1* SNPs G1199A, C1236T, G2677T and C3435T on overall survival in AML patients. The regression analysis were split into two models since the SNPs C1236T and G2677T are so closely linked that the effect of one SNP could not be distinguished from the other.

Model with C1236T				Model with G2677T			
Variable	P=	HR	(95% CI)	Variable	P=	HR	95% CI
Age at diag.	0.003	1.05	(1.02-1.08)	Age at diag.	0.002	1.05	(1.02-1.08)
<i>FLT3</i>	0.049	1.89	(1.00-3.57)	<i>FLT3</i>	0.111	1.69	(0.89-3.23)
<i>NPM1</i>	0.052	0.55	(0.31-1.01)	<i>NPM1</i>	0.105	0.61	(0.34-1.11)
G1199A [§]	0.372	1.48	(0.63-3.53)	G1199A [§]	0.542	1.31	(0.55-3.08)
C1236T C/C		1		G2677T G/G*		1	
C1236T C/T	0.007	0.32	(0.14-0.73)	G2677T G/T	0.001	0.25	(0.11-0.58)
C1236T T/T	0.005	0.24	(0.09-0.65)	G2677T T/T	0.003	0.22	(0.08-0.60)
C3435T C/C		1		C3435T C/C		1	
C3435T C/T	0.806	1.12	(0.46-2.69)	C3435T C/T	0.340	1.55	(0.63-3.84)
C3435T T/T	0.428	1.55	(0.53-4.57)	C3435T T/T	0.425	1.55	(0.53-4.55)

Note: [§] - The wild-type (G/G) was compared to the other genotypes (G/A and A/A). * - For G2677T/A the A-allele was excluded from the analysis due to low frequency. HR – Hazard ratio, 95% CI – 95% confidence interval for the hazard ratio.

Figure 1

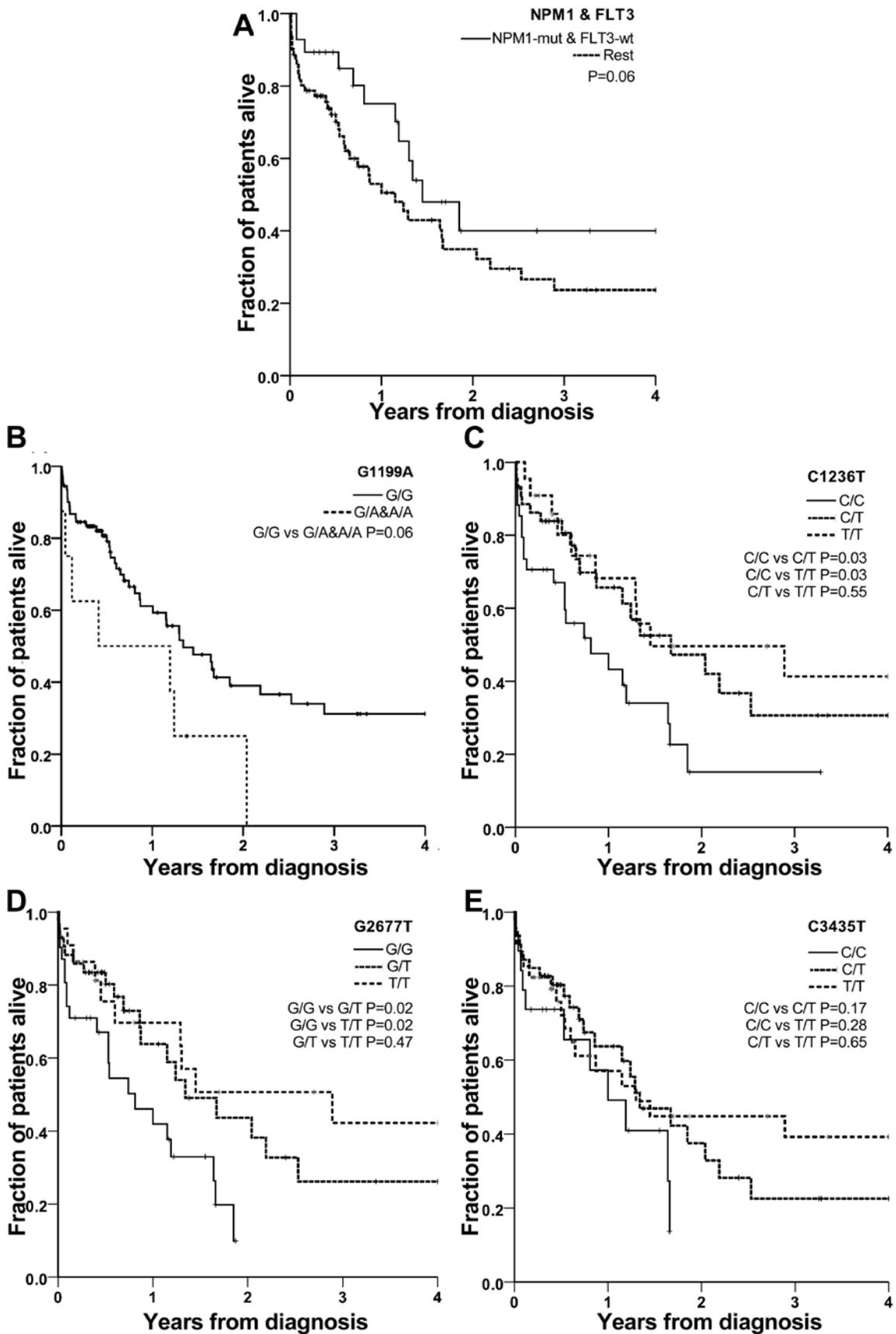


Figure 1. The overall survival of AML patients depending on *FLT3* and *NPM1* mutation status or *ABCB1* genotype. Survival was compared between A) patients carrying a *NPM1* mutation and *FLT3*-ITD wild-type versus the other patients, and patients carrying the different genotypes of the *ABCB1* SNPs B) G1199A G/G vs G/A&A/A, C) C1236T, D) G2677T and E) C3435T. The P-values represent the comparison of the genotypes by log-rank tests.

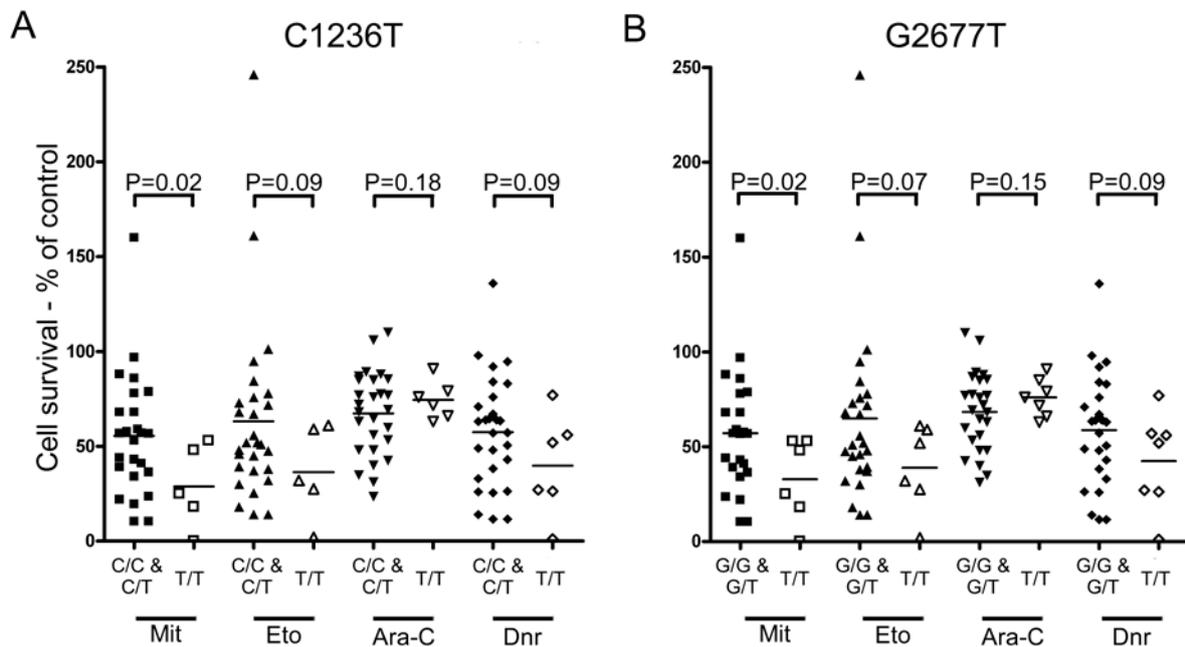


Figure 2. The *in vitro* sensitivity of leukemic cells from *FLT3*-ITD wild-type patients to chemotherapeutic drugs commonly used in the treatment of AML and its correlation to *ABCB1* SNPs A) C1236T and B) G2677T. The survival of the leukemic cells from patients carrying the 1236 or 2677 T/T genotypes was compared to the survival of cells from the other patients. Mit – mitoxantrone (n=29), Eto – etoposide (n=32), Ara-C – cytarabine (n=33) and Dnr – daunorubicin (n=33).