Pharmacogenomic Analysis of Cytogenetic Response in Chronic Myeloid Leukemia Patients Treated with Imatinib

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ABSTRACT

Purpose: **To better understand the molecular basis of cytogenetic response in chronic myeloid leukemia patients treated with imatinib, we studied gene expression profiles from a total of 100 patients from a large, multinational Phase III clinical trial (International Randomized Study of IFN-α *versus* STI571).**

Experimental Design: **Gene expression data for >12,000 genes were generated from whole blood samples collected at baseline (before imatinib treatment) using Affymetrix oligonucleotide microarrays. Cytogenetic response was determined based on the percentage of Ph^+ cells from bone marrow following a median of 13 months of treatment.**

Results: **A genomic profile of response was developed using a subset of individuals that exhibited the greatest divergence in cytogenetic response; those with complete response (0% Ph^+ cells; $n = 53$) and those with minimal or no response (>65% Ph^+ cells; $n = 13$). A total of 55 genes was identified that were differentially expressed between these two groups. Using a "leave-one-out" strategy, we identified the optimum 31 genes from this list to use as our genomic profile of response. Using this genomic profile, we were able to distinguish between individuals that achieved major cytogenetic response (0–35% Ph^+ cells) and those that did not, with a sensitivity of 93.4% (71 of 76 patients), specificity of 58.3% (14 of 24 patients), positive predictive value of 87.7%, and negative predictive value of 73.7%.**

Conclusions: **Interestingly, many of the genes identified appear to be strongly related to reported mechanisms of *BCR-ABL* transformation and warrant additional research as potential drug targets. The validity and clinical implications of these results should be explored in future studies.**

INTRODUCTION

Chronic myeloid leukemia (CML) is associated with the presence of the Philadelphia (Ph) chromosome, a cytogenetic abnormality that is identified in up to 95% of CML patients (1). The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22, which results in the formation of a fusion gene between the *BCR* gene on chromosome 22 and the *ABL* tyrosine kinase gene located on chromosome 9. This *BCR-ABL* fusion gene is a constitutively active tyrosine kinase capable of interacting with numerous downstream signaling targets that are important for regulating cell growth (2, 3). Imatinib (Gleevec/Glivec, formerly STI571; Novartis Pharmaceuticals, East Hanover, NJ) was developed as a specific inhibitor of the *BCR-ABL* tyrosine kinase. Results of Phase I and Phase II clinical trials have demonstrated imatinib to be highly effective in the treatment of CML patients, particularly those in the chronic phase of the disease (4, 5).

This pharmacogenomic study was conducted as part of the International Randomized Study of IFN-α *versus* STI571 (IRIS) study. A total of 1106 patients with newly diagnosed Ph^+ CML in chronic phase was enrolled in this Phase III clinical trial, with 553 patients each randomized to either imatinib or IFN-α plus cytarabine [IFN/1-β-D-arabinofuranosylcytosine (AraC)] as the initial therapy (6). The study design allowed for patients to crossover to the other treatment arm because of lack of response, loss of response, or intolerance of treatment. Patients were previously untreated with the exception of hydroxyurea, which was used in >80% of these patients. Cytogenetic response was monitored by calculating the percentage of Ph chromosome-positive (Ph^+) cells from bone marrow analyses performed at 3-month intervals. Results from the IRIS study following a median of 19 months of treatment have recently been reported by O'Brien *et al.* (7). They reported an estimated rate of major cytogenetic response (≤35% Ph^+ cells) at 18 months of 87.1% [95% confidence interval (CI), 84.0–90.0] and an estimated complete cytogenetic response (0% Ph^+ cells) rate of 76.2% (95% CI, 72.5–79.9) at 18 months.

Microarray technology that evaluates the signatures of thousands of individual genes at a time is growing rapid acceptance in the clinical oncology setting (8). This technology has been used to identify genetic factors that can differentiate between different classes of cancers (9–11), biomarkers of clinical response (12, 13), as well as genes that can predict the development of resistance to imatinib treatment in cases of acute lymphoblastic leukemia (14). The primary aim of this study was to use this gene expression profiling strategy to identify a genomic profile that is associated with cytogenetic response in chronic phase CML patients after 12 months of treatment. Gene expression profiling was performed on a subset of 100 patients

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that were randomized to imatinib as the first-line treatment, with a median length of treatment of 13 months. RNA was extracted from whole blood collected at baseline, with no additional selection to distinguish between leukemic and nonleukemic cells. Taking the most conservative approach, the classification of responders was limited to only those cytogenetic responses that were verified in at least one subsequent (≥4 weeks) bone marrow assessment. Thus, the percentage of responders reported in this study is lower than what has been previously published for the IRIS study (15).

MATERIALS AND METHODS

Patients. Blood for RNA extraction was collected from >200 patients from the United States enrolled in the IRIS clinical trial. The pharmacogenomics protocol was approved by Institutional Review Boards at each participating site. RNA of sufficient quantity and quality was extracted from 106 samples, which were collected at baseline before drug treatment from patients that were randomized to imatinib as first-line treatment. Of these 106 samples, one sample was eliminated due to the patient dropping out very early from the study. Response was determined as the best cytogenetic response detected by bone marrow assessment of ≥20 metaphases, which was confirmed in at least one subsequent (≥4 weeks) bone marrow analysis. This more conservative classification of cytogenetic response was chosen in an effort to define the clearest phenotypes for our analysis. An additional 5 samples were thus eliminated from additional analysis due to lack of confirmation of cytogenetic response.

To create the largest distinction between responders and nonresponders, the initial pharmacogenomic analysis was performed on a subset of the remaining 100 patients. Individuals that crossed over to the IFN/1-AraC treatment arm ($n = 1$) or that were discontinued from the study ($n = 2$) were not included in this initial analysis. Patients with 0% Ph^+ cells were classified as complete cytogenetic responders (CCyR; $n = 53$) and those with >65% Ph^+ cells were classified as nonresponders (NoCyR; $n = 13$). Thus, a total of 66 individuals were included in the selection of the genomic profile. Subsequent analysis of major CyR (MCyR; 0–35% Ph^+ cells) *versus* nonmajor response included all of the 100 patients, including those that dropped out or crossed over.

RNA Expression Profiling. RNA expression data were generated from each blood sample using high-density oligonucleotide microarrays (HG U95Av2; Affymetrix, Santa Clara, CA) that represent >12,000 known human genes and expressed sequence tags. Sample preparation and microarray processing were performed using protocols from Affymetrix. In brief, total RNA was extracted from frozen whole blood using Tri Reagent BD (Sigma, St. Louis, MO) and then purified through RNeasy Mini Spin Columns (Qiagen, Valencia, CA). Starting with 5–8 μg of purified total RNA, double-stranded cDNA was synthesized from full-length mRNA using Superscript Choice System (Invitrogen-Life Technologies, Inc., Carlsbad, CA). The cDNA was then transcribed *in vitro* using BioArray High Yield RNA Transcript Labeling kit (ENZO Diagnostics, Farmingdale, NY) to form biotin-labeled cRNA. The cRNA was fragmented and hybridized to the microarrays for 16 h at 45°C.

Arrays were washed and stained using an Affymetrix fluidics station according to standard Affymetrix protocols. Arrays were scanned using an Affymetrix GeneArray scanner and the data (.DAT file) captured by the Affymetrix GeneChip Laboratory Information Management System. The Laboratory Information Management System database was connected to an internal UNIX Sun Solaris server through a network filing system that allows for the average intensities for all probes cells (.CEL file) to be downloaded into an internal Oracle database. The fluorescence intensity of each microarray was normalized by global scaling to a value of 150 to allow for direct comparison across multiple arrays.

Quality of each array was assessed by evaluating factors such as background, percentage of genes present, scaling factor, and the 3′/5′ ratios of the housekeeping genes β-actin and *GAPDH*. Although there was a wide range in these quality control parameters for the samples analyzed in this study, there were no statistically significant differences in sample quality between the CCyR and NoCyR groups.

Data Analysis. Microarray data for the 66 samples was imported into GeneSpring version 4.1.5 software (Silicon Genetics, San Carlos, CA), and raw expression values were filtered such that at least 10% of the samples (7 of 66) had an average intensity value of 100 or greater above background. Additional filtering steps were performed using GeneSpring, Excel, and SAS version 8.2 (SAS Institute, Cary, NC) to identify a list of genes that most distinguished between the CCyR and NoCyR groups. A total of 55 genes fit the criteria of at least a 1.7-fold difference (arbitrarily chosen) between the two groups with $P < 0.05$ by nonparametric, one-way ANOVA.

From this list of 55 genes, we used a "leave-one-out" procedure to determine the optimum number of genes to use as the final prognostic set, as described by van't Veer *et al.* (12). In brief, genes were ordered by correlation (absolute value of Pearson correlation coefficient) between expression values and the response category (0 = NoCyR; 1 = CCyR). Starting with the 5 most highly correlated genes, one sample was taken out of the analysis, and the mean gene expression profile for each group (CCyR and NoCyR) was calculated from the remaining 65 samples. The predicted outcome for the left-out sample was determined by performing a Pearson correlation between the expression profile of the left-out sample with the mean CCyR and NoCyR profiles calculated using the 65 samples. This analysis was repeated using the remaining samples until all 66 samples had been left out once. The number of cases of correct and incorrect predictions was determined by calculating the number of false negatives (CCyR misclassified as NoCyR) and false positives (NoCyR misclassified as CCyR). The entire leave-one-out process was repeated after adding additional predictor genes from the top of the list until all 55 genes were used. The gene number that resulted in the fewest number of false negatives and false positives was chosen as the optimal genomic profile ($n = 31$).

The next step was to use this optimized set of genes to calculate an appropriate threshold value to use for an accurate classification of CCyR or NoCyR. It was empirically decided to compare individual samples to the NoCyR profile as opposed to the CCyR profile after comparing results from both. A Pearson correlation was used to correlate the expression profile of each

Table 1 Baseline demographic and hematological parameters for the 66 patients used to develop the genomic profile of cytogenetic response

Parameter	CCyR[a] (0% Ph^+ cells)		NoCyR (>65% Ph^+ cells)		
	n	Mean ± SD or %	*n*	Mean ± SD or %	*P*
Demographics					
Age (yrs)	53	47.3 ± 13.1	13	48.3 ± 8.9	0.821
Gender	53	64% M, 36% F	13	62% M, 38% F	1.000
Previous HU treatment	53	83% HU, 17% no HU	13	85% HU, 15% no HU	1.000
Hematological					
WBC count ($\times 10^9$/liter)	53	31.1 ± 30.7	13	40.1 ± 27.1	0.134
Platelet count ($\times 10^9$/liter)	53	402 ± 251	13	395 ± 270	0.784
Peripheral blasts (%)	53	0.27 ± 0.69	13	0.58 ± 1.16	0.195
Peripheral basophils (%)	53	3.30 ± 3.43	13	4.39 ± 5.96	0.612
Peripheral eosinophils (%)	53	2.13 ± 2.37	13	2.38 ± 2.40	0.666
Sokal risk score[b]	33	0.81 ± 0.34	8	1.28 ± 0.65	0.006

[a] CCyR, complete cytogenetic response; NoCyR, minimal or no cytogenetic response; *P* calculated by Fisher's exact or ANOVA; HU, hydroxyurea.
[b] Sokal risk score (16) was not available for all patients.

of the 66 samples to the mean NoCyR profile (calculated using all 13 of the 66 patients with NoCyR). A threshold correlation value was chosen such that <10% of NoCyR cases were misclassified as CCyR ($r = 0.54$).

Statistical Analyses. Statistical analyses such as Fisher's exact test, nonparametric, one-way ANOVA and calculation of odds ratios, along with 95% confidence limits, were calculated using SAS version 8.2. Statistical significance was established at $P < 0.05$.

RESULTS

Patients. To identify a list of genes that are differentially expressed between patients that respond to imatinib and patients that do not, 53 patients with complete cytogenetic response (CCyR; 0% Ph^+ cells), and 13 patients with minimal or no response (NoCyR; >65% Ph^+ cells) were studied. Patient demographics were similar for both the CCyR and NoCyR groups, with no significant differences in mean age or in gender distribution (Table 1). We also evaluated baseline values for several hematological factors, some of which have been identified as prognostic factors for major cytogenetic response such as percent peripheral blasts, WBC count, and platelet count (4). As demonstrated in Table 1, there were no significant differences in baseline hematological parameters between our responder and nonresponder groups, nor was there a difference in percentage of individuals previously treated with hydroxyurea. There was a significant difference in risk stratification using the Sokal score (16), which was not unexpected, with those individuals in the nonresponder group, demonstrating a higher mean Sokal score compared with those that achieved complete cytogenetic response.

Genomic Profile of Cytogenetic Response. We identified 55 genes that were significantly differentially expressed between the CCyR and NoCyR groups (Table 2). Using a leave-one-out methodology, we optimized this list to 31 genes. Fig. 1 displays the results of cluster analysis of these 31 genes, with the samples ordered by correlation coefficient. A threshold correlation was selected at a value that would minimize the number of false negatives to <10% (optimized specificity; $r = 0.54$). Using this threshold, an individual with a correlation coefficient of ≥0.54 (based on Pearson correlation with mean NoCyR expression profile) would be classified as a nonresponder, whereas an individual with $r < 0.54$ would be classified as a responder. As demonstrated in Table 3, this resulted in 50 of the 53 CCyR individuals being correctly classified as responders (sensitivity = 0.943), and 12 of the 13 NoCyR individuals being correctly classified as nonresponders (specificity = 0.923). Results of Fisher's exact test indicated a highly significant association between the clinical response status and the predicted response determined using the genomic profile ($P < 0.00001$). The calculated odds ratio of 200 (95% CI, 19.1–2096), although also highly significant, is likely inflated because it was calculated using the same data that was used to derive the genomic profile. Ideally, these results should be validated using an independent set of samples.

To determine the optimum set of genes that could differentiate between responders and nonresponders, we only used those patients who achieved complete cytogenetic response (0% Ph^+ cells) and those with minimal or no response (>65% Ph^+ cells), with the exclusion of 3 individuals who had crossed over to the IFN/AraC treatment arm or had been discontinued. Subsequent analysis was performed to evaluate all of the 100 individuals in this study using our genomic profile. For these analyses, the profile of the 31 genes for each of the samples was correlated against the mean NoCyR profile using the threshold correlation value of 0.54. Table 4 displays the breakdown of genomic classification by the cytogenetic response achieved following a median of 13 months of treatment. Results of analysis by Fisher's exact test indicated that there was a significant association between the response predicted by genomic profiling and the actual cytogenetic response ($P < 0.00001$). Follow-up analysis using this same genomic profile with clinical data collected following a median of 19 months of treatment indicated that this association remained significant ($P < 0.00001$; data not shown).

MCyR Categorization. Additional analysis was performed by grouping all individuals into one of two categories based upon their response status. Individuals with 0–35% Ph^+ cells (complete or partial response) were classified as having achieved MCyR, and those individuals that had >35% Ph^+ cells

Table 2 List of 55 genes differentially expressed between responders and nonresponders, grouped by gene function

Gene symbol	Affymetrix probe set	GenBank accession no.	Locus	Description	Fold Difference[a]	BCR-ABL[b]
Cell cycle/Cell proliferation						
AS3	32641_at	AB023196	13q12.3	Androgen-induced prostate proliferative shutoff associated protein	↑ 3.0	
CDC6	36839_at	U77949	17q21.3	CDC6 cell division cycle 6 homologue (*S. cerevisiae*)	↑ 1.7	1, 2
RAD9[c]	32158_at	U53174	11q13.1–q13.2	RAD9 homologue (*S. pombe*)	↓ 2.4	3
SMC1L1[c]	32849_at	D80000	Xp11.22–p11.21	SMC1 structural maintenance of chromosomes 1-like 1 (yeast)	↓ 2.4	
Cytoskeleton/Cell adhesion						
ADD2	36052_at	U43959	2p14–p13	Adducin 2 (β)	↑ 3.6	1
CXCR3[c]	34077_at	X95876	Xq13	Cytokine (C-X-C motif) receptor 3	↑ 3.1	1
TUBG1[c]	33346_r_at	M61764	17q21	Tubulin, γ 1	↓ 1.8	
Immune response						
KIR2DL3	36886_f_at	L41268	19q13.4	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	↑ 1.8	
Metabolism/Enzyme						
ACPP	617_at	M24902	3q21–q23	Acid phosphatase, prostate	↓ 1.9	
CYP4F3[c]	1305_s_at	D12620	19p13.2	Cytochrome P450, subfamily IVF, polypeptide 3 (leukotriene B4 ω hydroxylase)	↑ 1.8	
HPRT1	37640_at	M31642	Xq26.1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	↑ 1.8	
PPP2R2A	41167_at	M64929	8p21.1	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), α isoform	↑ 2.8	2, 3
RAB2[c]	623_s_at	M28213	8q12.1	RAB2, member RAS oncogene family	↓ 2.6	
VRK1	39980_at	AB000449	14q32	Vaccinia-related kinase 1	↓ 2.1	
Plasma membrane/Extracellular						
FVT1[c]	36120_at	X63657	18q21.3	Follicular lymphoma variant translocation 1	↓ 2.1	
HPS1[c]	38467_at	U96721	10q23.1–q23.3	Hermansky-Pudlak syndrome 1	↑ 1.8	
PCDH7	41534_at	AB006755	4p15	BH-protocadherin (brain-heart)	↑ 1.9	1
SNTB2	40589_at	U40572	16q22–q23	Syntrophin, β 2 (dystrophin-associated protein A1, 59kD, basic component 2)	↑ 2.0	
Signal transduction						
CRHR2[c]	33949_at	AF011406	7p15.1	Corticotropin releasing hormone receptor 2	↑ 7.1	
GIT2[c]	36741_at	D63482	12q24.1	G protein-coupled receptor kinase-interactor 2	↓ 2.7	1
GNAQ	38581_at	U40038	9q21	Guanine nucleotide binding protein (G protein), q polypeptide	↓ 1.8	1
GRP58[c]	38986_at	Z49835	15q15	Glucose regulated protein, 58 kDa	↓ 2.1	2
H963[c]	31919_at	AF002986	3q26.1	Platelet activating receptor homologue	↓ 2.1	
JAK1[c]	1457_at	M64174	1p32.3–p31.3	Janus kinase 1	↓ 2.1	2, 3
MYLK[c]	32847_at	U48959	3q21	Myosin, light polypeptide kinase	↓ 3.5	1, 3
OR2F1	31425_g_at	AC004853	7q35	Olfactory receptor, family 2, subfamily F, member 1	↑ 1.7	
PAK1[c]	1558_g_at	U24152	11q13–q14	p21/Cdc42/Rac1-activated kinase 1 (STE20 homologue, yeast)	↑ 2.7	1, 2, 3
PIK3R1[c]	1269_at	M61906	5q12–q13	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 α)	↑ 4.1	1, 2, 3
PIK3R3	322_at	D88532	1p33	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, γ)	↑ 4.5	1, 2, 3
PKIG[c]	34376_at	AB019517	20q12–q13.1	Protein kinase (cAMP-dependent, catalytic) inhibitor γ	↓ 2.4	
PPP1R12B	41137_at	AB007972	1q32.1	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	↑ 1.9	1
TNFSF14	31742_at	AF064090	19p13.3	Tumor necrosis factor (ligand) superfamily, member 14	↓ 2.1	3
TRAF5	1328_at	U69108	1q32	TNF receptor-associated factor 5	↑ 2.8	3
TRIP6[c]	39341_at	AJ001902	7q22	Thyroid hormone receptor interactor 6	↑ 2.6	1

Table 2 Continued

Gene symbol	Affymetrix probe set	GenBank accession no.	Locus	Description	Fold Difference[a]	BCR-ABL[b]
ZNF259[c]	32518_at	AF019767	11q23.3	Zinc finger protein 259	↑ 2.0	2
Transcription factor						
CBL[c]	34416_at	X57110	11q23.3	Cas-Br-M (murine) ecotropic retroviral transforming sequence (Casitas B-lineage lymphoma)	↑ 2.0	1, 2, 3
CREB1[c]	37535_at	M27691	2q32.3–q34	cAMP responsive element binding protein 1	↓ 1.8	2
REL	1856_at	X75042	2p13–p12	v-rel reticuloendotheliosis viral oncogene homologue (avian)	↓ 2.1	3
RNF4	35777_at	AB000468	4p16.3	Ring finger protein 4	↓ 1.8	
ZNF132	33597_at	U09411	19q13.4	Zinc finger protein 132 (clone pHZ-12)	↓ 1.9	
ZNF85[c]	36303_f_at	U35376	19p13.1–p12	Zinc finger protein 85 (HPF4, HTF1)	↑ 3.4	
Translation factor/RNA binding						
SFRS3[c]	351_f_at	D28423	11	Splicing factor, arginine/serine-rich 3, 5′UTR (sequence from the 5′cap to the start codon)	↓ 3.2	
TUFM[c]	39867_at	S75463	16p11.2	Tu translation elongation factor, mitochondrial	↓ 1.7	
Transporter						
AQP4[c]	40793_s_at	U34846	18q11.2–q12.1	Aquaporin 4	↑ 4.1	
KCNMB1	38298_at	U25138	5q34	Potassium large conductance calcium-activated channel, subfamily M, β member 1	↑ 2.1	
LAPTM4A	39019_at	D14696	2p24.3	Lysosomal-associated protein transmembrane 4 α	↓ 1.7	
TOMM34[c]	37050_r_at	AI130910	20	Translocase of outer mitochondrial membrane 34	↓ 3.0	
Unknown						
DKFZP564O0423	36078_at	AL080120	11q13.4	DKFZP564O0423 protein	↑ 1.8	
DKFZp586F2423	39692_at	AL080209	7q34	Hypothetical protein DKFZp586F2423	↓ 2.0	
FLJ10569	38226_at	W27152	8p21.2	Hypothetical protein FLJ10569	↓ 2.0	
KIAA0196	38419_at	D83780	8p22	KIAA0196 gene product	↑ 3.5	
KIAA0575	38875_r_at	AB011147	2p25.1	KIAA0575 gene product	↑ 3.9	
KIAA0708[c]	41614_at	AB014608	6p12.3	KIAA0708 protein	↓ 2.1	
LHFPL2[c]	37542_at	D86961	5q13.3	Lipoma HMGIC fusion partner-like 2	↓ 3.5	
LOC113251[c]	35180_at	AL050205	12q13.12–q13.13	c-Mpl binding protein	↓ 1.9	

[a] Fold difference in mean gene expression levels for responders (CCyR) *versus* nonresponders (NoCyR): ↑, higher in CCyR; ↓, lower in CCyR.
[b] Associated with mechanism of BCR-ABL-induced transformation: 1, cell adhesion; 2, mitogenic signaling; 3, inhibition of apoptosis (see text for references).
[c] Included as one of the 31 genes in the genomic profile of response (of 55 differentially expressed genes).

(minor, minimal, or no response) were classified as nonresponders (NoMCyR). Patients that were identified early on as having progressive disease ($n = 2$) were included in the NoMCyR group (see Table 4 for individual breakdowns). Results of comparing individuals with major cytogenetic response *versus* patients with no major response, using our genomic profile with a 0.54 correlation threshold, are presented in Table 3. The results of this analysis were highly significant, with a P of <0.00001 and a calculated odds ratio of 19.9 (95% CI, 5.9–67.1). However, it is important to note that this odds ratio is also likely to be inflated because it was calculated using some of the same data that was used to derive the initial genomic profile. Compared with the initial analysis of CCyR *versus* NoCyR, the specificity was notably decreased for the comparison of major *versus* nonmajor response (Table 3). This is likely due to the increased heterogeneity after the addition of the minor and progressive disease classes to the NoMCyR nonresponder category.

Association with Hydroxyurea Treatment. A large percentage of the patients in this trial had been previously treated with hydroxyurea before being treated with imatinib, including 87% of the 100 patients evaluated in this pharmacogenomics analysis (also see Table 1). Furthermore, 56% of these 100 patients received concurrent treatment with hydroxyurea during the first 6 months of treatment with imatinib. Results of analyses indicated that there was no significant association between the response predicted by genomic profiling and previous or concurrent hydroxyurea usage ($P > 0.2$; Fisher's exact text). Additionally, there was no association between hydroxyurea usage (either previous or concurrent) and actual cytogenetic response in the 100 patients studied for pharmacogenomic analysis (data not shown).

Association with Time to Progression. The primary objective of the IRIS study was to determine the time to progression of Ph^+ CML patients randomized to treatment with imatinib compared with patients randomized to treatment with

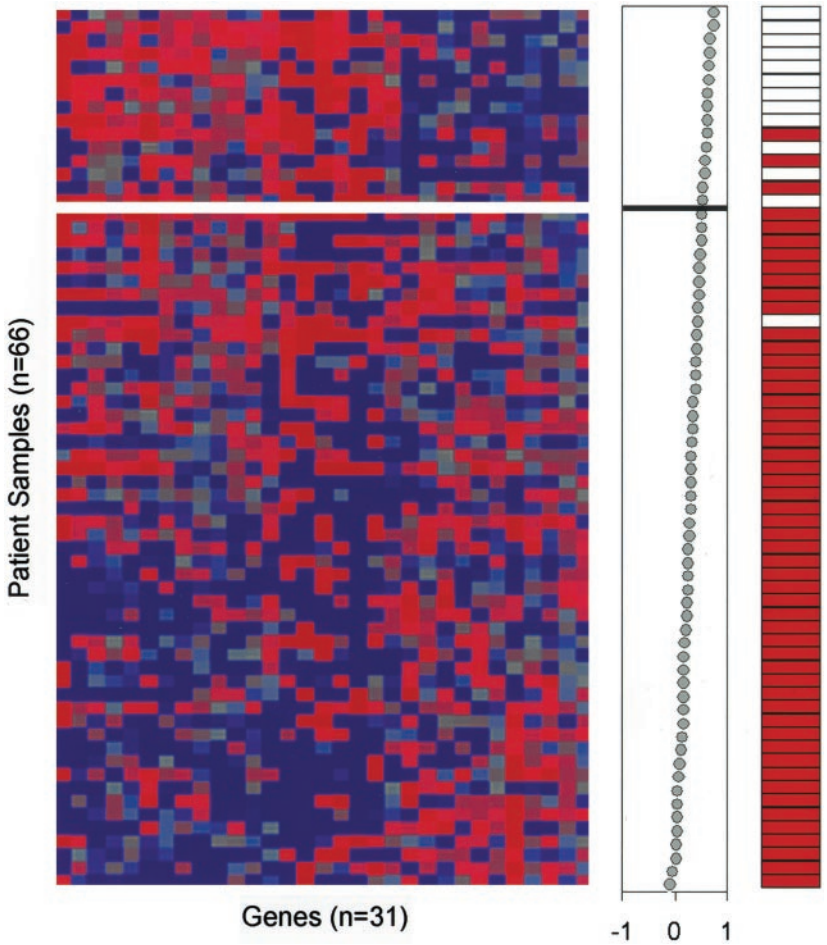

Fig. 1 Cluster of 31 genes comprising the genomic profile of response. Colors represent relative levels of expression, with blue representing low expression and red representing high expression. Samples are ordered according to correlation of gene expression with the mean cytogenetic nonresponders (NoCyR) expression profile, and clustering of genes was performed using the Pearson similarity method in GeneSpring. Correlation coefficients for each sample are plotted in the *middle panel*, with samples with the highest correlation to the NoCyR profile at the *top* and those with least correlation to the NoCyR expression profile at the *bottom*. The *right panel* represents the actual complete cytogenetic responder (CCyR) status, with solid (*red*) indicating CCyR and white representing patients with NoCyR. *Line* indicates the threshold correlation value that was determined to minimize false negatives to <10% ($r = 0.54$).

IFN (7, 16). At the time of this analysis, however, there were too few progression events in our subset of patients with which to perform any statistically relevant tests.

DISCUSSION

Using RNA from whole blood collected at baseline, we identified 55 genes that were differentially expressed between individuals with complete cytogenetic response and those with minimal or no cytogenetic response ($P < 0.05$). Using an optimized set of 31 of these genes, we were able to classify individuals as likely responders or nonresponders by correlating individual gene expression profiles with a reference genomic profile. Results for identifying those individuals that achieved a MCyR (0–35% Ph^+ cells) demonstrated a sensitivity of 0.934

Table 3 Frequency analysis and calculation of odds ratios for complete and major cytogenetic response[a]

Response status	*n*	Response profile ($r < 0.54$) Obs (Exp)	No response profile ($r \geq 0.54$) Obs (Exp)	Odds ratio (95% CI)	*P*	Sens.	Spec.	PV+	PV−
Complete (CCyR) *versus* NoCyR; $n = 66$[b]									
CCyR	53	50 (41)	3 (12)	200 (19.1–2096)	1.14×10^{-9}	0.943	0.923	0.980	0.800
NoCyR	13	1 (10)	12 (3)						
Major (MCyR) *versus* NoMCyR; $n = 100$									
MCyR	76	71 (62)	5 (14)	19.9 (5.9–67.1)	2.9×10^{-7}	0.934	0.583	0.877	0.737
NoMCyR	24	10 (19)	14 (5)						

[a] *r*, Pearson correlation coefficient; CI, confidence interval; Sens., sensitivity; Spec., specificity; PV+, predictive value positive; PV−, predictive value negative; Obs, observed frequencies; Exp, expected frequencies; CCyR, complete response (0% Ph^+ cells); NoCyR, minimal or no response (>65% Ph^+ cells); MCyR, major cytogenetic response (≤35% Ph^+ cells); NoMyR, no major response (>35% Ph^+ cells).

[b] Includes patients with complete, minimal or no response (see Table 4) minus 3 patients who crossed over or were discontinued.

Table 4 Genomic classification *versus* actual cytogenetic response[a]

Response	% Ph^+ cells	Total no. of patients	Response profile ($r < 0.54$) Obs	(Exp)	No response profile ($r \geq 0.54$) Obs	(Exp)
Complete	0%	54	51	(44)	3	(10)
Partial	>0–35%	22	20	(18)	2	(4)
Minor	>35–65%	7	6	(6)	1	(1)
Minimal	>65–95%	4	0	(3)	4	(1)
None	>95%	11	2	(9)	9	(2)
Progressive disease		2	2	(2)	0	(0)
Total		100	81	(81)	19	(19)
			$P = 6.17 \times 10^{-9}$ (Fisher's exact test)			

[a] *r*, Pearson correlation coefficient; Obs, observed frequencies; Exp, expected frequencies; PD, progressive disease.

and positive predictive value of 0.877 (Table 3), somewhat less than what was seen for the analysis of CCyR *versus* NoCyR. However, the specificity of 0.583 and negative predictive value of 0.737 for MCyR *versus* NoMCyR was considerably lower than that seen for the CCyR/NoCyR analysis (Table 3). This low specificity could be because of several factors such as the high heterogeneity of the NoMCyR group (>35% Ph^+ cells) and that some of these nonresponders would eventually achieve a MCyR given additional time. Another likely factor is that the genomic profile was derived from RNA taken from whole blood, with no selection between leukemic and nonleukemic cells, which is a considerable limitation of this current study.

As indicated in Table 2, the identified genes have many functions, including cell cycle regulators, transcription factors, and signal transduction genes. Interestingly, many of the genes identified appear to be functionally related to the pathogenesis of CML via the *BCR-ABL* oncogene. It is believed that the *BCR-ABL* oncogene can lead to malignant transformation via three major mechanisms: altered cell adhesion; constitutively active mitogenic signaling; and inhibition of apoptosis (see Refs. 1, 3, 17 for reviews).

Genes Associated with Cell Adhesion. β-Integrins and cytoskeletal elements play a major role in the adhesion process. Expression of *BCR-ABL* is believed to decrease the adhesion of the CML progenitor cells to bone marrow stroma cells and extracellular matrix, thus allowing them to escape normal cell proliferation regulation (3). This may be because a large portion of the *BCR-ABL* gene is associated with cytoskeletal elements, either directly by the COOH-terminal actin-binding domain or through several different adapter proteins (17).

A number of the genes that we identified that were differentially expressed between the responders (CCyR) and nonresponders (NoCyR) are also associated with the cell adhesion process, either through interaction with cytoskeletal elements or related to the integrin signaling pathway. These include *CBL* (18), *CXCR3* (19), *MYLK* (20), *PAK1* (21, 22), *PIK3R1/R3* (23), *PPP1R12B* (24), *ADD2* (25), *CDC6* (26), *GIT2* (27), *GNAQ* (28), *PCDH7* (29), and *TRIP6* (30). Furthermore, several of these genes have been shown to interact with each other such as *MYLK* with *PAK1* (22), *PAK1* with *PIK3* (23), and *PIK3* with *CBL* and *CXCR3* (19, 31). Myosin light chain phosphorylation is directly regulated by two of the genes in our list, which are phosphorylated by *MYLK* and dephosphorylated by *PPP1R12B* (24). As shown in Table 2, expression of *MYLK* was down-regulated while *PPP1R12B* was up-regulated in the CCyR group, suggesting that dephosphorylated myosin light chain is associated with better response to imatinib treatment.

Genes Associated with Mitogenic Signaling. An important step in the *BCR-ABL*-induced transformation of hematopoietic cells is the abrogation of the requirement for growth factors for cell proliferation and survival (17). Activation of several mitogenic signaling pathways have been implicated in the *BCR-ABL*-induced transformation, including the Ras, mitogen-activated protein kinase, Janus-activated kinase-signal transducers and activators of transcription (STAT), phosphatidylinositol 3′-kinase, and Myc pathways (3). Several of the genes that we identified as differentiating between responders and nonresponders have also been associated with these mitogenic pathways. These include *CBL* (32, 33), *CDC6* (34), *CREB1* (35), *GRP58* (36), *JAK1* (37), *PAK1* (38), *PIK3R1/R3* (31), *PPP2R2A* (39), and *ZNF259* (40).

Genes Associated with Inhibition of Apoptosis. The third potential mechanism for *BCR-ABL*-induced transformation of hematopoietic cells is through the inhibition of apoptosis. Possible pathways associated with this antiapoptotic response include the phosphatidylinositol 3′-kinase/Akt signaling pathway (41, 42), the STAT family of proteins (43, 44), and the nuclear factor-κB signaling pathway (45, 46). Genes from our genomic profile that fit this category include *CBL* (47), *JAK1* (43, 44), *MYLK* (48, 49), *PAK1* (50), *PIK3R1/R3* (41, 42), *PPP2R2A* (51), *RAD9* (52), *REL* (53, 54), *TNFSF14* (55), and *TRAF5* (56, 57). Several of these genes such as *CBL*, *MYLK*, *PAK1*, *PIK3* and *JAK1* show overlapping functions, with some also being involved in the altered cell adhesion and mitogenic signaling associated with *BCR-ABL* transformation. Two other genes, *TRAF5* and *TNFSF14* (also known as *LIGHT*), have an intermediate gene in common, namely the lymphotoxin-β receptor, which is another member of the tumor necrosis factor family of cytokines that have been implicated in apoptosis (58).

CBL. One gene of particular interest is *CBL*, the product of the c-Cbl (Casitas B-lineage lymphoma) oncogene, which is one of the most prominent targets of *BCR-ABL* (59, 60). *CBL* has been shown to function as an E3 ubiquitin ligase, recruiting ubiquitin-conjugating enzymes, and directing ubiquitination and degradation of activated receptor tyrosine kinases, although whether it plays a direct role in the ubiquitination of *BCR-ABL* itself is not yet known (61). *CBL* has been shown to be a substrate for many other signaling molecules in addition to

BCR-ABL, including growth factor receptors, cytokine receptors, hormone receptors, integrins, and various antigen/immunoglobulin receptors (32). *CBL* is a M_r 120,000 cytoplasmic protein consisting of an NH_2-terminal tyrosine kinase binding domain and a COOH-terminal domain that contains a RING finger domain, a proline-rich region, and a leucine zipper region (32). Another member of the CBL family, *CBLB* shares 50% overall homology with *CBL* but retains 98% identity in the tyrosine kinase binding and RING domains (62). In contrast to *CBL*, the *CBLB* isoform has been demonstrated to be down-regulated after *BCR-ABL* activation (63).

In our study, we found that patients that responded to imatinib treatment (CCyR group) displayed increased expression of *CBL* compared with the nonresponder (NoCyR) group. A similar expression profiling study was recently performed by Kaneta *et al.* (64). Using data from a Phase II clinical trial in Japan, they identified a list of 79 genes that were differentially expressed between CML patients that responded to imatinib therapy and those that did not respond. From this list of 79 genes, they identified *CBLB* to be down-regulated in their responder group. Again, this is consistent with studies that have shown that *CBL* and *CBLB* are differentially expressed in *BCR-ABL* transformed cells and that the two isoforms act through different signal transduction pathways (63). No other genes were similar between the two gene lists. Although different microarray technologies and probe sets were used (Affymetrix oligonucleotide arrays *versus* custom cDNA arrays), there are several other significant differences between the two studies that could explain why there was such a large disparity between the resulting lists of genes. One of these is the difference in sample preparation, with the Japanese group using Ficoll-separated mononuclear cells for RNA isolation (64), while we used whole blood for our RNA isolation procedure. There were also significant differences between the two patient populations analyzed, including the stage of disease, previous treatments, and even potentially race. In our study, the primarily Caucasian patients all had newly diagnosed CML in chronic phase with $<15\%$ blasts in peripheral blood and bone marrow and were previously untreated with exception of hydroxyurea. However, in the Japanese study, 2 of the 18 samples used to develop their gene list came from patients already in blast crisis and the status of previous treatments is not reported (64). Furthermore, the Japanese group used major cytogenetic response ($\leq 35\%$ Ph^+ cells) to classify their responder group (64), whereas we used only patients with complete cytogenetic response (0% Ph^+ cells) as the responder group to develop our gene list. Lastly, the Japanese study was very much exploratory, using samples from only 18 patient samples to derive their gene list, as opposed to the 66 patients used in our study.

In conclusion, our analysis has identified a group of 55 genes that are differentially expressed between patients that achieved CCyR after treatment with imatinib and those that had minimal or NoCyR. Using an optimized genomic profile consisting of 31 of these genes, we were able to distinguish between individuals that achieved a MCyR (0–35% Ph^+ cells) and those that did not (NoMCyR; $>35\%$ Ph^+ cells) following a median of 13-months of imatinib treatment (Table 3). However, the resulting odds ratio of 19.9 (95% CI, 5.9–67.1), although statistically significant, is likely to be inflated because the patient samples used to derive the genomic profile were also included in the analysis. These results would need to be validated in an independent study before any clinical application, preferably using RNA collected from presorted Ph^+-leukemic cells. The genes that we identified in this pharmacogenomic study appear to be strongly related to the reported mechanisms of *BCR-ABL* induced transformation and therefore constitute a foundation for further research in the pathophysiology of CML.

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APPENDIX

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