Gene expression changes associated with progression and response in chronic myeloid leukemia

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Communicated by E. Donnall Thomas, Fred Hutchinson Cancer Research Center, Seattle, WA, December 13, 2005 (received for review June 24, 2005)

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease with distinct biological and clinical features. The biologic basis of the stereotypical progression from chronic phase through accelerated phase to blast crisis is poorly understood. We used DNA microarrays to compare gene expression in 91 cases of CML in chronic (42 cases), accelerated (17 cases), and blast phases (32 cases). Three thousand genes were found to be significantly (P < 10−10) associated with phase of disease. A comparison of the gene signatures of chronic, accelerated, and blast phases suggest that the progression of chronic phase CML to advanced phase (accelerated and blast crisis) CML is a two-step rather than a three-step process, with new gene expression changes occurring early in accelerated phase before the accumulation of increased numbers of leukemia blast cells. Especially noteworthy and potentially significant in the progression program were the deregulation of the WNT/β-catenin pathway, the decreased expression of Jun B and Fos, alternative kinase deregulation, such as Arg (Abl2), and an increased expression of PRAME. Studies of CML patients who relapsed after initially successful treatment with imatinib demonstrated a gene expression pattern closely related to advanced phase disease. These studies point to specific gene pathways that might be exploited for both prognostic indicators as well as new targets for therapy.

Conflict of interest statement: No conflicts declared.

Ffreely available online through the PNAS open access option.

Abbreviations: CML, chronic myeloid leukemia; CCR, complete cytogenetic remission.

Data deposition: The sequence reported in this paper has been deposited in the Gene Ontology (GO) database (accession no. GSE4170).

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statistical significance cutoff of $P < 10^{-11}$ (Fig. 1 and Table 4, which is published as supporting information on the PNAS web site). This set of genes identified in the progression from chronic to blast phase is referred to here as the “phase reporter” gene set.

We examined the proposed biologic function of the genes associated with CML phases by applying a biological annotation program based on the gene ontology (GO) and KEGG annotations. The major functional groups of genes in the phase reporter gene set are shown in Table 5, which is published as supporting information on the PNAS web site. The functional groups most highly correlated with disease phase (accelerated/blast phase relative to chronic phase) included increased expression of nuclear genes, mitochondrial genes, RNA-binding genes, and protein biosynthesis genes, reflecting the increased proliferation and metabolism of progressive disease. Advanced-phase CML, compared to chronic phase, exhibited a decreased expression of genes involved in structural integrity and adhesion, as well as decreases in expression of genes involved in inflammatory and immune response. In addition, several protooncogenes and tumor suppressor genes are differentially expressed in advanced phase CML, including N- and H-ras, FLT3, yes, AF1q, CBFB, WT1, ORALOV1, Bcl-2, and PTPN11.

CML Appears to Be a “Two-Step” Disease. We examined whether the progression of CML best fit a three-step model as often described...
in the literature (chronic phase → accelerated phase → blast crisis), or whether the expression data were best explained by a two-step model (chronic phase → advanced phase; i.e., accelerated or blast phase). We compared the gene expression of accelerated phase cases versus blast crisis cases (Fig. 4, which is published as supporting information on the PNAS web site). The correlation of expression level between accelerated and blast phase gene expression was strong ($r = 0.81$). In addition, the correlation of gene expression between accelerated phase and cases of accelerated phase defined by new cytogenetic abnormalities alone (i.e., chronic phase morphology but additional chromosomal changes besides the Ph) was also high ($r = 0.61$). These observations suggest that the difference of gene expression between accelerated and blast phase is a quantitative change in expression rather a qualitative change.

**Identification of “Progression-Specific” Gene Expression.** We next investigated how the phase reporter gene set was influenced by the gene expression signature of leukemia blasts and how these CML blasts compared to normal immature CD34+ cells. First, we made a direct comparison of the gene expression signature of six samples of normal CD34+ cells with CML blast blast samples containing $70\%$ blasts (Fig. 5, which is published as supporting information on the PNAS web site). We found that the gene expression pattern of CML blast cells was very similar to normal CD34+ cells. To uncover gene expression unique to CML progression (that is, associated with phase of disease, but not normal CD34+ expression), we used two approaches. First, we found genes differentially expressed between CML blast crisis and normal immature CD34+ cells (Fig. 6, which is published as supporting information on the PNAS web site, 368 genes with ANOVA $P < 0.01\%$). Secondly, we mathematically subtracted the normal CD34+ signature from each of the CML sample and examined the resulting effect on the phase reporter signals (Fig. 2A, 386 genes with $P < 10^{-8}$, and Table 6, which is published as supporting information on the PNAS web site). These two approaches identified 103 overlapping genes found in both gene sets (Table 7, which is published as supporting information on the PNAS web site, hypergeometric $P$ value: $1.3 \times 10^{-9}$). Table 8, which is published as supporting information on the PNAS web site (identified by the second approach), shows the “top ten” genes up- and down-regulated that are associated with progression, independent of normal CD34+ expression, based on log 10 ratio of expression compared to the chronic phase pool. These genes suggest a deregulation of genes of transcriptional regulation (GLI2, WT1, FO8, FO8B), signal transduction (SOCS2, Ras2, IL8), and apoptosis (GAS2). Also significantly associated with progression was PRAME (Preferentially Expressed Antigen of Melanoma), a gene with unknown function, but associated with myeloid malignancy (13, 14). The expression levels of these genes are shown in Fig. 2B.

Gene functions of the progression gene set were organized by GO and KEGG functional annotation (Table 1; for the full list, see Tables 9–11, which are published as supporting information on the PNAS web site). Of special relevance was aberrance in genes involved in the WNT/β-catenin signaling pathway, alterations in nucleosome genes, and genes involved in differentiation and apoptosis. In all, 16 genes associated with the β-catenin pathway were significantly deregulated in progression (e.g., cadherin, protocadherin, PRICKLE1, CASK1, PLCB1, FZD2, LRP6, SMAD3, MF1, etc.; see Fig. 6, which is published as supporting information on the PNAS web site).

Progression appears to be associated with expression changes of several cytoskeletal and adhesion molecules. Thus, there is an increase in CD47, Creb1, and ITGA5 expression, and a decrease in proto-cadherin, cadherin, actin, and β-actin. In addition, the abl family member abl2 (ARG) was significantly up-regulated in disease progression; by contrast, abl1, which is both expressed from the normal chromosome 9 and the t(9, 22) translocation, was not differentially expressed with progression.

### Table 1. Functional annotation of “progression” genes

<table>
<thead>
<tr>
<th>Keyword</th>
<th>Number (%)</th>
<th>Examples</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosome</td>
<td>36 (10)</td>
<td>GASOD45G, FANCG, XR2</td>
<td>$2 \times 10^{-7}$</td>
</tr>
<tr>
<td>Wnt signaling</td>
<td>12 (21)</td>
<td>ROK13A, Cadherin, MD11, Prickle 1, FZD2</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>Nucleosome</td>
<td>27 (14)</td>
<td>CEPRPA, CEBPE, FOXO3A</td>
<td>$3 \times 10^{-6}$</td>
</tr>
<tr>
<td>Sugar</td>
<td>10 (22)</td>
<td>BSZ1A, HIST1H2AE</td>
<td>$3 \times 10^{-11}$</td>
</tr>
<tr>
<td>Metabolism</td>
<td>23 (26)</td>
<td>G6PD, CEBPA, CEPE</td>
<td>$4 \times 10^{-6}$</td>
</tr>
<tr>
<td>Myeloid differentiation</td>
<td>22 (22)</td>
<td>FOXO3A, MCL1</td>
<td>$2 \times 10^{-7}$</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>8 (10)</td>
<td>GAD405G, BCL2, FOXO3A, MCL1</td>
<td>$2 \times 10^{-7}$</td>
</tr>
<tr>
<td>DNA damage response</td>
<td>22 (22)</td>
<td>GASOD45G, FANCG, XR2</td>
<td>$2 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

*Bold functions are up-regulated in progression; italic functions are down-regulated.

*The number of genes present on the array with the specific keyword; in parentheses are the percentage of significant genes found differentially expressed among the keyword class.

**MZF- and EF1α-Controlled Genes Are Aberrantly Expressed in Progression.** Gene expression networks defined by specific promoter regulation were analyzed in the progression and phase reporter gene sets (Table 2). The most significantly network of genes showing aberrant control in progression with those that contained a MZF promoter or a EF1α promoter sequence ($P < 10^{-10}$ and $<10^{-11}$, respectively; Figs. 8 and 9, which are published as supporting information on the PNAS web site). Also significantly associated with progression were genes bearing SPI-B, Yin Yang, and AHRR-ARNT promoter sequences (all with $P$ values $<10^{-9}$).

**Gene Expression Profiles of Cases Treated with Imatinib.** We studied the gene expression of CML patients for whom imatinib therapy was ineffective. We examined 15 cases of CML treated with imatinib, including nine patients who initially achieved a CCR on imatinib therapy, but then relapsed back into an apparent chronic phase by morphologic examination; three cases who had achieved a complete hematologic but no cytogenetic response; and three late chronic phase patients. All but one of these chronic phase patients who relapsed after a CCR had a point mutation in abl1, presumably abrogating imatinib activity (Table 3). In addition, only one of the relapsing cases had additional cytogenetic lesions.

Fig. 3 shows the expression pattern of these 15 CML cases. We found that the cases that appeared in chronic phase after relapsing after an achievement of CCR had expression patterns similar to the boundaries of “most chronic” cases (bottom of the heat map) and “most advanced” gene expression (top of the heat map) for all the cases that appeared in chronic phase after relapsing after an achievement of CCR had expression patterns similar to advanced disease. This can be demonstrated by segregating all CML cases by the correlation of gene expression signature between the boundaries of “most chronic” cases (bottom of the heat map) and “most advanced” gene expression (top of the heat map) for all 3,000 genes in the phase reporter gene set. The majority of the poor response patients have gene expression profiles more consistent with advanced disease rather than chronic phase. Both cases with T315I mutations, which have been shown to have especially poor prognosis (8, 15), have expression signatures more similar to advanced disease than chronic phase (red arrows).
However, there were gene expression features that were unique to the imatinib relapers. Examples of genes unique to imatinib resistance failure are those of serine threonine kinases (CTRL, MAP21K14, CLK3), mitogen-activated protein kinase (MKNK2), the tyrosine kinase oncogene FYN, and the drug exporter ABCC3. Fig. 10, which is published as supporting information on the PNAS web site, compares relapsed imatinib cases against blast crisis cases, and several areas (boxed) indicate genes that are expressed in reverse direction in these two states (Tables 12 and 13, which are published as supporting information on the PNAS web site, lists genes in the first box. See Table 12 for the rest of the imatinib resistance genes).

Discussion

The biology and treatment of CML is dictated by the phase of disease, as the efficacy of all therapies (transplantation, IFN, or imatinib) are most successful when used during the chronic phase, in comparison to accelerated phase and blast crisis. Understanding the biology of progression may provide clinical diagnostic markers of progression and offer insights into new strategies for treatment. The data presented here suggest that the progression of chronic phase CML to advanced phase CML is a two-step process, with progression associated a block of differentiation and apoptosis, a shift toward turning on expression of genes involved in the nucleosome, with alterations in cell adhesion, and activation of alternative signaling pathways. In addition, it appears that relapse after initial successful treatment with imatinib may be associated with gene expression patterns similar to advanced phase CML, suggesting that the process of progression persists in a subpopulation of CML cells during therapy.

In our analysis, we first used unsorted CML samples to develop gene signatures associated with the phase of disease. This data set gives a more global picture of the transformation of disease, and is important because any diagnostic assay for progression would optimally be performed on whole blood or marrow, rather than selected cell subpopulations. To control for any gene signatures that might be common to an immature blast phenotype, we subtracted a normal CD34+ signature from the CML samples. This left us with a progression gene set that should be highly enriched in genes associated with CML progression, but not reflecting normal CD34+ cell gene expression. This gene set may be more indicative of the biology of CML progression, and give more insight to pathways that might be exploited by new therapeutic agents. Moreover, the progression gene set may contain genes able to distinguish early evidence of progression in immunophenotypically similar immature cells.

The demonstration that the gene expression pattern between accelerated and blast phases are very similar suggests that the crucial steps in progression are at the transition of chronic to accelerated phase, before obvious morphologic, cytogenetic, or clinical evidence of progression. This has clinical implications, because these patients might benefit from more aggressive therapy. In addition, the observation that gene signatures of blast crisis can be seen in accelerated phase patients by cytogenetic criteria only and blast crisis cases in remission (both of which have similarly low blast counts as patients with chronic phase disease), demonstrates two important points. First, it points out the difficulty of correlating

Table 3. Characteristics of cases treated with imatinib

<table>
<thead>
<tr>
<th>Code</th>
<th>Phase of disease at initiation of imatinib</th>
<th>Phase of disease at time of sample</th>
<th>Cytogenetics</th>
<th>Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph + ; NEW:t(1;12) in all cells</td>
<td>M244V</td>
</tr>
<tr>
<td>4</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>T315I</td>
</tr>
<tr>
<td>5</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>80% Ph +</td>
<td>F359V</td>
</tr>
<tr>
<td>6</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>M351T, F317L</td>
</tr>
<tr>
<td>8</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>M351T</td>
</tr>
<tr>
<td>10</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>E255K</td>
</tr>
<tr>
<td>11</td>
<td>Chronic post-allo BMT</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>T315I</td>
</tr>
<tr>
<td>12</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>L248V</td>
</tr>
<tr>
<td>14</td>
<td>Chronic</td>
<td>Relapsed chronic</td>
<td>100% Ph +</td>
<td>M351T, H396R</td>
</tr>
</tbody>
</table>

BMT, bone marrow transplantation.

Fig. 3. Gene expression in patients with resistance to imatinib failure cases. Cases were ranked and sorted by the correlation of summed gene expression, with cases representing the most "chronic phase-like" and most "blast crisis-like" forming the boundaries of gene expression patterns. Cases that had a poor response to imatinib are designated by a blue dot in the "IM-cases" box to the right of the heat map. The red arrows point to the two cases that had the T315I Abl mutation.
morphology with the biology of the disease. Secondly, the penetration of a progression gene expression signature into a “chronic phase” appearing bone marrow suggests that progression is not merely an absolute block of differentiation, but that abnormal gene expression signals persist in normal appearing differentiated cells. This is critically important because it provides the basis for testing for progression genes in unsorted bone marrow samples from “chronic phase” patients.

Bcr-Abl has a wholesale range of biological activities. Critical in the transformation process is the activation of the Ras/mitogen-activated protein kinase (MAPK) pathways, which has broad effects on changes in cell adhesion (through Rho), proliferation (MAPK pathway), and apoptosis (through Akt) (16, 17). Imatinib’s efficacy results from a blockade of these effects. Imatinib is less successful for advanced-phase disease; this may be because these leukemia have become less dependent on the pathways that imatinib blocks. Thus, we found that the MAPK pathway was relatively underexpressed in advanced disease compared to chronic phase, but other signaling pathways, including those involving cytokines (IL3RA, SOCS2), alternative ras pathways (Rras2), and those involved in cell adhesion (WNT/β-catenin) are activated. The activation of these pathways may allow progression even in the face of therapeutic blockade of Bcr-Abl activated pathways. In addition, abl2 (Abl related gene, or ARG) is also up-regulated in progression. The transformation may disrupt normal differentiation, promoting the progression of a progression gene expression signature into a “chronic phase” appearing bone marrow suggests that progression is not merely an absolute block of differentiation, but that abnormal gene expression signals persist in normal appearing differentiated cells.

It has previously been demonstrated that the probability of developing a point mutation depends largely on the time from diagnosis to initiation of therapy (8). This finding implies that the genetic mechanisms that lead to point mutations are relentless, and therefore the treatment of “late” chronic phase patients (i.e., > 1 year from diagnosis) may be undermined by genetic changes that have already occurred. Branford et al. (8) demonstrated that patients who developed P-loop point mutations had a very poor outcome, with approximately half dying within a year of relapse. These observations are in keeping with our demonstration that many imatinib failures have gene expression changes similar to advanced disease, despite their benign pathological appearance. The predictive power of marker responses to imatinib therapy for signs of progression to advanced disease.

Several of the genes found in the progression set might serve as early markers of progression in diagnostic assays, and may serve as therapeutic targets, as well. For example, PRAME was originally identified as a tumor antigen recognized by cytotoxic T cells against a melanoma surface antigen (13, 39). Like other antigens MAGE, BAGE, or GAGE, PRAME is expressed in some solid tumors; unlike these other antigens, however, it has been found to be overexpressed in >25% of leukemia, and has been found to be induced by Bcr-Abl in CML cell lines (14, 40). Indeed, PRAME overexpression has been described as one of the few features that characterize the transient myeloproliferative syndrome of Down’s syndrome from the progressive acute megakaryoblastic leukemia found in that disorder (41). Very recently, insights into the function of PRAME make it an attractive target for potential therapy. PRAME is a nuclear protein, which seems to act as a dominant repressor of retinoic acid receptor (RAR) signaling (42). In cell line models, the forced overexpression of PRAME blocked RAR-mediated differentiation, growth arrest, and apoptosis. Thus, therapeutic maneuvers to block the PRAME/RAR effect may allow for differentiation and cell death, and be particularly effective in attacking advanced phase CML.

In comparison to other types of leukemia, there have been few papers exploring the use of microarrays on the biology of CML (43–45). It is difficult to make a direct comparison of these studies and ours, given the different types of samples obtained (some unsorted, some AC133+ or CD34+ selected), the difference in the array platforms, etc. Nonetheless, in general, the functional changes of progression (changes in differentiation, apoptosis, cell adhesion, and inflammatory response) remained as common themes across these studies. Moreover, in keeping with a recent publication that found that the high expression of elastase (ELA2) was associated with a long period of indolent disease (45), in our data, ELA2-decreased expression is associated with progression.

These findings have the potential to influence therapy of CML. Patients who present with gene expression patterns suggestive of advanced phase disease might benefit by moving to more aggressive (i.e., transplantation) or other investigational therapies. Moreover, PCR assays of individual genes (or small sets of genes) may be used to monitor patients early in the course of imatinib therapy for signs of progression to advanced disease. Lastly, new targets to interfere with progression, or reverse imatinib resistance, may be exploited.
Materials and Methods

Patient Samples. All samples were obtained under the auspices of institutional review board approve protocols. Samples came from the Fred Hutchinson Cancer Research Center, the Southwest Oncology Group (SWOG) Myeloid Repository, the University of Oregon Health Sciences Center, the University of California, Los Angeles, or the University of Chicago. RNA extraction was either performed immediately, or in the case of samples stored in a liquid nitrogen repository, after thawing. All RNA samples were quality tested by analysis of ribosomal RNA peaks using an ABI Bioalyzer. The definition of chronic, accelerated and blast crisis was based on the criteria of Sokal et al. and the International Bone Marrow Transplant Registry (46, 47). Thus, chronic phase was defined as <10% blasts, the accelerated phase was defined as 10–30% blasts or <10% blasts with clonal evolution, and blast crisis was defined as >30% blasts.

Amplification, Labeling, and Hybridization. The procedures of RNA amplification, labeling, and the hybridization to arrays, as well as the specifics of the array platforms, has been published (48). Analytic Methods and Results. Each individual sample was hybridized to a pool of chronic phase samples. The log10 (Ratio) of intensity of individual samples to the pool were used for the subsequent analysis. Before selecting features by ANOVA, 25,000 genes were first screened for evidence of differential regulation by requiring P value of regulation <1% in more than three experiments, where P value of regulation is based on the platform error model. Features differentiating progression stages were selected by ANOVA test.

Functional Annotation of Gene Lists. Genes represented on the microarray were annotated by assignment to GO Biological Process or Molecular Function categories (www.ebi.ac.uk/GOA), or to KEGG pathways (www.genome.jp/kegg/ pathway.html). Gene lists (input sets) were queried for enrichment of members of specific functional classes or pathways relative to the background frequency. The significance (P value) of enrichment was computed by using the hypergeometric probability distribution. Reported in each case are the number of genes in the input set (input gene count), number of genes in a particular category or pathway in the input set (overlap gene count), and number of genes in a particular category among all genes present on the array (set gene count). The total number of unique genes on the array is 24,132.

Methods to Common Promote Site Analysis. The common promoter sites were based on the predictions derived from the database (oPOSSUM) by Wasserman et al. (www.cisreg.ca). The hypergeometric P value for enrichment of a particular binding site was computed by comparing the number of genes with the binding site from a signature gene set to that from a background set (i.e., all genes represented on the microarray).

Controls. We compared gene expression signatures from the sites contributing samples to confirm that there were no site-specific confounding the analysis.

Because some samples of blast crisis came from peripheral blood rather than bone marrow, we compared three samples in which simultaneous samples were available from bone marrow and peripheral blood. Gene expression was extremely well correlated (r = 0.97–0.99; Fig. 11, which is published as supporting information on the PNAS web site).

This work was supported in part by National Institutes of Health Grants CA-18029 and CA-85053 (to J.P.R.) and the Howard Hughes Medical Institute (B.D. and C.S.).

Supplemental Figure 1. Comparison of gene expression of accelerated and blast phase CML

![Scatter plot showing gene expression comparison with correlation = 0.81](image-url)
Supplemental Figure 2. Comparison of blast crisis and normal CD34+ gene expression.
Supplemental Figure 3. Expression differences between CML blasts and normal CD34+ cells

\filer1\informatics\analysis\CML\Sep03\CD_BC_reporter_121603.txt
Supplemental Figure 4. WNT/B-catenin pathway involvement in CML
Supplemental Figure 5. Gene expression of genes with MZF promoter sequences
Supplemental Figure 6. Gene expression of genes with delta EF1 promoter sequences
Supplemental Figure 7. Gene expression differences between imatinib failures and CML blast crisis.
Supplemental Figure 8. Correlation of bone marrow and peripheral blood expression

- Bone Marrow: $r = 0.96$
- Peripheral Blood: $r = 0.97$, $r = 0.99$