Prediction of risk of disease recurrence by genome-wide cDNA microarray analysis in patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy

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Abstract. Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) reveals very poor prognosis due to high incidence of relapse when treated with standard chemotherapy. Although >96% of patients with Ph+ALL achieved complete remission (CR) with imatinib-combined chemotherapy in a phase II clinical trial conducted by the Japan Adult Leukemia Study Group (JALSG), 26% of them experienced hematological relapse in a short time after achievement of CR. In this study, to establish a prediction system for risk of relapse, we analyzed gene expression profiles of 23 bone marrow samples from patients with Ph+ALL using cDNA microarray consisting of 27,648 cDNA sequences. Using the 19 randomly-selected test cases, we identified 16 genes that were expressed significantly differently between patients with (n=8) and without (n=11) continuous response; from the list of 16 genes, we selected the 6 'predictive' genes and constructed a numerical scoring system by which the two groups were clearly separated, with positive scores for the former and the negative scores for the latter. Scoring of 4 cases that were reserved from the original 23 cases predicted correctly their clinical responses. In addition, three cases whose BCR-Abl transcript levels failed to reduce sufficiently after induction therapy, also revealed negative scores. We also developed a quantitative reverse transcription-PCR-based prediction system that could be feasible for routine clinical use. Our results suggest that achievement of continuous response with imatinib-combined chemotherapy can be predicted by expression patterns in this set of genes, leading to achievement of 'personalized therapy' for treatment of this disease.

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Abbreviations: ALL, acute lymphoblastic leukemia; Ph+, Philadelphia chromosome-positive; CPM, cyclophosphamide; DNR, daunorubicin; VCR, vincristine; PSL, predonisolone; MTX, methotrexate; Ara-C, cytarabine; Dex, dexamethasone; mPSL, methylprednisolone; JALSG, Japan Adult Leukemia Study Group; CR, complete remission; EFS, event-free survival

Key words: imatinib, BCR-Abl, acute lymphoblastic leukemia, prediction, microarray, recurrence

Introduction

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) is characterized by a reciprocal translocation between chromosome 9 and chromosome 22 [t(9;22)(q34;q11)], leading to the formation of the BCR-Abl fusion protein. This translocation is observed in about 3-5% of pediatric ALL and 20-30% of adult ALL, and is usually associated with very poor prognosis (1,2). Recently-developed chemotherapy regimens for adult Ph-negative ALL are very effective; 70-90% of the patients reach complete response (CR) and 30-50% of them achieve long-term survival (3-7). Although patients with Ph+ALL reveal almost similar CR rates ranging 60-80% with the same chemotherapy regimens, most of them experience relapse and die of the disease; the 5-year survival rates are <10% for adults (8-11). However, their long-term event-free survival (EFS) rate is expected to

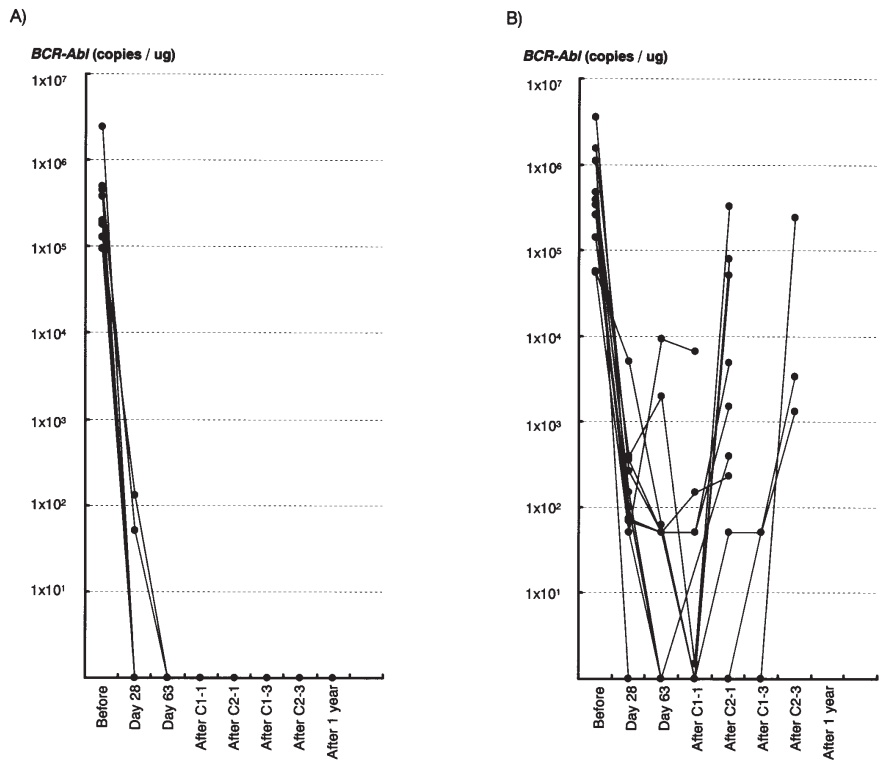

Figure 1. Molecular responses in 19 patients with Ph+ALL treated with imatinib-combined chemotherapy. Each line represents the BCR-Abl levels for patients with (A; group A) and without (B; group B) continuous response. The copy numbers of *BCR-Abl* in y-axis were measured by quantitative real-time RT-PCR. The day numbers in x-axis indicate days after initiation of the remission induction therapy. C1-1 indicates the first cycle of consolidation 1 (C1), C2-1 the first cycle of consolidation 2 (C2), C1-3 the third cycle of C1 and C2-3 the third cycle of C2.

be as high as 40% if they undergo allogeneic hematopoietic stem-cell transplantation (HSCT) during first CR (12-18).

The Abl-selective tyrosine kinase inhibitor imatinib, which binds specifically to the nucleotide-binding pocket in the catalytic domain of BCR-Abl (19), has been developed, and is becoming the standard therapeutic drug for patients with chronic myelogenous leukemia (CML) (20-22). For Ph+ALL harboring the same chromosome abnormality, imatinib alone was able to induce good response in a substantial proportion of Ph+ALL patients, but the response was not durable (17,23). Hence, several groups are currently exploring a combination of imatinib and chemotherapy, and encouraging results have been published (20,24-27). The Japan Adult Leukemia Study Group (JALSG) conducted a phase II clinical trial of imatinib-combined chemotherapy for adult patients with newly diagnosed Ph+ALL (27), and reported that CR was achieved in 77 of 80 patients (96.2%). Nevertheless, 20 of the 77 CR patients (26%) experienced relapse within a relatively short follow-up period.

Generally, allogeneic HSCT has been considered the only curative form of treatment available for adults with this disease. However, it is associated with high risk of treatment-related morbidity and mortality. In fact, among 23 deaths reported in the article (27), 13 (57%) were due to transplantation-related complications (27). Additionally, second generation tyrosine kinase inhibitors with more potent *in vitro* and *in vivo* activities are under development, and some of them have shown significant clinical efficacy in imatinib-resistant Ph-positive leukemias (28,29). Taken together, there is a growing importance of discriminating patients who should be treated with imatinib-combined chemotherapy and those who should be offered HSCT or second generation tyrosine kinase inhibitors at the early stage of their disease before experiencing recurrence.

In this study, we analyzed the gene expression profile of 26 Ph+ALL cases using cDNA microarray representing 27,648 genes, and established a system to predict a risk of disease recurrence during imatinib-combined therapy. We report identification of 6 genes that showed significantly different levels of expression between patients with or without recurrence within 4 months after achievement of CR. We suggest that such information may lead ultimately to our goal of 'personalized therapy'.

Materials and methods

Patients and treatments. The JALSG Ph+ALL202 trial was open for patients with newly diagnosed Ph+ALL aged 15-64 years. Eligibility criteria included adequate functioning of the liver, kidneys, and heart and an Eastern Cooperative Oncology Group performance status between 0 and 3. Written informed consent was obtained from all patients before registration (25,27).

The design of the trial was previously described in detail (27). RNAs were isolated from bone marrow cells collected before start of treatment, and were subjected to the multiplex reverse-transcription-PCR (RT-PCR) test and this microarray analysis. All patients positive for *BCR-Abl* were treated with imatinib-combined chemotherapy. For remission induction therapy, imatinib was administered from day 8 to day 63 in

combination with cyclophosphamide (CPM), daunorubicin (DNR), vincristine (VCR), and predonisolone (PSL). Consolidation therapy consisted of an odd course (C1) comprising high-dose methotrexate (MTX) and high-dose cytarabine (Ara-C), and an even course (C2) with single-agent imatinib for 28 days. C1 and C2 were alternated for four cycles each. After the completion of the consolidation therapy, patients received maintenance therapy consisting of VCR, PSL, and imatinib up to 2 years from the date they had attained CR. The daily dose of imatinib used in this study was 600 mg.

Of patients registered in the JALSG trial, 26 patients who met all of the following criteria were eligible for the present study: i) approval of the microarray study by the institutional ethics committee; ii) samples for which informed consent was obtained for this analysis; iii) HSCT was not performed during the remission induction or consolidation therapy; iv) the proportion of blast cells exceeded 70%; v) patients who survived longer than 2 months after the initiation of this treatment. According to the responses to the imatinib-combined chemotherapy, we categorized patients into 2 groups: patients whose *BCR-Abl* level in bone marrow became undetectable during the remission induction therapy and did not increase throughout the observation period (group A), and patients whose *BCR-Abl* level in bone marrow once dropped below the threshold (50 copies/μg total RNA) by quantitative real-time RT-PCR analysis, but increased within 4 months after achievement of CR (group B) as shown in Fig. 1.

RNA extraction and T7-based RNA amplification. Total RNAs from bone marrow cells of each Ph+ALL patient were extracted using QIAamp® RNA Blood Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. To guarantee the quality of RNAs, total RNAs extracted from each sample were electrophoresed on a denaturing agarose gel, and the quality was confirmed by the presence of rRNA bands. Extracted total RNA samples were treated with DNase I (Nippon Gene, Tokyo, Japan), and T7-based RNA amplification was then carried out as described previously (30). After two rounds of RNA amplification, we obtained 40-350 mg of amplified RNA for each case. As a control, a mixture of total RNA extracted from mononuclear cells of 11 healthy volunteers was used. RNAs amplified by this method accurately reflected the proportions in the original RNA source, as we had confirmed earlier by semiquantitative RT-PCR experiments (31), in which data from the microarrays were consistent with the results obtained by RT-PCR.

cDNA microarray. To obtain cDNAs for spotting on the glass slides, we performed RT-PCR amplification for each gene, as described previously (31). The PCR products were spotted on Alice glass slides® (GE Healthcare, Amersham Biosciences, Buckinghamshire, UK) with a high-density Microarray Spotter Lucidea (GE Healthcare); 9,216 genes were spotted in duplicate on a single slide. We prepared three different sets of slides (a total of 27,648 gene spots), on each of which the same 52 housekeeping genes and 2 negative control genes were spotted as well. The cDNA probes were prepared from amplified RNA using the method described previously (30). For hybridization experiments, 2.5 mg of amplified RNAs from each patient and from the control were reversely transcribed in the presence of Cy5-dCTP and Cy3-dCTP (GE Healthcare), respectively. Hybridization, washing, and detection of signals were carried out as described previously (30).

Quantification of signals. We quantified the signal intensities of Cy3 and Cy5 from the 27,648 spots and analyzed the signals by substituting backgrounds, using ArrayVision software (Imaging Research, Inc., St. Catharines, Canada). Subsequently, the fluorescence intensities of Cy5 (Ph+ALL) and Cy3 (control) for each target spot were adjusted so that the mean Cy5/Cy3 ratio of the 52 house-keeping genes became one. Because the data with low signal intensities are less reliable, we defined a cut-off value for the data on each slide as described previously (32) and excluded genes from further analysis when both Cy3 and Cy5 signal-intensities were lower than the cut-off value (32).

For genes in which either or both of the signal-intensities were above the cut-off value, we calculated the ratio of Cy5/Cy3 signals as a relative expression ratio using the raw data of each sample. However, if either of the Cy3 or Cy5 signal intensity was lower than the cut-off value, the Cy5/Cy3 ratio might be calculated to an extremely high or low and lead to make a significant false-influence for the further analysis. Hence, when either Cy3 or Cy5 signal intensity was less than the cut-off value, we adjusted the lower one to be half of each cut-off value plus the signal intensities, and then calculated the Cy5/Cy3 ratios.

Hierarchical clustering analysis. We used web-available software ('Cluster' and 'TreeView') written by M. Eisen (http://genome-www5.stanford.edu/MicroArray/SMD/restech.html) to create a graphic representation of the microarray data and to create a dendrogram of hierarchical clustering. Before the clustering algorithm was applied, the fluorescence ratio for each spot was first log-transformed and then the data for each sample were median-centered to remove experimental biases.

Extraction of genes for predicting the disease recurrence. We applied a random permutation test to identify genes that were expressed at a significantly different level in leukemic cells between group A and B. Mean (μ) and standard deviation (δ) were calculated from the log transformed relative expression ratios of each gene in patients with and without continuous response. A discrimination score (DS) for each gene was defined as follows:

$$DS = (\mu_A - \mu_B)/(\delta_A - \delta_B)$$

We carried out permutation tests to estimate the ability of individual genes to distinguish the two groups; samples were randomly permutated between the two groups 10,000 times. Because the DS data set of each gene showed a normal distribution, we calculated a P-value for the user-defined grouping (33). For the initial analysis, we applied the expression data for original 19 cases consisting of 8 in group A and 11 in group B. As the next step, we randomly excluded one case from each group and made 10 different

combinations consisting of 7 in group A and 10 in group B. We performed additional 10 permutation tests using the 10 combinations of the two groups.

Calculation of prediction score. We calculated a prediction score according to the procedure described previously (33). Each gene (g_i) votes for either case in group A or group B depending on whether the expression level (x_i) in the sample is closer to the mean expression level of group A or group B in reference samples. The magnitude of the vote (V_i) reflects the deviation of the expression level in the sample from the average of the two classes:

$$V_i = I\ x_i - (\mu_A+\mu_B)/2\ I$$

We summed the votes to obtain total votes for the group A (V_A) and group B (V_B), and calculated PS values as follows:

$$PS = [(V_A-V_B)/(V_A+V_B)] \times 100$$

reflecting the margin of victory in the direction of either patient with or without continuous response. PS values range from -100 to 100; a higher absolute value of PS reflects a stronger prediction.

Evaluation of the classification and leave-one-out method. We calculated the classification score (CS) using prediction scores of patients with (PS_A) and without (PS_B) continuous responses in each gene set, as follows:

$$CS = [\mu(PS_A)-\mu(PS_B)]/[\delta(PS_A)+\delta(PS_B)]$$

A larger value of CS indicates better separation of the two groups by the predictive-scoring system. For the leave-one-out test, one sample is withheld, the permutation P-value and mean expression levels are calculated using remaining samples, and the class of the withheld sample is subsequently evaluated by calculating its prediction score. We repeated this procedure for each of the 19 samples.

Quantitative reverse transcription-PCR. Aliquots of the same aRNA hybridized to the microarray slides from individual samples and from a mixture of pooled mRNA from peripheral blood of 11 healthy volunteers were reversely transcribed using oligo$(dT)_{12-18}$ primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative-RT-PCR were carried out using SYBR Premix Ex Taq (Takara, Ohtsu, Japan) on an ABI PRISM 7700 Sequence Detection system (Applied Biosystems, Foster, CA) as described previously (34). The sequences of primers used in the real-time PCR are shown in Table II. To normalize the expression of each gene, we selected as internal controls *PGK1*, *ACTB*, *CCT6A* and *SFRS4* from among the 52 housekeeping genes because they showed the smallest Cy5/Cy3 fluctuations in our microarray data. Because normalization to these four endogenous control genes (PGK1, ACTB, CCT6A and SFRS4) led to similar conclusions (data not shown), we subsequently recorded only the data normalized according to levels of PGK1 expression. For generation of standard curves we used a mixture of mRNAs derived from mononuclear fraction from 19 patients. Quantitative RT-PCR experiments were done in duplicate for all 6 'predictive' genes, and relative expression ratios of each sample were calculated. The normalized gene expression values were log-transformed (on a base 2 scale), in a manner similar to the transformation of microarray-based hybridization data.

Results

Patients characteristics. Characteristics of the 26 patients are summarized in Table I. There were 14 males and 12 females with the median age of 45.0 years (range, 15-63 years). Seven patients were positive for major BCR-Abl and 19 for minor BCR-Abl. All patients received the same remission induction, consolidation and maintenance therapy as described in Materials and methods. CR was achieved in 25 of 26 patients (96%). After a median follow-up of 26.7 months, relapse occurred in 12 patients. The probabilities of EFS and overall survival at 2 years were 48.7% (SE, 10.1%), and 60.0% (SE, 10.0%), respectively. As shown in Fig. 1, we categorized patients into two groups according to the responses to the imatinib-combined chemotherapy.

Identification of genes associated with disease recurrence. To extract genes that were differentially expressed between the two groups, we first analyzed initial 19 samples (8 in group A and 11 in group B) by comparing expression levels of 27,648 genes. We carried out a random permutation test to identify genes that showed significantly different expression levels between the two groups, and identified dozens of genes whose permutation P-values were $<1x10^{-3}$ and whose signal intensities were higher than the cut-off level in >60% of samples in at least one group (% presence >60). To further select the genes that more effectively distinguish the 2 groups, we performed 10 additional random permutation tests with 10 different combinations of 7 cases in group A and 10 cases in group B that were selected randomly, because the number of samples for this analysis was limited. As shown in Table III, *TNK2*, *GLTSCR2* and *AP2B1* were selected by all of the 10 tests; of course, the genes with smaller P-values in the primary random permutation test had a tendency to be selected frequently through these 10 additional tests. The additional random permutation tests further defined the 16-gene set, which were commonly selected by more than four additional permutation tests and isolated by the same criteria as described above ($p<1x10^{-3}$, % presence >60).

Establishment of predictive scoring system for disease recurrence. Using the 16-gene set that seemed to effectively distinguish the two groups, we calculated the prediction score of each sample by the weighted-vote method (34). Then we rank-ordered these candidates on the basis of the number of selected times by 10 permutation tests (Table III) and calculated prediction scores by the leave-one-out test for cross-validation. For the leave-one-out test, we withheld one sample and calculated the permutation P-values and mean expression levels using the remaining samples to identify genes that were the most powerful for separating the two groups.

Table I. Patient characteristics.

Age	Gender	Response to imatinib combined chemotherapy[a]	WBC count (per μl)	Bone marrow blast (%)	FAB type	Type of BCR-Ab	Copy number of BCR-Abl[b]
57	Female	Group A (learning)	3,520	72.3	L1	Minor	$4.40x10^5$
59	Female	Group A (learning)	19,000	92	L2	Major	$9.30x10^4$
28	Female	Group B (learning)	19,800	94	L2	Minor	$1.40x10^5$
20	Female	Group B (learning)	141,000	97.6	L2	Minor	$1.50x10^6$
48	Male	Group A (learning)	103,000	95	L2	Minor	$3.80x10^5$
40	Male	Group B (learning)	46,500	90	L2	Minor	$3.40x10^5$
34	Male	Group B (learning)	176,000	98	L2	Minor	$3.80x10^5$
62	Female	Group A (learning)	26,800	95.9	L2	Minor	$1.30x10^5$
63	Male	Group B (learning)	231,000	96	L2	Minor	$1.10x10^6$
57	Male	Group B (learning)	85,800	95	L2	Minor	$1.40x10^5$
63	Female	Group A (learning)	18,100	91.1	L1	Minor	$1.80x10^5$
52	Male	Group A (learning)	87,300	91.9	L2	Major	$2.00x10^5$
54	Female	Group B (learning)	13,700	96.2	L2	Minor	$4.70x10^5$
15	Male	Group B (learning)	75,100	97.2	L2	Minor	$5.60x10^4$
45	Male	Group A (learning)	3,300	98	L2	Minor	$4.90x10^5$
45	Male	Group B (learning)	139,000	98	L2	Minor	$5.50x10^4$
30	Male	Group B (learning)	7,500	96.4	L2	Major	$3.50x10^6$
35	Female	Group A (learning)	91,000	88.9	L2	Major	$2.40x10^6$
56	Female	Group B (learning)	23,400	94.6	L2	Minor	$2.60x10^5$
33	Male	Group A (test)	5,800	96.4	L2	Major	$7.20x10^6$
38	Female	Group A (test)	4,000	93	L2	Major	$3.90x10^6$
59	Female	Group A (test)	122,000	94	L2	Minor	$2.90x10^5$
37	Male	Group A (test)	18,800	92	L1	Minor	$4.40x10^5$
52	Male	Group C	54,500	90	L2	Minor	$7.60x10^5$
22	Female	Group C	114,000	94.8	L2	Major	$1.20x10^6$
21	Male	Group C	814,000	98	L2	Minor	$5.30x10^5$

[a]Group A, patients whose BCR-Abl level in bone marrow became undetectable during remission induction therapy and indicated no increase thereafter. Group B, patients whose BCR-Abl level in bone marrow dropped below the threshold (50 copies/μg total RNA) but increased within 4 months after achievement of CR. Group C, patients whose BCR-Abl level in bone marrow failed to decrease sufficiently after induction therapy. Test, samples used for validation of the prediction scoring system. [b]Copy numbers of BCR-Abl before initiaton of imatinib-combined chemotherapy were measured by quantitative real-time RT-PCR.

Table II. The primer sets.

Accession no.	Symbol	Forward primer	Reverse primer
Internal controls			
NM_000291	*PGK1*	5'-CAAATGGAACACGGAGGATAAAG-3'	5'-CACAGGAACTAAAAGGCAGGAAA-3'
NM_001101	*ACTB*	5'-CCCTGGAGAAGAGCTACGAG-3'	5'-TGAAGGTAGTTTCGTGGATGC-3'
AF385084	*CCT6A*	5'-AGGACCAAATAAGCACACACTCAC-3'	5'-CACACAGCCATCATCAATAGCA-3'
L14076	*SFRS4*	5'-GGAAGGTCGAGGAGAGAGTGAG-3'	5'-CTGGAGCGTGATTCTGATGG-3'
Predictive genes			
BC044310	*TNK2*	5'-TCTTCAGCTCCACAACCTAAGACA-3'	5'-GGTGCTACCGCTGTTCAAATC-3'
NM_015710	*GLTSCR2*	5'-CTTGTTGCAGAAGGGTTGAGG-3'	5'-AAGAAGCTCAGGCGGAAGG-3'
NM_001282	*AP2B1*	5'-GCTTCCCTCTTGGCTCATTC-3'	5'-GGTGTTTGCTTCTCCTGCTCTT-3'
NM_013286	*RBM15B*	5'-ACTGTCTGCTGTGGTTCTGTATTTT-3'	5'-GGATGTCTTCGATTCTTTTAGCTTG-3'
NM_024834	*C10orf119*	5'-ATTAGCAGTACCTTGGGCTGTTTT-3'	5'-TGCACCACCTCCTCCACTT-3'
AA196770	*ALS2CR4*	5'-TAGGCCAGGCATGGATCTTA-3'	5'-CCTCCAAAGGTGAGCTGGTA-3'

Table III. The 16 discriminating genes.

Accession no.	No. of selected times[a]	P-value[b]	Symbol	Gene name	Group[c]
BC044310	10	1.93×10^{-6}	*TNK2*	Tyrosine kinase, non-receptor, 2	-
NM_015710	10	1.31×10^{-5}	*GLTSCR2*	Glioma tumor suppressor candidate region gene 2	-
NM_001282	10	3.11×10^{-5}	*AP2B1*	Adaptor-related protein complex 2, ß 1 subunit	-
NM_013286	9	1.46×10^{-8}	*RBM15B*	RNA binding motif protein 15B	+
NM_024834	9	7.30×10^{-9}	*C10orf119*	Chromosome 10 open reading frame 119	+
AA196770	7	1.52×10^{-4}	*ALS2CR4*	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, ca	+
U31383	7	1.58×10^{-4}	*GNG10*	Guanine nucleotide binding protein (G protein), γ 10	+
NM_173060	7	1.63×10^{-4}	*CAST*	Calpastatin	-
AI039422	7	2.29×10^{-4}	*RAVER2*	Ribonucleoprotein, PTB-binding 2	+
T86339	6	3.38×10^{-5}	*TNC*	Tenascin C (hexabrachion)	+
AA609551	6	1.31×10^{-4}		Transcribed locus	+
BC075836	6	4.91×10^{-4}	*RBBP4*	Retinoblastoma binding protein 4	+
NM_016097	4	5.74×10^{-4}	*IER3IP1*	Immediate early response 3 interacting protein 1	+
BC015936	4	6.20×10^{-4}	*ASH2L*	Ash2 (absent, small, or homeotic)-like (*Drosophila*)	+
NM_021727	4	6.51×10^{-4}	*FADS3*	Fatty acid desaturase 3	-
NM_002733	4	6.55×10^{-4}	*PRKAG1*	Protein kinase, AMP-activated, γ 1 non-catalytic subunit	-

[a]Number of selected times as significantly discriminating genes in ten different random permutation tests. [b]P-values were calculated by random permutation tests with cases with continuous response (group A, n=8) and without (group B, n=11). [c]Plus indicates the genes up-regulated in group A and minus indicates the genes up-regulated in group B. Information was retrieved from Unigene database in National Center for Biotechnology information.

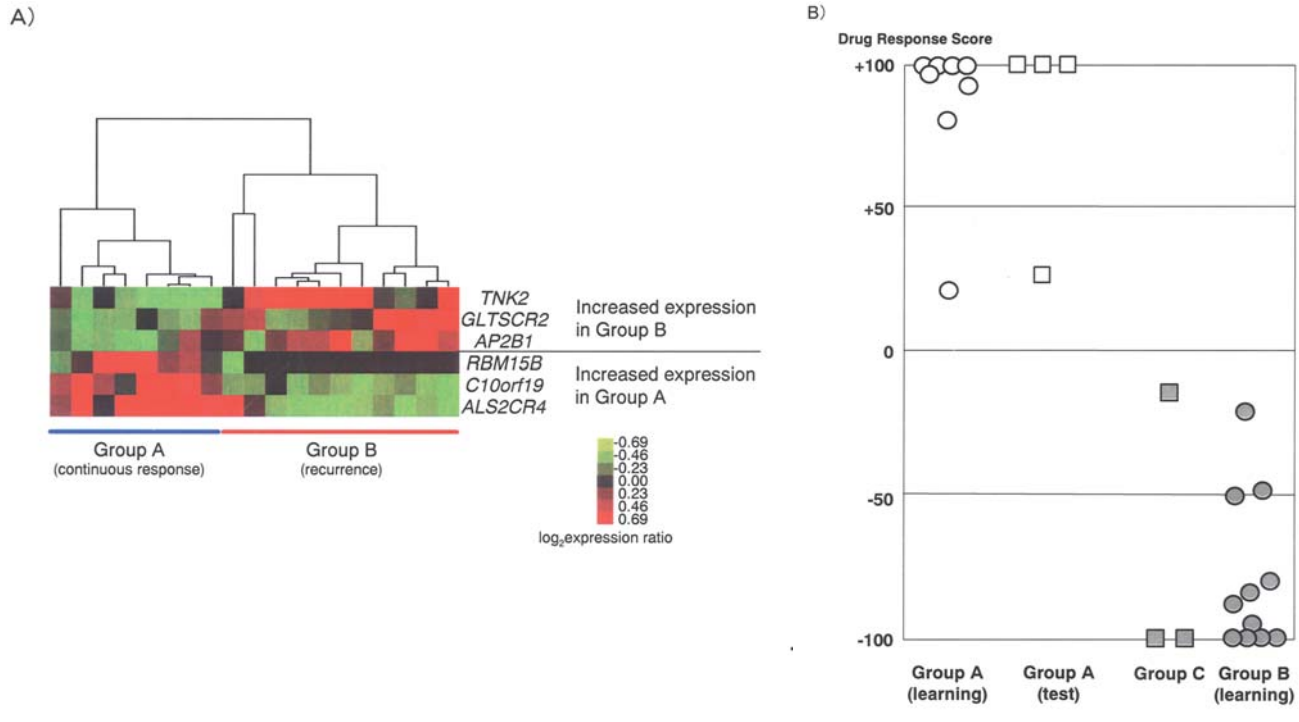

Figure 2. Clustering analysis of 6 predictive genes and distribution of prediction scores for 26 patients. (A) Hierarchical clustering of 19 'learning' cases using 6 predictive genes for recurrence. The dendrograms represent similarities in expression patterns among individual cases; longer branches indicate greater differences. All samples fell appropriately into one of two trees according to their response to imatinib-combined chemotherapy. Three genes were up-regulated in patients with continuous response, while the other three genes were up-regulated in patients without continuous response. (B) The prediction score for individual patients using the 6 discriminating genes. The symbol for white circles (cases with continuous response) and black circles (cases without continuous response) indicate scores in cross validation test of patients whose expression data were used for selecting discriminating genes (learning). The symbols for white squares represent scores for four test cases with continuous response, and black squares represent scores for three cases without sufficient decreases of *BCR-Abl* levels after induction therapy. High absolute values show high confidence.

Table IV. Correlation of microarray expression data with quantitative-PCR derived values.

Accession no.	Symbol	Pearson correlation coefficient	P-value	Spearman rank correlation	P-value
BC044310	*TNK2*	0.84	0.001	0.90	0.00006
NM_015710	*GLTSCR2*	0.73	0.003	0.70	0.006
NM_001282	*AP2B1*	0.65	0.002	0.50	0.029
NM_013286	*RBM15B*	0.72	0.03	0.80	0.01
NM_024834	*C10orf119*	0.66	0.003	0.72	0.001
AA196770	*ALS2CR4*	0.82	0.00003	0.84	0.00001

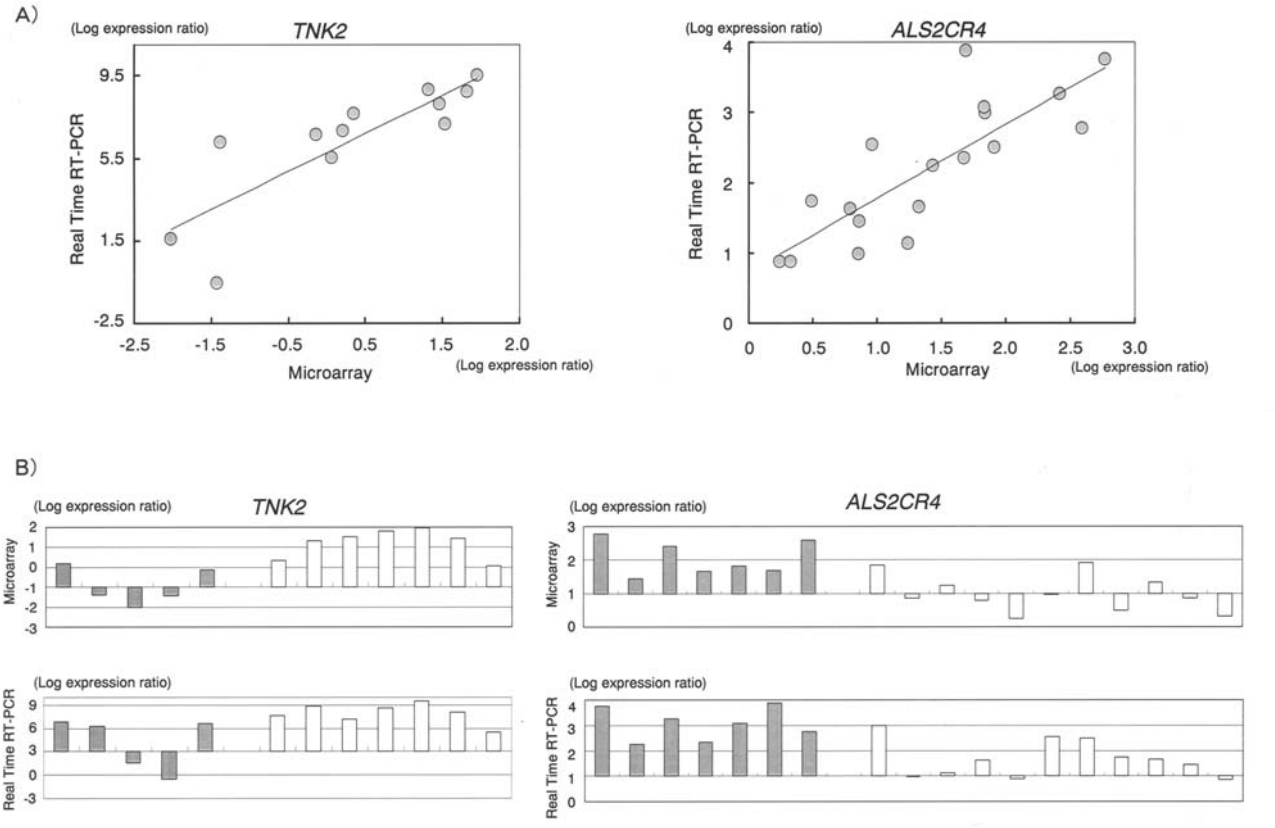

Figure 3. Comparison of microarray-expression data for two genes with quantitative RT-PCR data. (A) Correlation between microarray data for *TNK2* (left) and *ALS2CR4* (right) and values derived from quantitative RT-PCR experiments. Pearson correlation coefficients of *TNK2* and *ALS2CR4* were 0.84 (p=0.001) and 0.82 (p=0.00003) respectively. (B) Microarray (above) and quantitative RT-PCR (below) data for *TNK2* (left) and *ALS2CR4* (right). Black bars in the histogram represent expression of cases with continuous response; white bars represent expression of cases without continuous response.

We calculated the classification score (CS) using the prediction scores of eight group A and eleven group B samples in each gene set, and obtained the best separation of the two groups by using the top 6 genes in our candidate-gene list (Fig. 2 and Table III). A supervised hierarchical clustering analysis using this set of genes with Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm) yielded good separation of the two groups with regard to absence or presence of the recurrence within 4 months after CR (Fig. 2A).

Finally, to verify the prediction scoring system based on expression data for this set of 6 genes, we examined 4 'test' cases (Fig. 2B). We investigated gene expression profiles in each of the four test cases and then calculated a prediction score for each sample. As shown in Fig. 2B, all of the four cases in group A revealed positive scores indicating the prediction scores for the 4 test cases correctly reflected their clinical response although the number of the cases is too small to make any solid conclusion. Our data suggest that expression levels of these six genes or a part of them might play some roles in the molecular mechanism related to the disease recurrence after achievement of CR by the imatinib-combined chemotherapy. Moreover, we investigated gene expression profiles in each of three cases whose BCR-Abl transcript levels failed to reduce sufficiently after induction therapy (group C). Prediction scores for these three samples calculated from the expression levels of the six genes were negative as for the cases in group B (Fig. 2B).

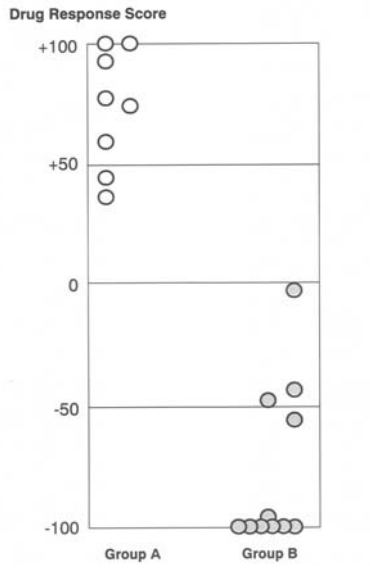

Figure 4. Quantitative reverse transcription-PCR-based prediction scoring system. Prediction scores for 19 cases using values derived from quantitative RT-PCR experiments with 6 predictive genes. White circles indicate scores for cases with continuous response (group A). Black circles indicate scores for cases without continuous response (group B).

Validation of expression measurements. To further validate the results of cDNA microarray analysis, we carried out real-time quantitative RT-PCR for the 6 predictive genes and four quantitative-control genes, PGK1, ACTB, CCT6A and SFRS4, using 19 original (learning) cases. We observed significant concordance between the results from the cDNA microarray and those of the quantitative RT-PCR experiments. As shown in Table IV, Pearson and Spearman rank correlation were positive and statistically significant for all of them. In particular, *TNK2* (tyrosine kinase, non-receptor, 2) was significantly more highly expressed in group B than in group A (Fig. 3). On the other hand, expression of *ALS2CR4* [amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4] was significantly lower in group B than in group A (Fig. 3). The quantitative RT-PCR data were significantly concordant with microarray expression data for both genes (*TNK2*: r=0.84, P<0.001; *ALS2CR4*: r=0.82, P<0.00003; Table IV).

Establishment of a quantitative reverse transcription-PCR-based predictive scoring system. To examine the possibility of adapting our prediction system for clinical use, we attempted to establish a scoring system based on quantitative RT-PCR results. We performed quantitative real-time RT-PCR of the 6 predictive genes for 19 learning cases (8 in group A and 11 in group B), and calculated the prediction score for each case. When we estimated these scores by the leave-one-out cross validation test, all cases were placed correctly according to their response to imatinib-combined chemotherapy (Fig. 4).

Discussion

Despite relatively high CR rates achieved by standard induction chemotherapies, nearly all patients with Ph+ALL experience relapse within a short period of time and die of the disease (9-11). The phase II clinical trial testing imatinib-combined chemotherapy demonstrated that remission induction therapy resulted in CR in 77 (96.2%) of 80 patients, but early relapse was observed in 20 cases (27). Imatinib-combined regimen appeared effective and feasible, but in some cases, treatment response does not last for a long period. Hence, patients who are at higher risk for relapse may benefit from receiving allogeneic HSCT soon after achievement of CR. However, at the same time, it should be noted that 13 among the 49 patients who received allogeneic HSCT died of transplantation-related complications. Although allogeneic HSCT is a curative form of treatment available for adults with this disease, the procedure itself can be a major cause of treatment failure. Additionally, development of novel tyrosine kinase inhibitors such as dasatinib and nilotinib will further expand the treatment options for this disease (28,29). Under these circumstances, we believe that prediction for response to imatinib-combined chemotherapy should be of great use in clinical decision making.

For such purpose, we analyzed the gene expression profile of Ph+ALL cells using cDNA microarray system consisting of 27,648 genes and attempted to establish the prediction system for risk of disease recurrence in patients treated with imatinib-combined chemotherapy. Although other groups have reported the relation between resistance of Ph+ALL to STI571 and gene-expression profiles, this report is the first that shows an ability to predict the response to imatinib-combined therapy by gene expression profiles (35).

By statistical analysis of gene-expression profiles of ALL samples, we identified 16 genes whose expression levels might be associated with the risk of recurrence. The list of 16 genes that showed significant differences between the two groups might provide insight into the biological mechanism(s) involved with treatment response. Among them, the top 6 genes we used for calculation of the prediction score, included tyrosine kinase, non-receptor, 2 (*TNK2*) and adaptor-related protein complex 2, ß 1 subunit (*AP2B1*) that were up-regulated in group B (patients without continuous response). *TNK2* is a tyrosine kinase that inhibits both its intrinsic and stimulated GTPase activity after binding to CDC42 (36-38). On the other hand, AP2B1 is a component of the adaptor complexes which link clathrin to receptors in coated vesicles (36-38). The interaction of *TNK2 in vivo* with clathrin and AP-2 participated in various trafficking pathways, underlying an ability to increase receptor-mediated transferrin uptake (37,39). It is well known that iron is essential for cell proliferation, and the iron was suggested to enhance the cell proliferation or anti-apoptotic effect (40-43). The up-regulation of *TNK2* and *AP2B1* might induce the iron uptake into the leukemic cells, and the iron might play an important role for re-growth of the leukemic cells.

The interaction between integrin ß 1, which is a subunit of very late antigen (VLA4), on leukemia cells and fibronectin on stromal cells in bone marrow is thought to be crucial in the development of minimal residual disease (MRD) which causes leukemia relapse after chemotherapy (44-47). The up-regulation of calpastatin which inactivates the calpain might inhibit the integrin-mediated adhesion

disassembly and play an important role in the development of MRD. On the other hand, calpastatin (CAST) that was observed to be up-regulated in group B (Table III) is a specific inhibitor of calpain (calcium-dependent cysteine protease) that is involved in several key aspects of migration through the integrin-mediated signaling (48-50).

Using cDNA microarray, we established a prediction scoring system on the basis of expression levels of the most-significantly-associated 6 genes (sub-selected from the 16 genes) that distinguished clearly patients with continuous response from those without. The prediction scoring system using the 6 genes also correctly indicated all of four 'test' cases with continuous response to imatinib-combined chemotherapy although validation of the system using a larger patient size is warranted. Moreover, three cases with insufficient decreases of BCR-Abl transcript levels after induction therapy (group C) were classified into group B (patients without continuous response) category by this prediction system. In any case, it should also be noted that the functions of 4 of our 6 predictive genes are still not well known. Therefore, further investigations will be needed to clarify their biological mechanisms associated with treatment outcome.

Recently other groups have predicted prognosis or chemosensitivity of tumors based on quantitative RT-PCR results for expression of genes selected on microarrays (51). To confirm the reliability of microarray data and investigate a possibility of more convenient prediction strategies in the routine clinical use, we also performed quantitative RT-PCR experiments using the 6 predictive genes for 19 learning cases. We confirmed statistically significant correlation between the data obtained from cDNA microarray and those of quantitative RT-PCR (Table IV; Fig. 3). Moreover, we verified that our quantitative RT-PCR-based prediction system could also correctly classify all of the 19 learning cases with regard to their response to treatment (Fig. 4). Although separation of the two groups by quantitative RT-PCR using a set of 6 genes was not so clear as that done by the cDNA microarray analysis, the results are still convincing to separate the two groups.

In conclusion, we are confident that the system based on microarray-derived expression profiles or quantitative RT-PCR would provide a good prediction for disease recurrence for patients with Ph+ALL treated with imatinib-combined chemotherapy and it would bring significant clinical benefits, although a larger scale study is certainly warranted. Our data suggest that the goal of 'personalized medicine' prescribing the correct treatment to each patient with Ph+ALL, may be achievable by selecting specific sets of genes by the approach shown here.

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