Original Study

Effect of *ABCG2*, *OCT1*, and *ABCB1* (*MDR1*) Gene Expression on Treatment-Free Remission in a EURO-SKI Subtrial

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Abstract

Within the EURO-SKI trial, 132 chronic phase CML patients discontinued imatinib treatment. RNA was isolated from peripheral blood in order to analyze the expression of MDR1, ABCG2 and OCT1. ABCG2 was predictive for treatment-free remission in Cox regression analysis. High transcript levels of the ABCG2 efflux transporter (>4.5‰) were associated with a twofold higher risk of relapse.

Introduction: Tyrosine kinase inhibitors (TKIs) can safely be discontinued in chronic myeloid leukemia (CML) patients with sustained deep molecular response. *ABCG2* (breast cancer resistance protein), *OCT1* (organic cation transporter 1), and *ABCB1* (multidrug resistance protein 1) gene products are known to play a crucial role in acquired pharmacogenetic TKI resistance. Their influence on treatment-free remission (TFR) has not yet been investigated. **Materials and Methods:** RNA was isolated on the last day of TKI intake from peripheral blood leukocytes of 132 chronic phase CML patients who discontinued TKI treatment within the European Stop Tyrosine Kinase Inhibitor Study trial. Plasmid standards were designed including subgenic inserts of OCT1, ABCG2, and ABCB1 together with *GUSB* as reference gene. For expression analyses, quantitative real-time polymerase chain reaction was used. Multiple Cox regression analysis was performed. In addition, gene expression cutoffs for patient risk stratification were investigated. **Results:** The TFR rate of 132 patients, 12 months after TKI discontinuation, was 54% (95% confidence interval [CI], 46%-62%). ABCG2 expression (‰) was retained as the only significant variable ($P = .02$; hazard ratio, 1.04; 95% CI, 1.01-1.07) in

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multiple Cox regression analysis. Only for the ABCG2 efflux transporter, a significant cutoff was found ($P = .04$). Patients with an ABCG2/GUSB transcript level >4.5‰ (n = 93) showed a 12-month TFR rate of 47% (95% CI, 37%-57%), whereas patients with low ABCG2 expression (≤4.5‰; n = 39) had a 12-month TFR rate of 72% (95% CI, 55%-82%). **Conclusion:** In this study, we investigated the effect of pharmacogenetics in the context of a CML treatment discontinuation trial. The transcript levels of the efflux transporter ABCG2 predicted TFR after TKI discontinuation.

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Introduction

Tyrosine kinase inhibitor (TKI) discontinuation in chronic myeloid leukemia (CML) constitutes an important pillar in future CML treatment.[1] Several studies showed that TKIs can safely be discontinued in CML patients with sustained deep molecular response (DMR).[2-4] So far, DMR and treatment duration were shown to be predictive for successful treatment-free remission (TFR) whereas age, risk scores, and sex were not known to have an influence.[3] In addition, molecular levels of DMR (molecular response 4-log reduction [MR^4], molecular response 4.5-log reduction) were not reported to be predictive for TFR.[2,5] Until now, TFR biomarker research focused mainly on immune surveillance and immune exhaustion. Natural killer cells and CD86+ cells proved to be predictive for relapse-free survival.[6-8] However, the effect of pharmacogenetic factors has not yet been analyzed in the recent context of discontinuation trials.

The breast cancer resistance protein (*ABCG2*), organic cation transporter 1 (*OCT1*), and multidrug resistance protein (*ABCB1*) gene products are known to play a crucial role in acquired pharmacokinetic drug resistance and DMR in nilotinib, imatinib, and dasatinib treatment of CML patients.[9,10] The human organic cation transporter 1 (OCT1, human organic cation transporter 1, or solute carrier family 22 member 1) is involved in the absorption, distribution, and elimination of endogenous compounds, toxins, and other xenobiotics that are positively charged at physiological pH.[11] OCT1 activity was shown to predict overall survival and TKI response in CML patients.[12,13] Some studies showed that its gene expression predicts TKI response[14 pp 1,15] but the results could not be reproduced in several other studies.[16,17] Thus, this correlation is still a matter of debate. ABCB1 (P-glycoprotein, multidrug resistance protein 1 [MDR1]) and ABCG2 (breast cancer resistance protein) are 2 members of the adenosine triphosphate-binding cassette family of membrane transporters, prominent for their role in multidrug resistance. Imatinib, dasatinib, and nilotinib were shown to be substrates as well as competitive inhibitors in different extents.[18-23] ABCB1 expression has been reported to predict TKI response.[23-25 pp 1] A recent meta-analysis showed that ABCG2 polymorphisms were potential CML response predictors.[26] In this study, we aimed to investigate whether aberrant gene expression of these influx and efflux channels predispose for CML relapse after TKI discontinuation.

Methods

Samples and Study Design

This analysis included 132 CML patients from the European Stop Tyrosine Kinase Inhibitor Study (EURO-SKI) trial, a prospective multicenter TKI discontinuation trial (NCT01596114).[3] In accordance with the Declaration of Helsinki, written informed consent was obtained from all patients. According to the EURO-SKI inclusion criteria, patients with MR^4 duration of at least 1 year and TKI treatment of at least 3 years were enrolled. TFR is on the basis of survival without loss of major molecular response and is expressed as molecular relapse-free survival rate in the Kaplan−Meier analysis.

Gene expression analyses were performed on leukocyte RNA isolated from peripheral blood samples of patients screened in our center and 10 healthy individuals (male n = 5; female n = 5) served as controls. Blood samples were collected at the last day of TKI intake (baseline).

Molecular Analysis

The gene expression levels of breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog (ABL) and ABL1 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) from total leukocyte RNA of peripheral blood samples. BCR-ABL transcript levels were measured using standard plasmid dilutions, as described previously.[27] Ratios derived from BCR-ABL/ABL1 were converted to the International Scale.

The qRT-PCR reactions for quantifying *ABCB1*, *ABCG2*, *OCT1*, and *GUSB* (beta-glucuronidase 1, reference gene) consisted of (per 20-µL reaction mix): 4 µL LightCycler 480 Probes Master Mix (Roche Diagnostics, Indianapolis, IN), 0.5 µL reverse primer, 0.5 µL forward primer, 0.25 µL anchor probe, 0.25 µL sensor probe (TIB Molbiol, Berlin, Germany), and 2 µL cDNA or plasmid dilution (see Supplemental Table 1 in the online version: primer sequences). The cycle settings were the following: 10 minutes denaturation at 95°C, 40 cycles of 10-second denaturation at 95°C, 20 seconds annealing at 60°C, and 30 seconds elongation at 72°C.

Cloning of Plasmid Standards

The plasmid was cloned by transforming and cultivating *Escherichia coli* one shot Top10F' (Invitrogen, Carlsbad, CA). The preparation of the plasmid DNA was done using HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid linearization was performed using the Xbal restriction enzyme. For absolute quantification of the ABCB1, ABCG2, and OCT1 transcript levels, the 5-log series of plasmid dilutions were amplified using qRT-PCR. For this reason, the pEX-A2 plasmid design (ID: 3321214, Eurofins, Luxembourg) contained an 861-base pair insert, on the basis of

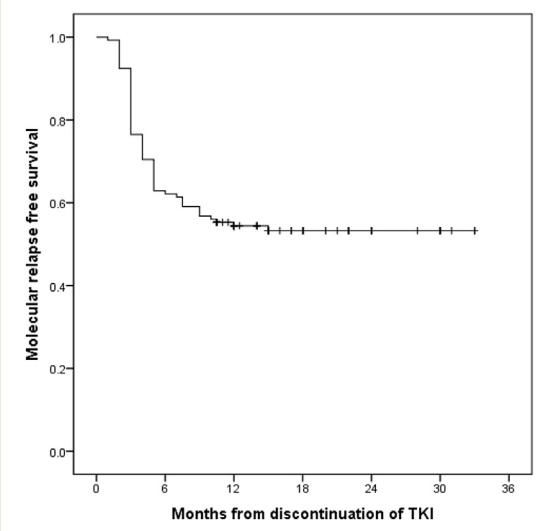
subgenic stretches of *ABCB1*, *ABCG2*, *OCT1*, and *GUSB* (reference gene) with their specific primer sequences.

The 5-log plasmid dilutions with a range from 40 to 400,000 transcripts were detected in all qRT-PCR runs. ABCG2, ABCB1, and OCT1 transcripts were detectable in every sample and their expression rate is presented as "gene of interest/GUSB" ratio.

Statistical Analysis

The hazard ratio (HR) of the efflux and influx channel transcript levels was estimated using Cox regression analysis. Significance was determined using the Wald test. Cutoffs for ABCG2, OCT1, and ABCB1 were sought for in 10,000 bootstrap samples of size n = 132.[28] In line with the minimal *P* value approach, within each sample the log-rank test was used to identify a threshold that separated 2 groups of patients with most different relapse probabilities.[29] While adjusting for multiple testing using the Bonferroni correction for a candidate threshold, the differences in the relapse probabilities of the resulting groups had to be statistically significant.[30] Association between variables was analyzed using the Mann–Whitney *U* test or Spearman rank correlation coefficient, whichever was applicable. Two related samples were compared using the Wilcoxon signed rank test. Relapse was defined as the first loss of major molecular response at any time. The study has an exploratory character. Apart from cutoff selection using the minimal *P* value method, multiplicity was not considered. For the 2-sided *P* values, the unadjusted significance level of .05 was applied to all statistical tests. Calculations were done using SAS (version 9.4, SAS Institute Inc, Cary, NC), the programming software R (version 3.2.2, R Foundation for Statistical Computing, Vienna, Austria), and SPSS (version 20, IBM Corp, Armonk, NY). To identify a minimal *P* value, the R package "party" (version 1.0-25) was applied.

Figure 1 Molecular Relapse-Free Survival Probabilities of 132 EURO-SKI Patients. After 12 Months, the Survival Probability Was 54% (95% CI, 46%-62%). Median Observation Time Was 17 Months



Results

Patient Characteristics

In our cohort, 132 chronic phase CML patients discontinued TKI treatment. The molecular relapse-free survival rate at 12 months resulted in 54% (95% confidence interval [CI], 46%-62%; Figure 1). Median observation time was 17 months. Median of MR^4 and TKI treatment duration was 4.3 and 7.6 years, respectively. Patients were exclusively treated with imatinib. Further patient characteristics are summarized in Table 1.

ABCB1, ABCG2, *and* OCT1 *Expression Levels*

Throughout the cohort, *ABCB1* showed the highest gene expression in peripheral blood leukocytes (70%; range, 18-512) whereas *ABCG2* showed the lowest transcripts levels (0.7%; range, 0-4). Twelve months after TKI discontinuation, the efflux transporters ABCG2 as well as ABCB1 and the influx transporter OCT1 presented no significant differences between relapsed and non-relapsed patients ($P > .05$). The comparison with healthy controls revealed significant overexpression of ABCB1 ($P < .03$) and suppression of OCT1 ($P < .03$) transcripts in chronic phase CML patients (Figure 2). The efflux transporter ABCG2 showed no significant changes in gene expression between EURO-SKI patients and healthy controls (Figure 2A).

We further investigated the effect of TKI withdrawal upon gene expression. After TKI discontinuation, in 40 patients analyzed, a significant downregulation of *ABCB1* at the day of relapse was shown ($P = .017$; Figure 3C). The other channels showed no such transcript level adaption after imatinib discontinuation ($P > .05$; Figure 3A and B).

Multiple Regression Analyses, Cutoff Investigation, and Risk Stratification

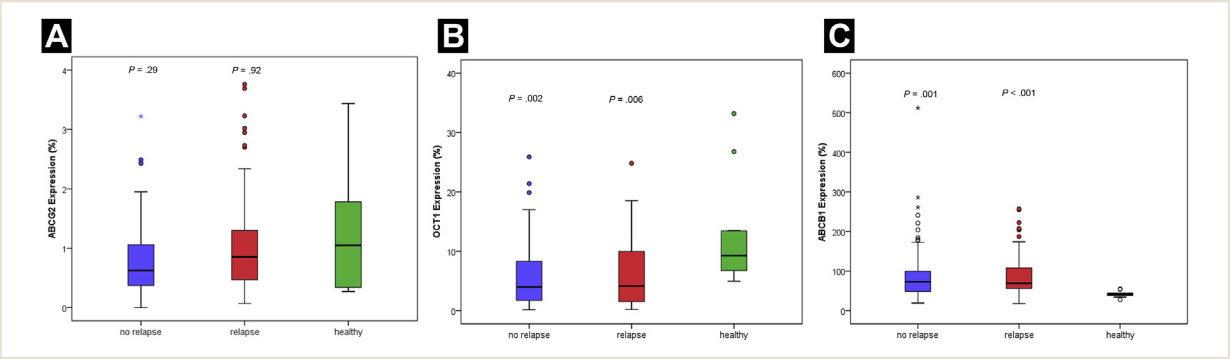
Prognostic significance of transcript levels was assessed using multiple Cox regression analysis, considering age and sex as well as the clinically relevant factors MR^4 duration and TKI duration as candidate variables. Prognostic scores (eg, European Treatment and Outcome Study, Sokal, Euro, and European Treatment and Outcome Study long-term survival) could not be considered because they were not sufficiently documented. *ABCG2* expression (‰) was retained as the only significant variable in the model ($P = .02$; HR, 1.04; 95% CI, 1.01-1.07). Adjusted for multiple testing and most often chosen in the bootstrap samples, the threshold

Table 1 Clinicopathologic Characteristics of the 132 EURO-SKI Patients

Patient Characteristic	Median Value (Range) or Percentage
Age, Years	62 (27-87)
Male Sex	52
TKI Duration, Years (n = 130)	7.6 (3-14)
MR^4 Duration, Years	4.3 (1-13)
ABCG2 Expression, %	0.7 (0-4)
OCT1 Expression, %	4.1 (0.2-26)
ABCB1 Expression, %	70 (18-512)

Abbreviations: EURO-SKI = European Stop Tyrosine Kinase Inhibitor Study trial; MR^4 = molecular response 4-log reduction; TKI = tyrosine kinase inhibitor.

Figure 2 Transcript Levels of *ABCG2* (A), *OCT1* (B), and *ABCB1* (C) of Patients at the Last Day of Tyrosine Kinase Inhibitor (TKI) Intake (Baseline) Compared With Healthy Individuals (n = 10). Patients Were Divided Into 2 Groups: 1 Group With Loss of Major Molecular Response Within 12 Months After Discontinuation of Therapy and 1 Group Without Loss. *P* Values Refer to the Comparison of Each Patient Group With the Healthy Controls Using the Mann–Whitney *U* Test



4.5‰ resulted in a significant risk stratification for the ABCG2 efflux transporter ($P = .04$; Figure 4). Patients with an ABCG2/GUSB transcript level >4.5‰ (n = 93) had a 12-month relapse-free survival rate of 47% (95% CI, 37%-57%), whereas patients with lower ABCG2 expression (≤4.5‰; n = 39) showed a 12-month TFR of 72% (95% CI, 55%-82%; Figure 4). ABCG2/GUSB transcript level >4.5‰ showed approximately a twofold higher risk of relapse after treatment discontinuation (HR, 1.92; 95% CI, 1.02-3.61). Cutoff analysis of OCT1 and ABCB1 provided no gene expression threshold with significant risk stratification.

All genes were tested for correlation with the BCR-ABL international scale transcript level before stopping as well as with TKI and MR[4] duration. No significant correlation was found (Spearman correlation $P > .05$).

Discussion

The present study was designed to determine the effect of pharmacogenetics on response after CML treatment discontinuation. On the basis of our findings, ABCG2 expression as opposed to OCT1 and ABCB1 (MDR1) was an independent predictor of TFR after TKI discontinuation. Accordingly, patients with a high expression of the ABCG2 efflux transporter (>4.5‰) were shown to have a twofold higher risk of relapse. These findings seem to be reasonable from a biological point of view, according to the hypothesis that a higher gene expression of ABCG2 might increase TKI efflux on the protein level and impair treatment.

In- and efflux transporter expression analyses of CML patients after long-term TKI treatment (median TKI duration, 7.6 years; median DMR duration, 4.3 years) have not been reported in the literature before. The results of this study showed that patients in DMR presented a different expression level of ABCB1 and OCT1, compared with healthy controls.

The overexpression of ABCB1 (MDR1) and suppression of OCT1 might confer a survival or selection advantage. ABCB1 seemed to respond most sensitively to TKI exposure, because imatinib withdrawal led to a significant downregulation or normalization of its transcript levels. In contrast, the expression of ABCG2 was similar to healthy controls. An in vivo study showed that ABCG2 is indirectly regulated by thephosphatidylinositol-4,5-

Figure 3 Transcript Levels of *ABCG2* (A), *OCT1* (B) and *ABCB1* (C) of EURO-SKI Patients (n = 40) on the Day of Treatment Discontinuation and on the Day of Loss of Major Molecular Response. *P* Values Refer to the Comparison of the 2 Groups Using the Wilcoxon Signed Rank Test

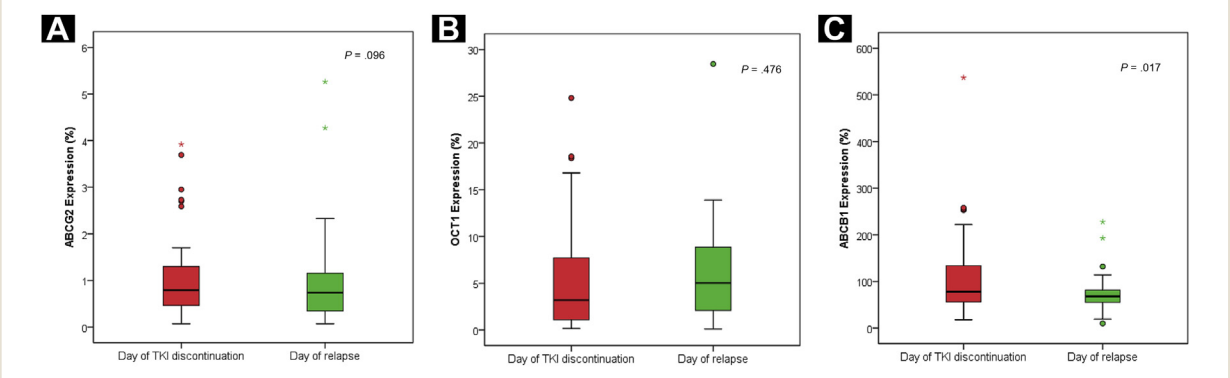
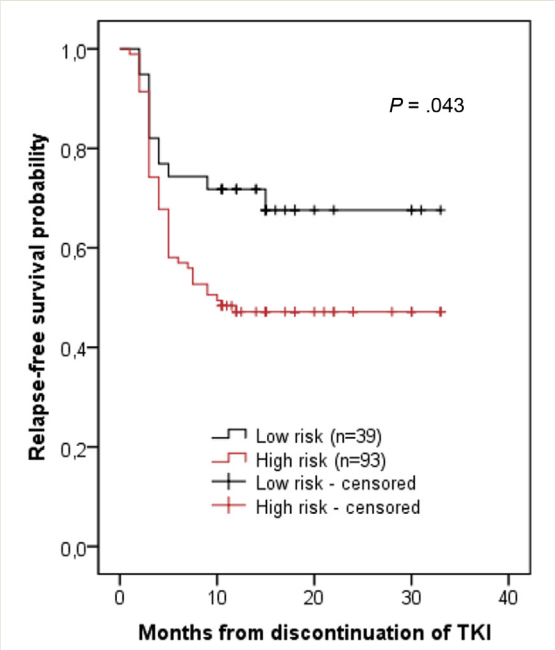


Figure 4 Molecular Relapse-Free Survival Probabilities of 132 EURO-SKI Patients Stratified in Accordance With Their *ABCG2/GUSB* Ratio on the Day of Treatment Discontinuation. Patients With *ABCG2/GUSB* Ratio >4.5‰ (Red) Showed a Higher Risk of Relapse Comparison With Patients With *ABCG2* Transcripts ≤4.5‰ ($P = .04$, Log Rank Test)



Abbreviation: TKI = tyrosine kinase inhibitor.

bisphosphate 3-kinase, RAC-alpha serine/threonine-protein kinase pathway, which is inhibited by imatinib-induced reduction of phosphorylated RAC-alpha serine/threonine-protein kinase.[31 pp 2] Inhibition of this pathway led to a post-transcriptional reduction of ABCG2 protein expression. An in vitro long-term exposure study showed a decrease in ABCG2 transcript level over time in K562 cells upon imatinib and dasatinib exposure.[32] In the present study most BCR-ABL positive cells had likely been eradicated and "normal" hematopoiesis was analyzed. The effect of TKI long-term exposure on in- and efflux transporters in BCR-ABL negative cells is yet not well understood. Our data confirmed the lack of auto-induction of ABCG2 after long-term imatinib exposure, as shown by Gardner et al in a murine model.[33] This study further presented a decline of ABCG2 protein expression after weeks of TKI exposure in liver and intestinal tissue. A possible explanation might be a TKI-mediated enhanced ABCG2 mRNA lysosomal degradation.[34,35]

In- and efflux transporter expression did not correlate with TKI and MR^4 duration. Concerning ABCB1, recent data showed that a high-fold rise of the ABCB1 mRNA expression from diagnosis to day 22 of imatinib therapy predicted patient response.[24] Thus our data cannot exclude a possible correlation between the in- and efflux transporter expression and TKI and MR^4 duration, because aberrant OCT1 and ABCB1 expression might develop early upon TKI exposure and correlation might get lost after years of MR^4 and TKI treatment.

With regard to the *ABCG2* gene expression, we showed that differential expression of a noninvasive biomarker can be detected using a sensitive quantification method on the basis of plasmid standards and may be used for TFR prediction. However, our findings need further validation by an independent cohort. Indeed, patients with high *ABCG2* expression showed similar TFR rates (47%) than the overall study population (54%). However, patients with low *ABCG2* expression may be eligible for shorter TKI treatment duration before therapy discontinuation. Furthermore, the impact of the in- and efflux transporters has to be reevaluated separately for each TKI. To improve the prediction of relapse-free survival, we suggest *ABCG2* be integrated in a multiple marker panel, because gene signatures might better reflect the multifactorial process of TFR.

Conclusion

This study was set out with the aim of investigating the effect of pharmacogenetics in a CML treatment discontinuation trial. *ABCG2* gene expression as opposed to *OCT1* and *ABCB1* (*MDR1*) predicted TFR after TKI discontinuation. High transcript levels of the ABCG2 efflux transporter (>4.5‰) were associated with a twofold higher risk of relapse.

Clinical Practice Points

- To date there exist no non-invasive molecular biomarkers predicting treatment-free remission after TKI discontinuation. In addition, it is little known about the pharmacogenomics in TKI discontinuation.
- The results of this study showed that patients in DMR after several years of TKI treatment presented differential expression of ABCB1 and OCT1, compared to healthy controls. However, only ABCG2 was predictive for treatment-free remission in Cox regression analysis. High transcript levels of the ABCG2 efflux transporter (>4.5‰) were associated with a twofold higher risk of relapse.
- ABCG2 may be a valuable biomarker to be integrated in a multiple marker screening for TFR prediction.

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Disclosure

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Supplemental Data

Supplemental table accompanying this article can be found in the online version at https://doi.org/10.1016/j.clml.2018.02.004.

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