ASXL1 and BIM germ line variants predict response and identify CML patients with the greatest risk of imatinib failure

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Key Points

- Germ line variants in *ASXL1* and *BIM* are strong biomarkers of response to imatinib in chronic phase CML.
- A combined Sokal risk and *ASXL1* and *BIM* variant model identified a subgroup of patients with the greatest risk of treatment failure.

Scoring systems used at diagnosis of chronic myeloid leukemia (CML), such as Sokal risk, provide important response prediction for patients treated with imatinib. However, the sensitivity and specificity of scoring systems could be enhanced for improved identification of patients with the highest risk. We aimed to identify genomic predictive biomarkers of imatinib response at diagnosis to aid selection of first-line therapy. Targeted amplicon sequencing was performed to determine the germ line variant profile in 517 and 79 patients treated with first-line imatinib and nilotinib, respectively. The Sokal score and *ASXL1* rs4911231 and *BIM* rs686952 variants were independent predictors of early molecular response (MR), major MR, deep MRs (MR⁴ and MR^{4.5}), and failure-free survival (FFS) with imatinib treatment. In contrast, the *ASXL1* and *BIM* variants did not consistently predict MR or FFS with nilotinib treatment. In the imatinib-treated cohort, neither Sokal or the *ASXL1* and *BIM* variants predicted overall survival (OS) or progression to accelerated phase or blast crisis (AP/BC). The Sokal risk score was combined with the *ASXL1* and *BIM* variants in a classification tree model to predict imatinib response. The model distinguished an ultra-high-risk group, representing 10% of patients, that predicted inferior OS (88% vs 97%; $P = .041$), progression to AP/BC (12% vs 1%; $P = .034$), FFS ($P < .001$), and MRs ($P < .001$). The ultra-high-risk patients may be candidates for more potent or combination first-line therapy. These data suggest that germ line genetic variation contributes to the heterogeneity of response to imatinib and may contribute to a prognostic risk score that allows early optimization of therapy.

Introduction

The majority of chronic myeloid leukemia (CML) patients are now effectively treated using BCR-ABL1 tyrosine kinase inhibitor (TKI) therapy. The first-generation TKI imatinib induces high rates of molecular response (MR) and improved survival and has a well-documented safety profile.^{1,2} The second-generation TKIs nilotinib and dasatinib lead to more rapid molecular responses than imatinib and may be associated with a decreased risk of blastic transformation. However, they are associated with idiosyncratic toxicities, and there are no overall survival (OS) differences among the 3 TKIs.^{3,4} All 3 drugs

are currently approved for first-line therapy, and the optimal agent for each individual patient is selected after balancing the risk of disease progression and expected toxicities.^{5,6} No current algorithm can accurately determine the risk of disease progression at diagnosis.

Time-dependent milestone molecular responses have excellent correlation with long-term outcomes regardless of the TKI used: the *BCR-ABL1* value after 3, 6, or 12 months of TKI treatment can determine whether therapy has failed, which mandates an early change of treatment in an attempt to reduce the risk of disease progression and death.^{5,6} However, response-based molecular monitoring is limited because it does not aid selection of first-line therapy, and there is a lack of evidence that salvage approaches in this setting are effective.

Currently, clinical scoring systems at the time of CML diagnosis, such as Sokal, Hasford, European Treatment and Outcome Study (EUTOS), or European Long-Term Survival (ELTS) risk scores, provide important prognostic information, are used to stratify risk,⁷⁻¹⁰ and are integral to any risk-adapted treatment strategy.¹¹ However, these scoring systems by themselves are of limited sensitivity and specificity. Their predictive power might be improved in combination with emerging prognostic biomarkers, which may further aid rational selection of optimal first-line treatment and increase optimal responses to TKI therapy.

Biological factors, such as a patient's germ line genetic variation, may play a role in the kinetics of leukemic cell decline and thus affect an individual's response to TKIs. Recently, extensive efforts have been made to identify molecular biomarkers for improved prediction of early response. Indeed, an inherited deletion polymorphism in *BIM* (*BCL2L11*), which is common in East Asians, has been reported to be associated with intrinsic imatinib resistance and inferior clinical responses, which may be overcome by more potent inhibitors.¹² *BIM* is a pro-apoptotic member of the *BCL2* intrinsic apoptosis pathway. Treatment with TKIs leads to upregulation of proapoptotic proteins, of which *BIM* is a key protein, and initiation of leukemic cell death.¹³⁻¹⁵ In addition, a common synonymous variant in the BH3 functional domain of *BIM* has been associated with inferior responses to imatinib and delayed decline of *BCR-ABL1*.¹⁶ Interestingly, this variant has also been associated with lower OS for children with acute lymphoblastic leukemia in which *BIM* mediates apoptosis of leukemic cells induced by corticosteroids.¹⁷ The variant favored the generation of an isoform that lacked the proapoptotic BH3 domain.

Germ line genetic variation in genes in other biological pathways have also been associated with response to TKI treatment, including hedgehog signaling (*SMO*), and pharmacogenetic processing of imatinib, such as *SLC22A1* (*OCT1*), *ABCG2*, *ABCB1* (*MDR1*), and *CYP3A5*.¹⁸⁻²² To date, none of these genetic markers of response have translated into routine clinical management of CML patients. Moreover, clinically relevant genes with prognostic relevance, such as *ASXL1*,²³ *TET2*,²⁴ *RUNX1*,²⁵ *IKZF1*,²⁶ and *TP53*,²⁷ predict response to therapy when somatically altered in hematopoietic diseases. These genes may also harbor germ line and somatic variants that have prognostic relevance for CML patients treated with TKIs.

Next-generation sequencing (NGS) technologies now facilitate large-scale multiplexing, which allows simultaneous directed amplification and sequencing of genomic regions of interest. We used

Table 1. The genes and regions targeted by the sequencing panel

Gene	Region
Intrinsic apoptosis*	
<i>BCL2A1</i> (<i>A1</i>)	
<i>BAD</i>	
<i>BAK1</i>	
<i>BAX</i>	
<i>BCL2</i>	All exons and splice sites, 3' and 5' UTR regions
<i>BCL2L2</i> (<i>BCLW</i>)	
<i>BCL2L1</i> (<i>BCLX</i>)	
<i>BCL2L11</i> (<i>BIM</i>)	
<i>BMF</i>	
<i>MCL1</i>	
Hematologic malignancy*	
<i>ASXL1</i>	Exon 12
<i>CBL</i>	Exons 8 and 9
<i>CDKN2A</i>	All exons
<i>GATA2</i>	Exon 5
<i>IDH1</i>	Exon 4
<i>IDH2</i>	Exon 5
<i>IKZF1</i>	Exons 3-6 and 3' UTR
<i>KRAS</i>	Exon 2
<i>NRAS</i>	Exon 2
<i>PAX5</i>	Exon 3
<i>PDGFRA</i>	Exons 12, 14, 18
<i>RB1</i>	All exons
<i>RUNX1</i>	All exons
<i>TET2</i>	All exons
<i>TP53</i>	All exons
<i>WT1</i>	All exons
Variant association*	
<i>IFNG</i>	rs2069705
<i>IL15</i>	rs10519613 and rs17007695
<i>MDM2</i>	rs2279744
<i>PTCH1</i>	rs41316950
<i>SMO</i>	rs1061285
Drug metabolism*	
<i>ABCG2</i>	rs2231142
<i>CYP2C19</i>	rs3758581
<i>CYP3A5</i>	rs776746
<i>GSTM1</i>	rs74837985
<i>NAT2</i>	rs1799930
<i>SLC22A1</i> (<i>OCT1</i>)	rs628031 and rs35191146
<i>SLCO1B1</i>	rs4149056
<i>UGT2B7</i>	rs7668258

*The genomic coordinates of the targeted regions are provided in supplemental Table 1.

targeted NGS to investigate germ line genetic variation in *BCL2* family members of the apoptotic pathway, drug metabolism genes, and genes implicated in hematologic malignancies to determine

Table 2. Univariate analysis of baseline factors and the *ASXL1* rs4911231 and *BIM* rs686952 variants in imatinib-treated patients

Factor	No. of patients	3-mo EMR		12-mo MMR		48-mo MR ⁴		48-mo MR ^{4,5}		48-mo FFS		48-mo progression to AP/BC		48-mo OS	
		3%	P	%	P	%	P	%	P	%	P	%	P	%	P
Overall response	528	77	<.0001	48	<.0001	50	.011	31	.005	74	<.0001	4	.259	95	.677
Sokal risk group															
Low	215	87		56		55		37		83		2		96	
Intermediate	161	74		47		50		32		72		6		94	
High	130	65		38		43		20		59		6		94	
Sex			.12		.026		.009		.074		.311		.210		.058
Female	225	77		57		56		34		75		3		97	
Male	303	77		42		46		28		71		5		93	
<i>BCR-ABL1</i> transcript type			.212		.701		.068		.018		.0741		.782		.393
e13a2	198	79		43		47		25		78		4		96	
e14a2	229	76		54		54		38		73		3		95	
e13a2 and e14a2	96	77		45		54		25		65		6		92	
Median age, y			.43		.24		.38		.12		.672		.520		.523
≤48	269	77		46		49		27		73		5		95	
>48	259	77		51		51		34		73		3		94	
Imatinib starting dose, mg			.005		.682		.088		.264		.054		.797		.164
400	90	67		43		50		24		71		2		98	
600	313	80		49		55		34		75		4		92	
800	125	79		51		40		29		71		5		96	
<i>ASXL1</i> rs4911231 genotype			.029		.012		.013		<.0001		.002		.200		.622
Good risk	155	85		56		59		43		83		2		96	
Poor risk	353	75		46		47		26		71		4		94	
<i>BIM</i> rs686952 genotype			.002		<.0001		<.0001		<.0001		.035		.057		.347
Good risk	249	84		55		60		41		80		2		96	
Poor risk	259	73		44		43		22		70		5		93	

Figure 1. Good- and poor-risk genotypes were identified for the *ASXL1* rs4911231 and *BIM* rs686952 variants. Univariate analysis of the achievement of MR^{4.5} as stratified by (A) the *ASXL1* rs4911231 and (B) the *BIM* rs686952 variant genotype identified good- and poor-risk genotypes which were strongly associated with MR^{4.5}.

association with response to first-line imatinib and nilotinib treatment. Two germ line variants, 1 in *BIM* and another in *ASXL1*, were identified that when combined with the Sokal risk score, more precisely predicted outcome than Sokal alone.

Methods

Patients

Consecutive patients treated with first-line imatinib ($n = 528$) or nilotinib ($n = 84$) who were molecularly monitored at our laboratory were evaluated for inclusion in the study. The median follow-up (estimated by reverse Kaplan-Meier) of the patients treated with first-line imatinib or nilotinib was 45 months (range, 1-135 months) and 23 months (range, 1-25 months), respectively. This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

Molecular analysis

BCR-ABL1 transcript levels were measured by using real-time quantitative polymerase chain reaction, as previously published.²⁸ Genotype analysis was performed for patients with archived samples available for testing (517 of 528 imatinib-treated patients and 79 of 84 nilotinib-treated patients). Posttreatment samples at various time points (with undetectable or minimal *BCR-ABL1*) were selected for molecular analysis. Somatic variation was detected by using this method; however, the predictive value of somatic variants was not assessed in this study.

Early MR (EMR) was defined as *BCR-ABL1* $\leq 10\%$ at 3 months. Confirmed major MR (MMR), MR⁴, and MR^{4.5} were defined as *BCR-ABL1* values of $\leq 0.10\%$, $\leq 0.01\%$, and $\leq 0.0032\%$ on the International Scale, respectively, at 2 consecutive measurements. The time to MMR, MR⁴, and MR^{4.5} was the duration between the start of therapy and the month of the first of the 2 consecutive measurements that met the response criteria.

The primer panel was designed by using the Ion AmpliSeq Designer. Libraries were prepared by using the Ion AmpliSeq Library Kit 2.0 and IonXpress barcodes. The Ion Proton System and Torrent Suite v4.4 were used for sequencing and variant calling, respectively (Thermo Fisher Scientific). The Sequenom iPLEX absorption, distribution,

metabolism, excretion (ADME), and/or toxicity PGx Panel v1.0 (Agena Bioscience) was performed per the manufacturer's instructions.

Development and validation of a targeted NGS panel for variant genotyping

To identify germ line variants of potential prognostic value, a custom sequencing panel of 474 amplicons targeting 39 genes (Table 1) involved in the intrinsic apoptotic pathway, drug metabolism, and hematologic malignancy was designed (supplemental Tables 1 and 2).

Genotypes were called at variant sites with minor allele frequencies $> 1\%$, as reported by the Single Nucleotide Polymorphism Database (dbSNP) ($n = 267$). Strict filtering was undertaken to eliminate uninformative or poor-quality genotype data, leaving 113 variants for association testing (supplemental Table 3). To validate the genotype data, the AmpliSeq and ADME panel genotyping results for 7 variants in 150 patients which overlapped between studies were compared. Genotype calls were, on average, 99.2% (range, 98.2%-100%) concordant between the methodologies (supplemental Table 4), confirming the high quality of the genotype data.

Statistical analysis

All statistical analyses were completed in R. Linkage disequilibrium (LD) analysis was performed by using the LDheatmap package, and tests for Hardy-Weinberg equilibrium used the HWE.chisq function from the genetics package. Haplotype blocks were determined by using HapMap data (version 3, release R2) in Haploview v4.2.²⁹ Pearson's χ^2 test was used to assess the frequency distribution of variant genotypes between groups.

Fine and Gray models were used to assess the cumulative incidence of MRs (for which achievement of the response was the event of interest, and competing risks were any event that led to permanent cessation of first-line imatinib or nilotinib^{30,31}) and also progression to accelerated phase or blast crisis (AP/BC) in which death in chronic phase was the competing risk. Associations of variables with molecular outcomes at univariate and multivariate levels were examined by using the cmprsk package in R, with significance determined by the Wald test. Akaike information criterion was used for model selection in the multivariate Fine and Gray regression.

Table 3. Univariate analysis of the *ASXL1* rs4911231 and *BIM* rs686952 variants in nilotinib-treated patients

Factor	No. of patients	12-mo MMR		24-mo MR ⁴		24-mo MR ^{4,5}		24-mo FFS	
		%	<i>P</i>	%	<i>P</i>	%	<i>P</i>	%	<i>P</i>
ASXL1 rs4911231 genotype			.28		.048		.31		.537
Good risk	23	78		66		32		86	
Poor risk	55	67		42		20		92	
BIM rs686952 genotype			.52		.77		.17		.877
Good risk	33	70		45		31		91	
Poor risk	43	70		50		16		88	

Failure-free survival (FFS), in which failure events included lack of milestone responses at 3, 6, and 12 months; loss of MR, hematologic response, or cytogenetic response; acquisition of BCR-ABL1 mutations; clonal chromosomal abnormalities in Philadelphia chromosome cells; progression to AP/BC; death⁵; and OS were analyzed by using the Kaplan-Meier method. Both FFS and OS were measured from the date of TKI commencement. The log-rank test was used to assess the significance of differences in survival distributions. Cox proportional hazards were calculated, and proportionality assumptions were checked by using the *coxph* and *cox.zph* functions from the survival package.

Time point estimates were made at 12 months for MMR and at 48 months for MR⁴, MR^{4,5}, FFS, progression to AP/BC, and OS in imatinib-treated patients and at 12 months for MMR and at 24 months for MR⁴, MR^{4,5}, and FFS in nilotinib-treated patients.

Landmark analysis assessed rates of MMR, MR⁴, MR^{4,5}, and FFS among patients grouped by *BCR-ABL1* at 3 months ($\leq 10\%$ and $> 10\%$) or by classification tree risk group. Patients with the event of interest by 3 months were excluded from the landmark analyses of MMR, MR⁴, or MR^{4,5}. Landmark analysis of FFS excluded patients with an event and those who were censored by 3 months.

Random forests analysis was implemented using the *rfsrc* function from the randomForestSRC package.^{32,33} The classification tree model was produced by using the *rpart* package in R and was pruned to optimize the prediction accuracy in 10-fold cross-validation. Bootstrap subsampling cross-validation was used to quantify the predictive discrimination of each model using the concordance index (C-index).

Variant functional prediction analysis

SNPnexus software was used to investigate the potential functional significance of the *ASXL1* and *BIM* variants and variants in linkage disequilibrium with the risk variants.^{34,35} TargetScan (v7.0) prediction software was used to identify loss/gain/modification of microRNA (miRNA) binding sites at *ASXL1* 3' untranslated region (UTR) sites.³⁶ Noncoding RNA profiling by array data sets publicly available on the Gene Expression Omnibus database were analyzed by using GEO2R differential expression analysis of mature miRNAs.^{37,38}

Results

Sokal risk score was predictive of imatinib response

Baseline predictors were assessed for prediction of EMR, time to MMR, MR⁴, MR^{4,5}, FFS, progression to AP/BC, and OS. The Sokal score was the only baseline variable that consistently predicted for EMR, MMR, MR⁴, MR^{4,5}, and FFS (Table 2). Female sex predicted for MMR ($P = .026$) and MR⁴ ($P = .009$), but not EMR, MR^{4,5}, or FFS. *BCR-ABL1* transcript type (e14a2) predicted only for MR^{4,5} ($P = .018$; Table 2). Imatinib starting dose predicted only for EMR ($P = .005$). None of the assessed baseline variables predicted for progression to AP/BC or OS (Table 2).

Identification of germ line variants important for prediction of response to imatinib

Random forests analysis was undertaken to identify germ line variants that could be combined with the Sokal score to predict

Table 4. Multivariate analysis of Sokal risk score, *ASXL1* rs4911231, and *BIM* rs686952

	EMR		MMR		MR ⁴		MR ^{4,5}		FFS	
	RR	P	RR	P	RR	P	RR	P	HR	P
Sokal risk group*										
Low	1		1		1		1		1	
Intermediate	0.8	.078	0.7	.006	0.87	.35	0.81	.28	1.62	.042
High	0.57	<.001	0.62	<.001	0.65	.01	0.5	.004	2.81	<.001
<i>ASXL1</i> rs4911231										
Good risk	1		1		1		1		1	
Poor risk	0.85	.15	0.75	.013	0.75	.041	0.61	.005	1.88	.006
<i>BIM</i> rs686952										
Good risk	1		1		1		1		1	
Poor risk	0.72	.002	0.71	.001	0.6	<.001	0.52	<.001	1.45	.046

*Relative risks (RRs) and hazard ratios (HRs) are compared with the Sokal low-risk group.

Figure 2. A risk classification tree model identified a subgroup of ultra-high-risk patients with the highest risk of experiencing therapy failure and disease progression. (A) A classification tree based on Sokal risk score and the *BIM* and *ASXL1* variant genotypes to predict 48-month FFS was generated by recursive partitioning using the Rpart package. The final classification tree produced 7 terminal nodes, which were readily distinguished into 4 risk categories; favorable, low, high and ultra-high. Each node box

response to imatinib at diagnosis. The Sokal score and 4 variants (*BIM* rs686952, *ASXL1* rs4911231, *ASXL1* rs2295763, and *ASXL1* rs2295764) were the only variables that had high association with outcomes in multiple models (EMR, MMR, MR⁴, MR^{4.5}, and FFS; supplemental Table 5). The 3 *ASXL1* variants were in high linkage disequilibrium (r^2 , 0.93-0.99; supplemental Figure 2). The variant minor allele frequencies detected in the imatinib-treated cohort were *BIM* rs686952 (A, 28.9%), *ASXL1* rs4911231 (C, 43.8%), *ASXL1* rs2295763 (C, 42.2%), and *ASXL1* rs2295764 (G, 42.3%), which were similar to those in the dbSNP 147 database (24.3%, 40.5%, 40%, and 40%, respectively). The marker *ASXL1* variant with the strongest association with imatinib response (*ASXL1* rs4911231) was chosen for subsequent analyses.

Germ line variants in *BIM* and *ASXL1* were predictive of response to imatinib but not nilotinib

To further examine the association of *BIM* rs686952 and *ASXL1* rs4911231 variants with outcome, patients were stratified into good- vs poor-risk groups on the basis of genotyping information in univariate analyses. The T/T genotype of *ASXL1* rs4911231 and the A/A and A/C genotypes of *BIM* rs686952 were associated with superior achievement of MR^{4.5} (Figure 1), and were condensed into good-risk genotypes and compared with poor-risk genotypes for all subsequent comparisons. Univariate analysis of achievement of the MRs and FFS demonstrated that the *BIM* and *ASXL1* good-risk variants were both significantly associated with superior achievement of MR and FFS (Table 2). Neither variant predicted for progression to AP/BC or OS (Table 2).

A median of 23 months of follow-up data were available for a cohort of 83 patients treated with first-line nilotinib, among whom 79 were evaluable for outcome assessment according to the *BIM* and *ASXL1* variant genotypes. In contrast to the imatinib cohort, a marginally significant difference was detected only for MR⁴ for the *ASXL1* variant ($P = .048$) and no differences were detected for the *BIM* variant (Table 3). There was no difference in genotype frequencies between the variant and nilotinib-treated patients for the *BIM* (28.9% vs 27.0%; $P = .431$) or the *ASXL1* variant (43.8% vs 42.3%; $P = .968$). These results, although they were in a smaller patient cohort, suggest that the poor risk associated with the *BIM* and *ASXL1* variants may be abrogated by treating with a more potent TKI such as nilotinib.

Combining the Sokal score with the *BIM* and *ASXL1* variants showed superior predictive power to that of Sokal alone

To assess the prognostic power of combining the *BIM* and *ASXL1* variants with the Sokal risk group, multivariate models were developed for the prediction of EMR, MMR, MR⁴, MR^{4.5}, and FFS. The Sokal risk group and the *BIM* and *ASXL1* variants each maintained significance ($P < .05$) in multivariate models for the prediction of MMR, MR⁴, MR^{4.5}, and FFS (Table 4), demonstrating that these variables are independent predictors of long-term response to imatinib at diagnosis. Importantly, the combination of the Sokal score with the *ASXL1* and *BIM* variants improved

prediction of imatinib response at diagnosis (supplemental Table 6).

To further explore the interactions between the Sokal risk score and the *ASXL1* and *BIM* variants, we applied a classification tree model for prediction of FFS. Sokal and the *BIM* and *ASXL1* variants were retained in the final classification model after minimization of the 10-fold cross-validation error. The classification tree produced 7 terminal nodes which could readily be grouped into 4 risk categories: favorable, low, high, and ultra-high (Figure 2A). Kaplan-Meier analysis of these 4 risk groups showed highly significant stratification between these risk categories for the prediction of FFS as expected (97%, 80%, 67%, and 44%, respectively; nominal $P < .001$; Figure 2B). Importantly, this model also strongly predicted for EMR ($P = .002$; Figure 2C), MMR ($P < .001$; Figure 2D), MR⁴ ($P = .012$; Figure 2E), and MR^{4.5} ($P < .001$; Figure 2F), demonstrating that the model retained significance across clinical outcomes.

Bootstrap cross-validation was undertaken to assess and compare the predictive power of the multivariate models combining Sokal, *BIM* rs686952, and *ASXL1* rs4911231 variants. The Sokal score univariate model provided a modest discriminatory power (C-index, 0.62; range, 0.47-0.74). Importantly, the addition of the *BIM* and *ASXL1* variants to the Sokal score significantly improved the predictive power in cross-validation assessments of predictive performance score in Cox multivariate and classification tree models for FFS (Figure 3A). Moreover, prediction of progression to AP/BC was improved by addition of the risk variants to Sokal (Figure 3B-C).

Predictive models incorporating the Sokal score and the *ASXL1* and *BIM* variants identified a subgroup of patients with the greatest risk of treatment failure

The FFS of the 130 Sokal high-risk patients was 59%. Among these, additional information from the 2 variants allowed further risk stratification: 34 patients were reclassified as low risk, 45 as high risk, and 51 as ultra high risk with 75%, 64%, and 44% FFS, respectively (nominal $P = .012$). We could thus identify a subgroup of patients with high Sokal scores who had the greatest risk of treatment failure. Furthermore, these patients classified as ultra high risk were significantly more likely to progress to AP/BC compared with all other patients (12% vs 1%; $P = .034$; Figure 4A) and had inferior OS (88% vs 97%; $P = .041$; Figure 4B). These results demonstrate a further refinement for risk stratification when variant genotyping information is added to Sokal risk scores.

The combined Sokal, *ASXL1* variant, and *BIM* variant classification tree risk model identified patients at ultra high risk among those with subsequent EMR

Numerous studies have demonstrated the strong association between EMR and long-term outcomes. In our study, patients who

Figure 2. (continued) displays the relative risk of the node compared with the whole population, the number of events/sample size at that node, and the percentage of observations used at that node. (B) Kaplan-Meier FFS survival plot based on the classification tree risk categories. (C-F) Cumulative incidence of (C) EMR, (D) MMR, (E) MR⁴, and (F) MR^{4.5} as stratified by the classification tree risk groups, as defined in panel A. CI, 95% confidence interval.

Figure 3. Comparison of the predictive power of Sokal and ASXL1 and BIM variant genotypes and their combinations. (A) The data set was randomly split into training and test sets 100 times and the concordance index (C-index) was calculated. The C-index is a non-parametric measure to quantify the discriminatory power of a predictive model, in which a C-index of 0.5 indicates no predictive discrimination, and a C-index of 1 indicates perfect predictive accuracy. For each model, during each of the 100 times of random splitting, 80% of the total samples were used to train the model, and the remaining 20% were used as the test set for C-index calculations. Both the Cox multivariate and classification tree multivariate models showed performance superior to that of the Sokal risk score univariate model (one-sided Wilcoxon signed rank test $P < .001$). The boundaries of the box mark the first and third quartile, with the median in the center and whiskers extending to 1.5 interquartile range from the boundaries. The horizontal black dashed line marks the C-index equivalent to random guess (C-index = 0.5). Cumulative incidence of progression to AP/BC, stratified by (B) Sokal risk group and (C) classification tree risk group.

achieved EMR at 3 months of imatinib treatment was a very strong predictor of MMR ($P < .001$), MR^4 ($P < .001$), $MR^{4.5}$ ($P < .001$), FFS ($P < .001$), progression to AP/BC ($P < .001$), and OS ($P < .001$) compared with patients who did not achieve EMR. Interestingly, the majority (31 [62%] of 51 evaluable patients) of the

ultra-high-risk patients identified in the risk classification tree achieved the EMR milestone. EMR achievers ($n = 410$) were stratified by their baseline classification tree risk group. The ultra-high-risk patients by definition had significantly inferior achievement of long-term outcomes: 12-month MMR ($P = .009$), 48-month MR^4

Figure 4. The classification tree risk model identified a subgroup of ultra-high-risk patients with the highest risk of experiencing therapy failure and disease progression. (A) Progression to AP/BC and (B) OS was assessed in Sokal high-risk patients ($n = 130$), as stratified by ultra-high-risk group and the “other” risk groups (combined high-, low-, and favorable-risk groups as a result of a low number of events).

($P = .004$), $MR^{4.5}$ ($P = .002$), FFS ($P < .001$), and progression to AP/BC ($P = .026$) (supplemental Figure 3). Multivariate analysis demonstrated that the classification tree risk model of variables assessed at baseline was an independent predictor of outcome (Table 5).

The potential biological role of the *ASXL1* and *BIM* risk variants

Given the strong association with clinical outcome, we sought to investigate the putative biological function of the *ASXL1* and *BIM* risk variants. The *ASXL1* risk variant is a synonymous variant in exon 12 of *ASXL1*, and the linked variants (rs2295763 and rs2295764) are located in the 3' UTR of *ASXL1*. A putative functional role for these 3 variants could not be identified by in silico assessments. Functional predictions were expanded to variants within the *ASXL1* haplotype block. That analysis identified that *ASXL1* rs2295762, which was in high linkage with the *ASXL1* risk variants ($r^2 = 0.98$), is located in an evolutionary conserved region of the 3' UTR, and is predicted to create microRNA-34a (miR-34a) and miR-486-3p seed sites (supplemental Table 7).

The *BIM* rs686952 risk variant is located in intron 11. No potential functional significance was identified. The analysis was expanded to

variants located in the *BIM* haplotype block. Most notably, *BIM* rs724710, a synonymous variant located in the highly conserved BH3 functional domain, was in high linkage with the *BIM* risk variant ($r^2 = 0.92$) and was previously reported to be associated with imatinib response (supplemental Table 7).¹⁶ The *BIM* BH3 domain variant was genotyped in our cohort, and subsequent univariate analysis showed that this variant was significantly associated with MMR ($P = .022$), MR^4 ($P = .012$), and $MR^{4.5}$ ($P = .016$), but not FFS ($P = .212$) or progression to AP/BC ($P = .303$).

These in silico functional predictions have identified 2 variants (*ASXL1* rs2295762 and *BIM* rs724710) in linkage with the risk variants identified by our statistical analysis, which are of potential biological significance. These variants are candidates for further functional assessment to investigate their putative biological impact and role in modulating response to imatinib.

Discussion

Improved risk stratification at diagnosis of CML could aid choice of the most appropriate first-line TKI for optimal outcome. With the availability of generic imatinib and potentially lower drug costs, more potent TKIs could be reserved for those at highest risk of treatment

Table 5. Multivariate analysis of the classification tree risk groups and EMR status at 3 months

	MMR		MR^4		$MR^{4.5}$		FFS	
	RR	P	RR	P	RR	P	HR	P
Classification tree baseline risk group								
Favorable	1		1		1		1	
Low	0.77	.13	0.74	.2	0.55	.012	1.89	.062
High	0.62	.011	0.68	.11	0.43	.002	2.34	.021
Ultra-high	0.41	<.001	0.29	.001	0.21	.002	2.86	.005
EMR status at 3 months of imatinib treatment, %								
≤10	1		1		1		1	
>10	0.21	<.001	0.16	<.001	0.09	<.001	1.53	<.001

Relative risks and hazard ratios for the classification tree are compared with the favorable-risk group.

failure and lower probability of reaching milestone MR. Early achievement of MRs is associated with subsequent deep MR,³⁹ which is a prerequisite for treatment-free remission. With treatment-free remission potentially moving into mainstream clinical practice with the intention of discontinuing TKIs as a standard treatment protocol,^{40,41} it is desirable to improve the number of patients who achieve MRs. We have identified a small cohort of imatinib-treated patients with the highest risk of treatment failure, disease progression, and low probability of MR by combining the Sokal risk score and inherited genetic biomarkers. Two germ line variants were identified as strong biomarkers of response to imatinib: an intronic variant in the proapoptotic protein *BIM* and a synonymous variant in *ASXL1*, a putative epigenetic modifier. Previous molecular biomarkers available at diagnosis have not shown such a consistent association with molecular and survival outcomes.¹⁸⁻²² By definition, all of the ultra-high-risk patients had high Sokal risk but they represented only 39% of the total Sokal high-risk group.

EMR is currently the strongest predictor of long-term outcomes and is incorporated into the National Comprehensive Cancer Network (NCCN) and European LeukemiaNet (ELN) guidelines. The NCCN recommends a therapy switch for patients who fail to achieve EMR at 3 months, whereas the ELN recommends switching therapy at 6 months.^{5,6} However, there is little evidence that changing therapy after a therapy failure event can effectively salvage these patients. In some patients, disease progression may occur within the first 6 months of diagnosis, with little time to implement effective salvage therapy. Identification of very high-risk patients at diagnosis for novel interventions may be the only way to rescue these ultra-high-risk patients. In contrast, favorable-risk patients may achieve optimal outcomes with the lowest potency inhibitor, although other high-risk patients will require more potent inhibition upfront to achieve optimal outcomes. Indeed, our data suggest that the more potent inhibitor, nilotinib, may overcome the adverse outcome identified for some patients treated with imatinib. The lack of association may also be the result of the smaller sample size for the nilotinib cohort. Therefore, analysis of the significance of these variants in a larger cohort of nilotinib-treated patients with longer follow-up data are required to confirm these findings.

Important variables were initially selected using nonparametric (random forests and recursive partitioning) models, which do not explicitly conduct hypothesis tests during model selection; thus, correction for multiple testing was not required. However, semi-parametric (univariate and multivariate analysis) statistical tests were used to confirm the significance of the variables that were deemed important.

Risk groups were initially constructed from an analysis of FFS, a composite end point of outcomes. This allowed validation of the prognostic significance of each of the complements of the failure events when they were investigated separately, which indeed was the case (Figure 2). The bootstrapping method of internal validation provides a nearly unbiased estimate of predictive accuracy of relatively low variance and has the additional advantage of using the entire data set for model assessment.⁴² Importantly, bootstrap cross-validation demonstrated that when the *BIM/ASXL1* variants were combined in a multivariate model (Cox or classification tree) with the Sokal score, a significant increase in the discriminatory capability of the prognostic model was observed compared with the Sokal score alone. It is possible that this analysis carries the risk of

overfitting, but our robust statistical models of cross-validation techniques and bootstrapping were designed to mitigate this risk. In addition, we have referred to nominal *P* values when testing for differences in FFS between risk groups that were constructed from classification trees built to predict FFS.

BIM is a proapoptotic member of the *BCL2*-family, which is crucial for the intrinsic apoptotic response to imatinib. The *BIM* risk variant was in high linkage with a synonymous variant (rs724710) in the BH3 functional domain. This variant is reported to be associated with imatinib response.¹⁶ How this variant modifies *BIM* function is yet to be elucidated, but synonymous variants can have functional effects by altering the dynamics of translation through different codon usage, by changing messenger RNA secondary structure that alters protein expression, or by altering RNA splicing by interfering with a splice site or splicing enhancers.⁴³ Certainly, BH3-mimetic drugs can overcome the imatinib resistance conferred by the *BIM* deletion polymorphism.¹² Furthermore, a stapled *BIM* peptide was recently shown to activate BH3-specific cell death to overcome apoptotic resistance in hematologic malignancies.⁴⁴ Further investigation into the mechanism of action of the *BIM* risk variants would reveal whether these therapeutic strategies would be effective for enhancing response.

ASXL1 exon 12 is a region commonly mutated in hematologic cancers, and mutations in this region are associated with aggressive disease and poor outcomes in acute myeloid leukemia and myelodysplastic syndrome.^{23,45,46} *ASXL1* is an epigenetic regulator that binds members of the polycomb repressive complex 2 (PRC2). This complex mediates gene silencing by inducing trimethylation of histone H3-lysine 27, and mutations in *ASXL1* impair PRC2 function.⁴⁷ We hypothesize that a variant that introduces an miRNA binding site causing downregulation of *ASXL1* expression may also contribute to poor outcomes in CML patients. In silico predictions identified a 3' UTR *ASXL1* variant (rs2295762) that is predicted to introduce miR-34a and miR-486-3p seed sites. Interestingly, miR-486 is reported to be involved in normal erythropoiesis and modulates drug resistance in CML.⁴⁸ Most notably, miR-486-5p was reported to modulate response to imatinib in CML progenitor cells.⁴⁸ The mechanism by which these variants affect response awaits further clinical and experimental evaluation. Alternatively, it is plausible that other variants in linkage disequilibrium with the *ASXL1* and *BIM* variants that were not detected using our targeted sequencing approach are responsible for the associations detected by this study.

Several variants in genes responsible for the pharmacologic activity of imatinib have been reported as predictors of imatinib response in CML, including *SLC22A1* (*OCT1*) rs628031 and 3 *MDR1* (*ABCB1*) variants (rs1128503, rs2032582, and rs1045642). However, inconsistent prognostic significance for each of these variants has been reported between studies, which limits their clinical application.^{21,49-51} A meta-analysis of outcomes in 1826 CML patients from 12 studies confirmed that the *ABCB1* rs1128503 variant has prognostic value in Asian populations.⁵² We did not observe any association between these 3 *ABCB1* variants with response to imatinib, although we may have different patient populations.

In conclusion, this study has identified germ line variants as strong biomarkers of imatinib response when combined with the Sokal score. Assessment of germ line genetic variation at diagnosis may

contribute to an enhanced prognostic score to aid therapeutic decision-making. The utility of this risk score should be determined in prospective clinical trials.

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Authorship

Contribution: J.E.M. designed and performed the research, analyzed data, and wrote the manuscript; L.P. designed and performed the research and contributed to preparation of the manuscript; S.B.

designed the research, analyzed data, and contributed to manuscript preparation; T.P.H., W.T.P., D.S., and H.S.S. contributed to the experimental design and manuscript preparation; D.T.Y. and J.R. analyzed data, provided advice on statistical analysis, and contributed to preparation of the manuscript; P.P.S.W. and A.W.S. contributed to data analysis; and D.J.P. and J.T. contributed to data analysis and provided statistical advice.

Conflict-of-interest disclosure: D.T.Y. received funding and honoraria from and has served as a consultant on an advisory board of Novartis Oncology and Bristol-Myers Squibb. J.R. holds shares in Novartis. T.P.H. received research funding and honoraria and is an advisory board member for Novartis Oncology, Bristol-Myers Squibb, and Ariad Pharmaceuticals. S.B. received research funding from Novartis Oncology, Ariad Pharmaceuticals, and Otsuka; honoraria from Novartis Oncology, Bristol-Myers Squibb, Otsuka, Qiagen, and Ariad; and is an advisory board member for Novartis Oncology and Qiagen. The remaining authors declare no competing financial interests.

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