

## Differential Gene Expression Patterns and Interaction Networks in *BCR-ABL*-Positive and -Negative Adult Acute Lymphoblastic Leukemias

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### A B S T R A C T

#### Purpose

To identify gene expression patterns and interaction networks related to *BCR-ABL* status and clinical outcome in adults with acute lymphoblastic leukemia (ALL).

#### Patients and Methods

DNA microarrays were used to profile a set of 54 adult ALL specimens from the Medical Research Council UKALL XII/Eastern Cooperative Oncology Group E2993 trial (21 p185<sup>BCR-ABL</sup>-positive, 16 p210<sup>BCR-ABL</sup>-positive and 17 *BCR-ABL*-negative specimens).

#### Results

Using supervised and unsupervised analysis tools, we detected significant transcriptomic changes in *BCR-ABL*-positive versus -negative specimens, and assessed their validity in an independent cohort of 128 adult ALL specimens. This set of 271 differentially expressed genes (including *GAB1*, *CIITA*, *XBP1*, *CD83*, *SERPINB9*, *PTP4A3*, *NOV*, *LOX*, *CTNND1*, *BAALC*, and *RAB21*) is enriched for genes involved in cell death, cellular growth and proliferation, and hematologic system development and function. Network analysis demonstrated complex interaction patterns of these genes, and identified *FYN* and *IL15* as the hubs of the top-scoring network. Within the *BCR-ABL*-positive subgroups, we identified genes overexpressed (*PILRB*, *STS-1*, *SPRY1*) or underexpressed (*TSPAN16*, *ADAMTSL4*) in p185<sup>BCR-ABL</sup>-positive ALL relative to p210<sup>BCR-ABL</sup>-positive ALL. Finally, we constructed a gene expression- and interaction-based outcome predictor consisting of 27 genes (including *GRB2*, *GAB1*, *GLI1*, *IRS1*, *RUNX2*, and *SPP1*), which correlated with overall survival in *BCR-ABL*-positive adult ALL ( $P = .0001$ ), independent of age ( $P = .25$ ) and WBC count at presentation ( $P = .003$ ).

#### Conclusion

We identified prominent molecular features of *BCR-ABL*-positive adult ALL, which may be useful for developing novel therapeutic targets and prognostic markers in this disease.

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### INTRODUCTION

A better understanding of the biology of acute lymphoblastic leukemia (ALL) and advances in ALL therapy have led to long-term survival rates of approximately 80% in children. However, only 30% to 40% of adults with this disease achieve long-term disease-free survival.<sup>1</sup> The relatively poor outcome in adult ALL has been explained by an increased frequency of high-risk molecular subtypes with more aggressive clinical behavior and greater drug resistance, poorer tolerance and compliance with treatment, and less effective treatment regimens compared with childhood ALL.<sup>2</sup> A particularly poor prognosis is associated with the t(9;22) translocation and the presence of the *BCR-ABL* fusion transcript,

which increases in frequency with age, from 2% to 5% in children to 20% to 40% in adults.<sup>3-5</sup> This molecular alteration exists in several forms and encodes multiple oncogenic products. Although the majority of chronic myelogenous leukemia (CML) patients express p210<sup>BCR-ABL</sup>, both p185<sup>BCR-ABL</sup> and p210<sup>BCR-ABL</sup> can be found in ALL patients. These two isoforms arise from distinct breakpoints in *BCR* on chromosome 22 and *ABL1* on chromosome 9, resulting in the fusion of exon 1 of *BCR* and exon 2 of *ABL1*, and fusion of exons 13/14 of *BCR* and exon 2 of *ABL1*, respectively.<sup>6</sup>

Several in vitro studies have demonstrated higher tyrosine kinase activity, increased transforming potential, and distinct downstream targets of p185<sup>BCR-ABL</sup> compared with p210<sup>BCR-ABL</sup>,

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suggesting that these two isoforms have different biologic and possibly clinical properties.<sup>7</sup> The clinical implications of these findings are controversial, and although a majority of clinical studies failed to demonstrate significant differences of the two isoforms with regard to clinical outcome in adults with ALL,<sup>4,8,9</sup> some recent reports indicate that these two isoforms could be associated with different clinical phenotypes of adult ALL.<sup>10</sup>

In this report, we performed a comprehensive analysis of the gene expression profiles in a set of 37 *BCR-ABL*-positive (21 and 16 of which expressed p185<sup>BCR-ABL</sup> and p210<sup>BCR-ABL</sup>, respectively) and 17 *BCR-ABL*-negative adult ALL specimens from the Medical Research Council (MRC) UKALL XII/Eastern Cooperative Oncology Group (ECOG) E2993 trial.<sup>11</sup> First, we aimed to determine gene expression signatures and molecular alterations potentially involved in the complex mechanisms of *BCR-ABL*-mediated neoplastic transformation, and which could explain aggressive behavior of *BCR-ABL*-positive adult ALL. Second, we evaluated differential gene expression in the two major isoforms of *BCR-ABL*, to determine whether these two isoforms are associated with distinct transcriptional changes.

## PATIENTS AND METHODS

### Patient Samples

Total RNA samples were isolated from cryopreserved pretreatment leukemia cells from 54 adult ALL patients entered onto the MRC UKALL XII/ECOG E2993 trial, as described previously.<sup>12</sup> Written informed consent was obtained from all patients, and the study was approved by the Institutional Review Board of Stanford University (Stanford, CA). All patients were immunophenotypically classified as early pre-B ALL. *BCR-ABL*-positive patients were selected based on the absence of additional cytogenetic abnormalities. *BCR-ABL*-negative patients had a normal diploid karyotype (at least 20 normal metaphases) and were negative for the most common ALL transcripts, *MLL/AF4*, *E2A/PBX1*, and *TEL/AML1*, as well as *FLT3*-gene internal tandem duplications.

### Microarray Procedures

Gene expression profiling was performed using Stanford human cDNA arrays containing 41,421 cDNA elements, corresponding to 24,472 different UniGene cluster IDs. Hybridized array signals were processed using the 3DNA Array 900 Expression Array Detection Kit according to the manufacturer's instructions (Genisphere, Hatfield, PA). This indirect, two-step labeling procedure uses dendrimer-based signal amplification<sup>13</sup> and avoids biases associated with commonly used sample amplification protocols.<sup>14</sup> Details of these procedures are presented in the Appendix (online only).

### Data Filtering and Transformation

Primary data were uploaded to the National Center for Biotechnology Information Gene Expression Omnibus (Series record: GSE5314), and to the Stanford Microarray Database,<sup>15</sup> where all subsequent low-level analysis procedures were performed. Data were first log-transformed and normalized using local, intensity-dependent normalization.<sup>16</sup> Nonflagged elements with signal-to-background ratios of at least 2.0 in either channel were selected based on their presence in at least 80% of samples. Finally, we selected only the clones with at least three-fold differential expression, in at least three samples, compared with their mean expression level across all the samples, resulting in 10,485 clones used for subsequent comparisons.

### Unsupervised and Supervised Data Analysis

Unsupervised hierarchical clustering was performed in Cluster and visualized in Treeview.<sup>17</sup> Supervised data analysis was performed in R (build 2.2.1; <http://www.r-project.org>) using a nonparametric *t* test as described previously.<sup>18</sup> A two-sample Welch *t* statistic was computed for each gene in each comparison, and the statistical significance of the differential expression was

estimated by 1,000 permutations of the class labels. The false discovery rate was estimated using *q*-value computation.<sup>19</sup> Nearest centroid classification was performed using the freeware *pamr* package.<sup>20</sup>

### Survival Analysis

Kaplan-Meier survival analysis, log-rank tests, and Cox proportional hazards analysis were performed in R. Overall survival (OS) was measured from the on-study date until date of death regardless of cause, with censoring of patients alive at last follow-up. Comparisons of baseline clinical variables were made using a Fisher's exact test (categorical variables) or Wilcoxon test (continuous variables).

### Interaction Networks and Functional Analysis

Gene ontology and gene interaction analyses were executed using Ingenuity Pathways Analysis tools 3.0 (<http://www.ingenuity.com>). The gene lists containing Entrez GeneIDs as clone identifiers, as well as fold-change or Wald score values from corresponding supervised analyses, were mapped to their corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). These so-called focus genes were then used in the network generation algorithm, based on the curated list of molecular interactions in IPKB. Significance for the enrichment of the genes in a network with particular biologic functions was determined by the right-tailed Fisher's exact test, using a list of all the genes on the array as a reference set. Details of these procedures are presented in the Appendix.

### Cross-Platform Validation

Affymetrix gene expression data for 12,625 probe-sets in 128 adult ALL samples were obtained from <http://www.bioconductor.org/docs/papers/2003/Chiaretti/chiaretti2/index.html>. Primary data were annotated and normalized as described previously.<sup>21</sup> The log<sub>2</sub>-transformed and mean-centered file was used in all subsequent cross-platform analyses.

### Quantitative Reverse Transcription Polymerase Chain Reaction

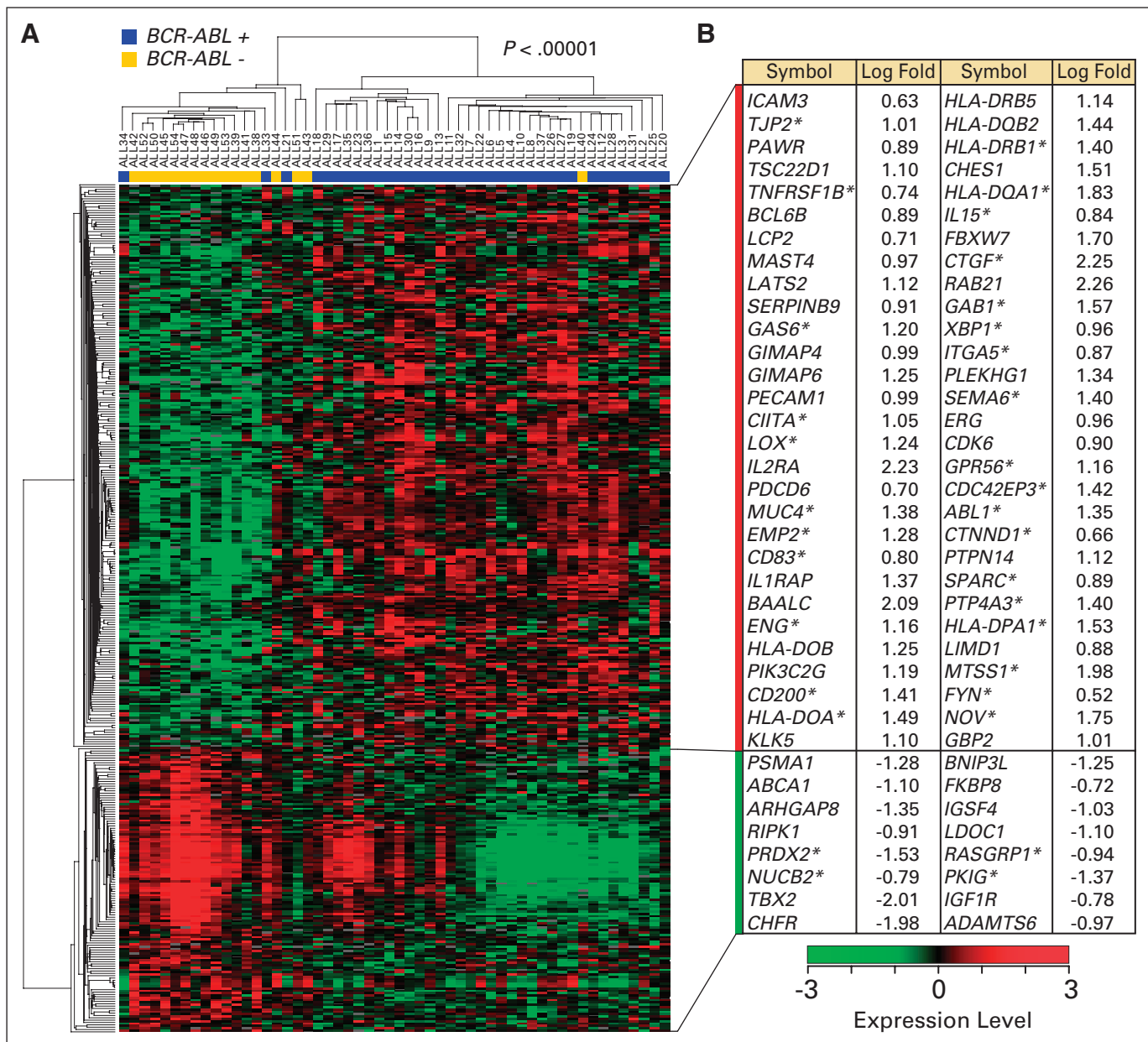
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the ABI Prism 7900HT Sequence Detection System using SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Details of the qRT-PCR procedures are presented in the Appendix.

## RESULTS

### *BCR-ABL*-Associated Gene Expression Signatures and Interaction Networks

We performed microarray-based gene expression analysis of a set of 37 *BCR-ABL*-positive and 17 *BCR-ABL*-negative ALL specimens. In supervised analysis, we identified 363 clones as differentially expressed in *BCR-ABL*-positive ALL compared with *BCR-ABL*-negative ALL (241 overexpressed and 122 underexpressed). Unsupervised two-way clustering of these clones distinguished the two subclasses of ALL with an overall accuracy of 93% (Fig 1A). They correspond to 271 unique Entrez GeneIDs, and are enriched for three highly relevant functions: cellular growth and proliferation (57 genes; *P* = .004 to 0.044), cell death (49 genes; *P* = .0007 to 0.049), and hematologic system development and function (40 genes; *P* = .00004 to 0.049). Selected genes from this set and their corresponding log fold-change values are displayed in Figure 1B.

Interaction patterns of these differentially expressed genes were examined in the context of the curated list of published molecular interactions in IPKB. To harness the power of this database maximally to reveal underlying biologic networks, we analyzed data from a total of 617 clones in the network-generation algorithm. These included 363 clones significant at *q* less than 0.05 and 254 clones with *q* more than or equal to 0.05 and less than 0.10. The top scoring network from

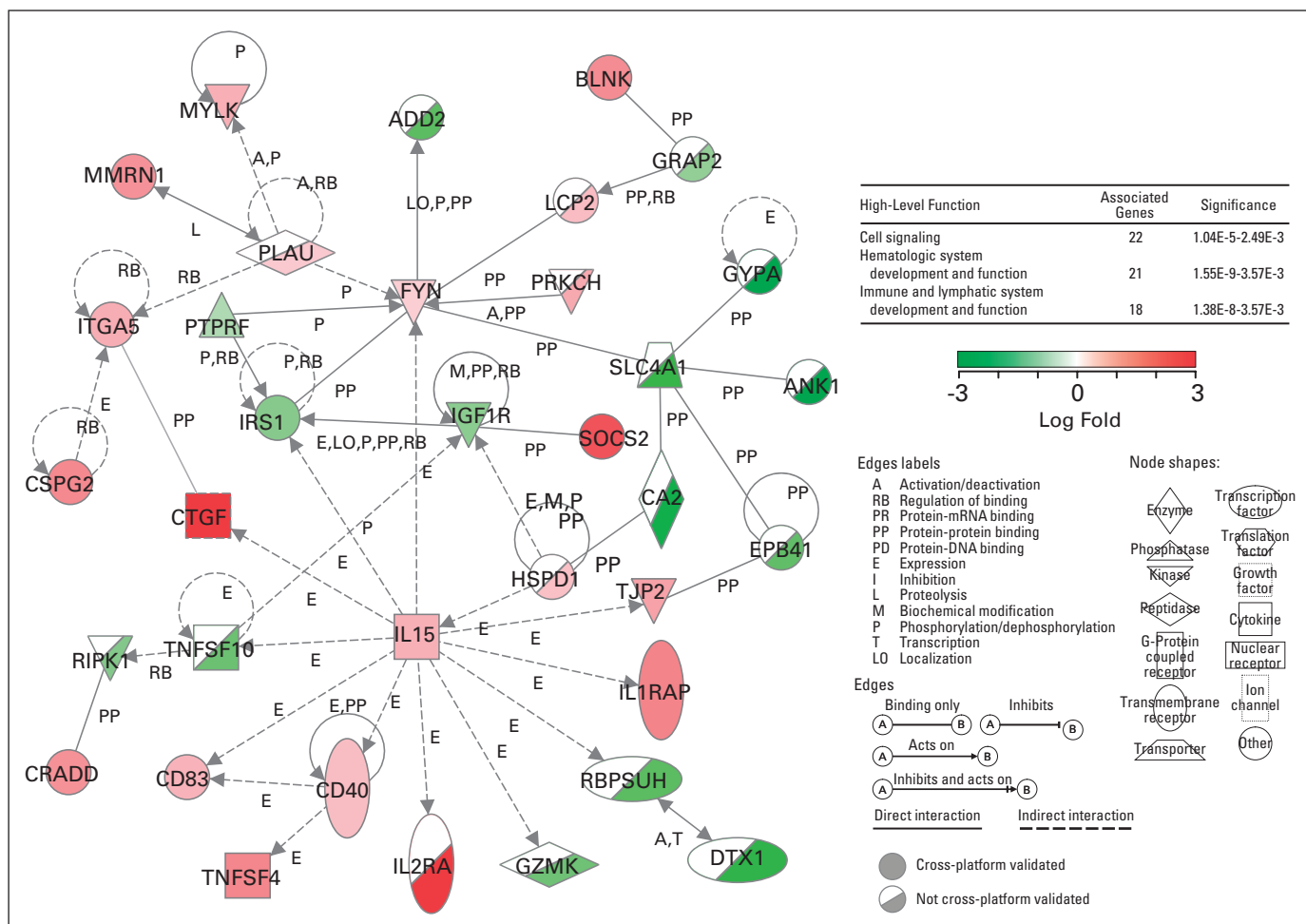


**Fig 1.** (A) Unsupervised hierarchical clustering of the 363 clones with significant differential expression in BCR-ABL-positive versus BCR-ABL-negative acute lymphoblastic leukemia; (B) names and log fold-change values of selected genes; (\*) cross-platform validated genes.

this analysis is displayed in Figure 2. Analysis of the node connectivity of its elements identifies cytokine *IL15* and the Src-family tyrosine kinase *FYN* as the only nodes with more than five edges, indicating that these two genes have hub-like properties, potentially having the highest biologic importance. In addition to *IL15* and *FYN*, the majority of other genes in this interaction network are implicated directly in hematologic system development and function (*IL2RA*, *IL1RAP*, *TNFSF4*, *CD40*, *CD83*, *BLNK*, *LCP*, *SOCS2*, and *HSPD1*; all overexpressed in BCR-ABL-positive ALL).

To assess the validity of the detected BCR-ABL gene expression signature, we performed qRT-PCR validation of the selected differentially expressed genes (Fig 3A), as well as cross-platform validation of the whole set of genes using previously published gene expression

profiles of 128 adult ALL samples. Hierarchical clustering of these samples with the genes found to be differentially expressed in our data set was performed using 251 probe sets corresponding to 168 unique Entrez GeneIDs that overlapped with 271 unique Entrez GeneIDs represented by the 363 clones in our BCR-ABL gene expression signature. This analysis separates the initial sample population into two main clusters, one of which contains the majority of BCR-ABL-positive samples (Fig 3B). Direct comparison of these 168 genes by a nonparametric *t* test confirms differential expression of 70 genes (42%). Similarly, direct comparison of overlapping genes at *q* less than 0.10 confirms differential expression of 110 genes (40%). A total of 18 nodes (51%) in Figure 2, including both hubs (*FYN* and *IL15*), are also validated by this cross-platform comparison.



**Fig 2.** The top-scoring network of interactions among the differentially expressed genes in *BCR-ABL*-positive versus *BCR-ABL*-negative acute lymphoblastic leukemia. Node colors and shapes correspond to fold-change values and functional classes of the gene products, respectively. The table lists high-level functions with statistically significant enrichment.

### Differential Gene Expression in *p185<sup>BCR-ABL</sup>*-Positive and *p210<sup>BCR-ABL</sup>*-Positive ALL

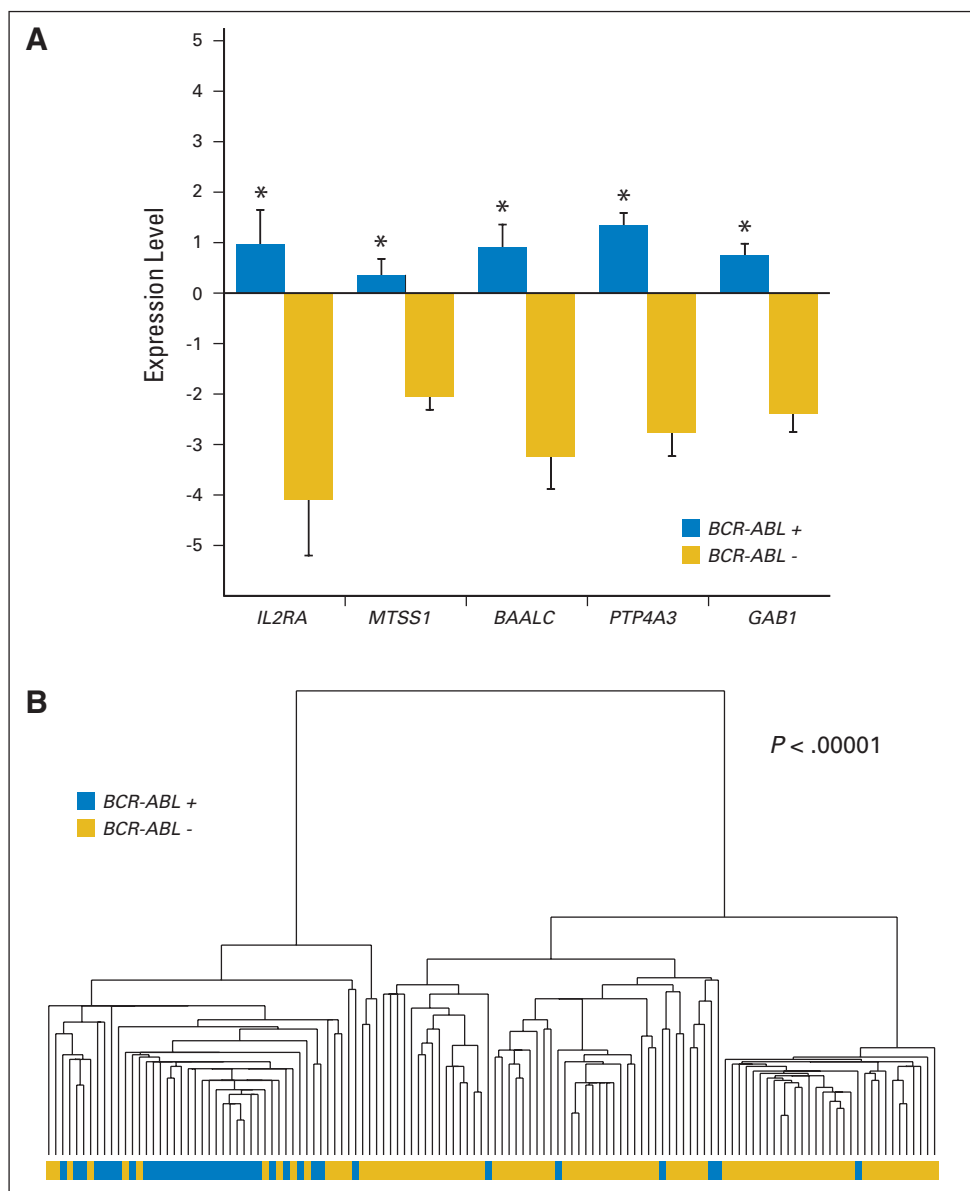
Gene expression profiles of 37 *BCR-ABL*-positive adult ALL samples in this data set (21 and 16 of which expressed *p185<sup>BCR-ABL</sup>* and *p210<sup>BCR-ABL</sup>*, respectively) were also used to assess genes and/or signaling pathways differentially regulated by the *BCR-ABL* isoforms. Supervised analysis of these samples demonstrated differential gene expression of 14 clones. These include *Cbl*-interacting protein *Sts-1*, *PILRB*, *RCBTB2*, *GNAI1*, and *SPRY1*, all of which are overexpressed in *p185<sup>BCR-ABL</sup>*-positive ALL, as well as *TSPAN16* and *ADAMTSL4*, which are overexpressed in *p210<sup>BCR-ABL</sup>*-positive ALL. Hierarchical clustering of this set of genes clearly separates these two sample groups (Fig 4A). We have also estimated a prediction accuracy of this set of genes using nearest-centroid classification. In a 10-fold cross-validation, we obtained a misclassification rate of 5% (Fig 4B).

Given that the *p210<sup>BCR-ABL</sup>* isoform has a Dbl homology domain with guanine-nucleotide exchange factor activity for Rho, Rac, and Cdc42 guanosine triphosphatases (GTPases),<sup>22</sup> we queried IPKB for all the Rho family GTPases and their known interaction partners and repeated our supervised analysis. However, of 106 clones in this analysis, none demonstrated statistically significant differential expression across the two groups of *BCR-ABL*-positive samples.

### Survival Analysis in *BCR-ABL*-Positive ALL

Kaplan-Meier analysis of the *p185<sup>BCR-ABL</sup>*-positive versus *p210<sup>BCR-ABL</sup>*-positive ALL patients did not reveal a statistically significant difference in OS (Fig 4C). They also were not different in their age, WBC count at diagnosis (WBC), complete remission rate, or expression of myeloid markers CD13/CD33 ( $P > .05$ ).

We then constructed a gene expression profiling-based predictor that does correlate with survival in our set of *BCR-ABL*-positive specimens. As a first step, we performed Cox proportional hazards analysis for each gene in the data set and used Wald scores from this analysis as a measure of their correlation with OS. In the second step, we performed molecular interaction analysis of the 524 clones with the top 5% of Wald scores using Ingenuity Pathways Analysis. The top scoring network from this analysis included 27 interacting genes (Fig 5A). Among these are *GAB1*, *CD34*, *GNAQ*, *RASGRP1*, *NRG3*, and *SELL*, all of which were correlated positively with survival, as well as *GRB2*, *RAPGEF1*, *MRAS*, *GRAP2*, *IRS1*, and *RUNX2*, which are correlated negatively with survival. The most over-represented function in this network is cellular differentiation (14 genes;  $P = 3.17 \times 10^{-6}$ ). Finally, the first principal component of the gene expression profile of these 27 genes was computed and used as a risk score for each sample. Importantly, our gene selection was not based solely on the maximal



**Fig 3.** (A) Quantitative reverse transcription polymerase chain reaction analysis of selected genes in 54 samples ( $n = 54$ ; mean and SEM of *HPRT*-normalized and median-centered levels; (\*)  $P < .05$ ). (B) Unsupervised hierarchical clustering of an independent set of 128 adult ALL specimens using 168 genes from our BCR-ABL gene expression signature represented in the validation set.

correlation with survival in the training set, but also relied on the known molecular interaction patterns of the candidate genes.

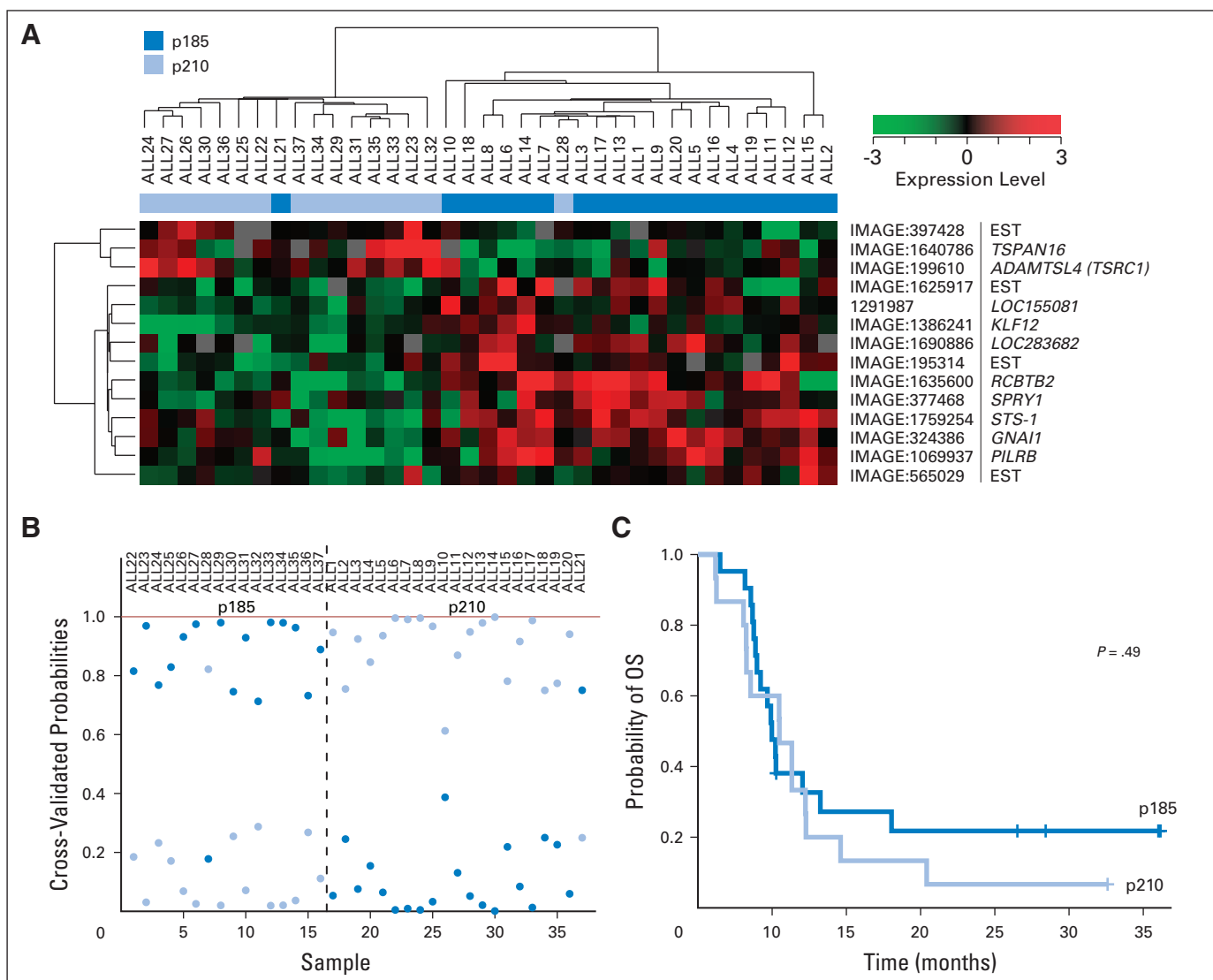
A statistically significant difference in OS ( $P = .008$ ; Fig 5B) was observed in samples with positive and negative first principal component (FPC) scores. The median OS in the group of samples with FPC less than zero was 14.5 months, whereas in the group of samples with FPC more than zero, the median OS was 7.8 months. To assess the interdependence of FPC score and other possible risk factors in BCR-ABL-positive ALL, we performed multivariate Cox proportional hazards analysis using the FPC score, patient age, and WBC as covariates in the model. According to this analysis, FPC score and  $\log(\text{WBC})$  were identified as independent predictors of OS in this data set (FPC score,  $P = .0001$ ;  $\log(\text{WBC})$ ,  $P = .003$ ; age,  $P = .25$ ).

## DISCUSSION

Expression profiles associated with various subtypes of ALL have been studied extensively by microarrays, primarily focused on childhood

ALL.<sup>21,23-30</sup> Although genes suitable for classification of childhood ALL are capable of distinguishing the respective adult ALL subentities, gene expression profiles have demonstrated significant differences between childhood and adult BCR-ABL-positive ALL.<sup>27,29</sup>

Our study identified 271 differentially expressed genes in BCR-ABL-positive versus BCR-ABL-negative adult ALL. Their functional analysis suggests that a substantial portion of these genes could be contributing factors in BCR-ABL-driven leukemogenesis. Their potential biologic relevance is supported further by the qRT-PCR validation, and especially by the differential patterns found in an independent set of 128 adult ALL samples. A relatively large overlap of 40% among assessable differentially expressed genes in the two unrelated studies deserves particular attention. Moreover, an evaluation of our BCR-ABL-positive ALL gene expression signature in a recently published CML study<sup>31</sup> revealed considerable overlap in the gene expression patterns of BCR-ABL-positive ALL and CML blast crisis. Using the subset of genes found on both microarray platforms, our



**Fig 4.** (A) Unsupervised hierarchical clustering of the 14 clones with differential expression in p185<sup>BCR-ABL</sup>-positive and p210<sup>BCR-ABL</sup>-positive acute lymphoblastic leukemia; (B) decision margins from 10-fold cross-validation of the nearest-centroid classifier based on these 14 clones; (C) Kaplan-Meier analysis of overall survival (OS) in the p185<sup>BCR-ABL</sup>-positive versus p210<sup>BCR-ABL</sup>-positive ALL. EST, expressed sequence tag.

signature was able to distinguish completely CML blast crisis from CML chronic phase by hierarchical clustering (data not shown).

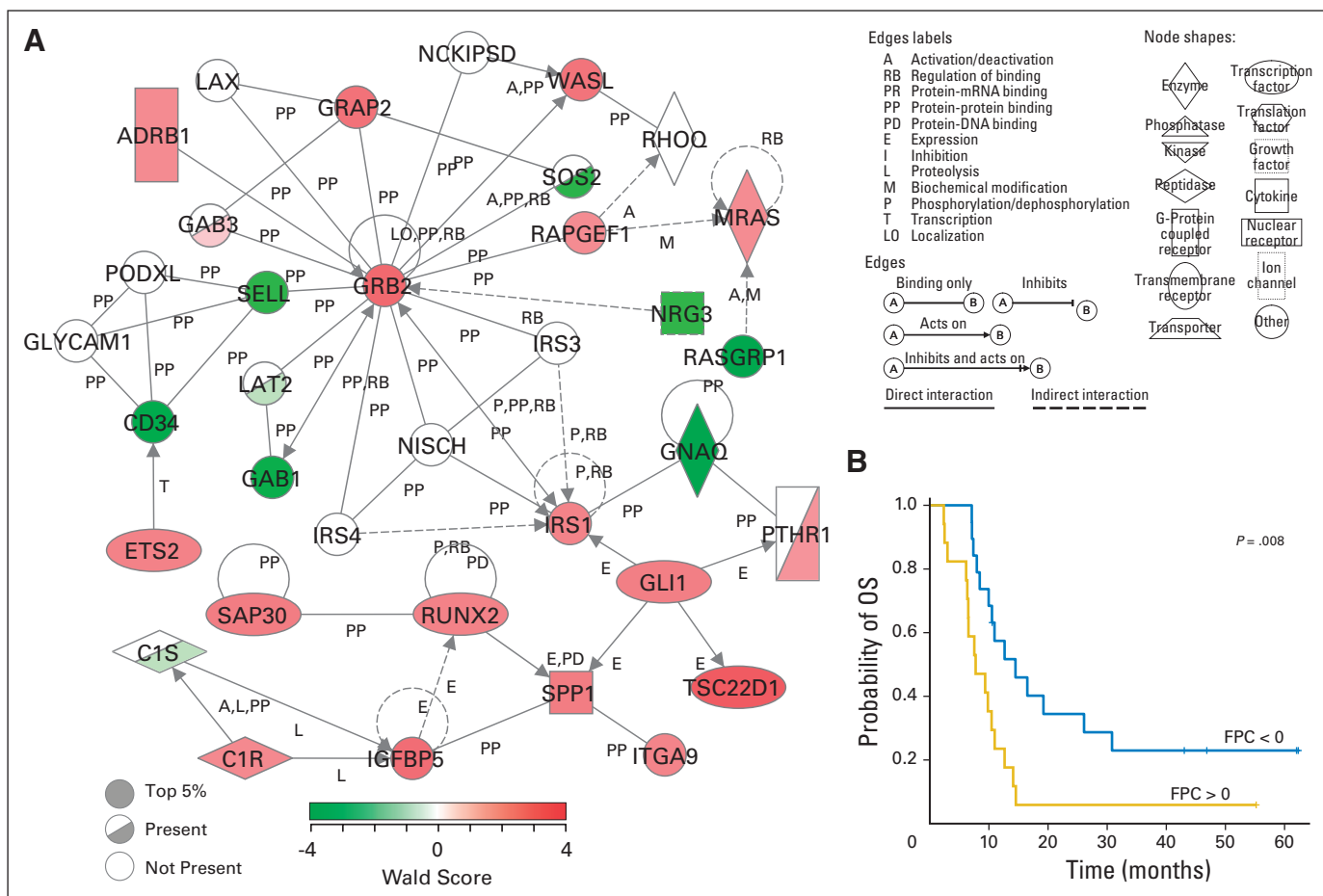
One of the validated genes, *GAB1*, a GRB2-associated binding protein 1, is a member of the Gab family of docking proteins. Although formation of the GRB2-SOS-GAB2 complex is one of the critical events in BCR-ABL signaling in CML, impaired function of GAB2 results in only partially diminished lymphoid leukemogenesis.<sup>32,33</sup> Overexpression of *GAB1* in ALL cells might provide an alternative to *GAB2*.

Another consistent feature of BCR-ABL-positive adult ALL samples is overexpression of the class II major histocompatibility complex (MHC) genes, potentially associated with upregulation of several genes involved in transcriptional activation and cell surface trafficking of MHC molecules, such as *CIITA*, a master transactivator of the MHC class II genes.<sup>34</sup> A possibly related finding is an increased expression of *CD83*. Soluble CD83 is elevated in a number of hematologic malignancies, and can suppress both in vitro and in vivo antitumor

responses, potentially contributing to the aggressive nature of BCR-ABL-positive ALL.<sup>35</sup> An additional mechanism of immune escape of BCR-ABL-positive ALL could be an increased expression of *SERPINB9*, which inactivates the cytotoxic protease granzyme B and prevents cytotoxic T lymphocyte-mediated apoptosis.<sup>36</sup>

The aggressive phenotype of BCR-ABL-positive ALL could be associated with highly overexpressed genes involved in cell adhesion, invasion, and angiogenesis (including *ITGA5*, *TJP2*, *ENG*, *MUC4*, *PTP4A3*, *NOV*, *MTSS1*, *CTNND1*, and *LOX*). Lysyl oxidase (*LOX*), in particular, is responsible for the invasive properties of hypoxic human cancer cells and is required for focal adhesion kinase activity.<sup>37</sup> Focal adhesion kinase is associated with enhanced blast migration, increased cellularity, and poor prognosis in acute myeloid leukemia,<sup>38</sup> and could have a similar role in BCR-ABL-positive ALL.

Our study adds to previous reports by reconstructing interaction networks of the identified candidate genes. The most prominent network was constructed around *FYN* and *IL15*. These highly connected



**Fig 5.** The top-scoring survival-related network depicting a gene expression- and interaction-based predictor of overall survival (OS) in *BCR-ABL*-positive acute lymphoblastic leukemia (ALL), (A) with node colors and shapes corresponding to the Wald-score and functional class of the gene product, respectively; and (B) Kaplan-Meier analysis of OS in the *BCR-ABL*-positive ALL stratified by their first principal component (FPC) prediction score.

hubs might be excellent candidates for targeted disruption of the critical interaction networks, providing novel strategies for treatment of neoplasms associated with the numerous and often redundant molecular alterations. In this context, the dual tyrosine kinase inhibitor dasatinib, which targets imatinib-resistant *BCR-ABL* as well as *FYN* and other members of the Src-family of nonreceptor tyrosine kinases,<sup>39</sup> might have activity in the treatment of *BCR-ABL*-positive adult ALL, beyond its role as a *BCR-ABL* inhibitor. All of the above genes are expressed in both p185<sup>BCR-ABL</sup>-positive and p210<sup>BCR-ABL</sup>-positive ALL. A small number of genes were differentially expressed across the two isoforms. Although two cell adhesion modulators, *TSPAN16* and *ADAMTSL4*, are overexpressed in p210<sup>BCR-ABL</sup>-positive ALL, p185<sup>BCR-ABL</sup>-positive ALL samples show a high expression of several known cell signaling regulators, such as *PILRB*, *STS-1*, and *SPR1*.<sup>40-42</sup>

Contrary to prior in vitro studies,<sup>7</sup> no differences were found in the expression of the Rho family of GTPases or their interaction partners. We found overexpression of some of these genes, such as *CDC42EP3* and *PLEKHG1*, in both *BCR-ABL*-positive groups. It appears that these downstream targets can be activated independent of the Dbl homology domain found in p210<sup>BCR-ABL</sup>. Indeed, it has been shown that both p185<sup>BCR-ABL</sup> and p210<sup>BCR-ABL</sup> form heterotetramers

with *BCR*, allowing for *Dbl* homology domain-mediated effects to be present even in p185<sup>BCR-ABL</sup>-positive leukemia cells.<sup>22</sup>

Finally, our survival analyses provide additional insights into this aggressive disease. No difference was seen in the overall survival of patients with p185<sup>BCR-ABL</sup>-positive versus p210<sup>BCR-ABL</sup>-positive ALL in our set of samples. A multigene survival predictor that did correlate with survival had multiple elements participating in *BCR-ABL*-associated signaling pathways. *GRB2*, *GAB1*, *GRAP2*, *RAPGEF1*, *MRAS*, *ETS2*, *IGFBP5*, and *RASGRP1* are involved in RAS signaling, but the nature of that association in our samples is not clear, especially because of the seemingly opposite effects of *GRB2* and *GAB1* on OS of the patients in the study. *GLI1* and *IRS1* have strong antiapoptotic effects through upregulation of *Bcl-2* and downregulation of *FasL*.<sup>43,44</sup> *RUNX2* overexpression interferes with T-cell differentiation; it causes enhanced cell migration, invasion, and survival of adherent cell types,<sup>45</sup> and it upregulates *PI3K* subunits and *AKT*, possibly amplifying *PI3K* signaling<sup>46</sup>—another critical component of *BCR-ABL*-induced expression of *SPP1*, which is an adverse prognostic factor in our study. Of note, it has been demonstrated that overexpression of *SPP1* correlates with drug resistance in CML cells.<sup>47</sup> Additional experiments are necessary to validate our survival predictor and to

elucidate potential mechanisms linking its gene elements with leukemic cell behavior.

In conclusion, we have defined and validated a set of candidate genes likely to play a role in BCR-ABL–driven leukemogenesis. We have also assessed differential expression associated with the two BCR-ABL isoforms. Finally, we constructed a gene expression–based survival predictor, which could shed light on determinants of outcomes of BCR-ABL–positive adult ALL. These findings may be useful for developing novel therapeutic targets and prognostic markers in this disease.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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## **Appendix**

The Appendix is included in the full-text version of this article, available online at [www.jco.org](http://www.jco.org). It is not included in the PDF version (via Adobe® Reader®).