Gene expression signatures associated with the in vitro resistance to two tyrosine kinase inhibitors, nilotinib and imatinib

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The elucidation of the molecular mechanism of TKI resistance has broad clinical implications such as the early identification of resistant cases, personalized modulation of drug regimens and facilitating the screening of new targets for therapeutic intervention. In this study, we established TKI-resistant in vitro cell line models by exposing K562 cell lines to nilotinib (doses of 50 and 250 nM) and imatinib (a dose of 800 nM). The expression profiles of TKI-resistant sublines and susceptible K562 parental cell lines were obtained using high-throughput oligonucleotide microarray. We identified gene candidates whose activation may provide survival benefits when endogenous Bcr-Abl oncoprotein becomes inactivated by TKI, thereby allowing for the acquisition of resistance phenotype. Pathway analysis also identified a number of molecular functions activated in the resistant clones, which may provide additional clues about the molecular changes in resistant clones. The transcriptome analysis of TKI-resistant cell lines and their functional analysis in this study may advance the understanding of the human TKI resistance and may provide valuable insights into the development of effective clinical strategies.
of the mechanisms behind TKI-resistance and facilitate the development of effective diagnostic and therapeutic strategies.

Materials and methods

Cell lines resistant to TKI
Among the Bcr-Abl-positive cell lines, we selected erythroid leukemic K562 cell lines that do not show Bcr-Abl overexpression accompanying the acquisition of imatinib resistance. To construct TKI-resistant K562 sublines, the K562 cell lines were exposed to three conditions, 50 and 250 nM of nilotinib and 800 nM of imatinib. The culture conditions and related experimental protocols are described elsewhere. To rule out the mutation-based resistance acquisition, the BCR-ABL loci of three resistant K562 sublines were screened by nucleotide sequencing, and the absence of major clinically relevant point mutations including T315I was confirmed for all three sublines. The expression level of BCR-ABL kinase was also checked using real-time reverse transcriptase PCRs to rule out the resistance by transcriptional upregulation as described previously.

Microarray analysis
RNA was extracted from parental and TKI-resistant K562 cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. We used Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) to assess the quantity and quality of extracted RNA. For high-throughput expression profiling, we used Applied Biosystems Human Genome Survey Microarray Version 2.0 (Applied Biosystems, Foster, CA, USA) representing 28000 human genes. For hybridization, digoxigenin-UTP-labeled cRNA was generated and linearly amplified from 5 μg of total RNA using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit V2.0. Array hybridization, chemiluminescence detection and image acquisition were performed using Applied Biosystems Chemiluminescence Detection Kit.

Table 1 Primers for RT-qPCR

| Gene | Amplicon size (bp) | Sequence |
|------|-------------------|----------|
| AURKC | 180 | Sense | 5′-AATGTGTACCTGGCTCGGCTCAAG-3′ |
| | | Anti-sense | 5′-CCCGCGTGACATCGGAAATAGTT-3′ |
| BTK | 146 | Sense | 5′-AAAGCAGTTCCTTGACCAACAGAACCCTA-3′ |
| | | Anti-sense | 5′-ACCGGACTGGAATAGTT-3′ |
| FYN | 128 | Sense | 5′-ACGCAAGAAGGGTGCAGAACGTCG-3′ |
| | | Anti-sense | 5′-TCCTTTTGGTAAGTTCCCC-3′ |
| SYK | 147 | Sense | 5′-ATGGAAAGTTCCTGATGGCGACGGCA-3′ |
| | | Anti-sense | 5′-AGAACGTCGCAACCTGCTTTCCCT-3′ |
| YES1 | 157 | Sense | 5′-AAGCTCGACTGTGACTGCTCC-3′ |
| | | Anti-sense | 5′-GCGGGCAGCGCATCTACGAC-3′ |
| GAPDH | 301 | Sense | 5′-CCACCCGGGACGTTTATACTGTTTATTA-3′ |
| | | Anti-sense | 5′-CAAGGGGGCTCAGAAATCATCA-3′ |

Abbreviation: RT-qPCR, real-time quantitative PCR.

Figure 1 Unsupervised hierarchical clustering of the 455 genes, which showed differential expression between TKI-resistant K562 sublines and TKI-susceptible parental K562.
and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer according to manufacturer’s protocol. The hybridization was performed three times per sample. Images of each array hybridization were collected using the AB 1700 Analyzer equipped with high resolution, large-format CCD camera, including two short chemiluminescence images with 5 s exposure length for gene expression analysis. Two fluorescent images were obtained for feature finding and processed for spot normalization. Images were auto-gridded and the chemiluminescent signals were quantified. After the background subtraction, spot intensity data were quantile normalized.

Analysis of expression profiles
Using the signal values corrected for background intensities, the probes were filtered out and 15,000 genes were used for the subsequent analysis. For clustering, we performed one-way analysis of variance (ANOVA) and selected the genes under the

Figure 2 Gene expression patterns of 12 gene clusters categorized from the 455 differentially expressed genes.
P-value < 0.10. According to the expression similarities, 12 K-means gene clusters were grouped using the software of Cluster and Treeview.\textsuperscript{21} For pathway analysis, we collected functional gene sets from three public databases, GO, KEGG and GenMAPP.\textsuperscript{22–24} Too large (> 200 genes) or small gene sets (< 10 genes) were excluded, and a total of 642 functional gene sets were used for subsequent enrichment analysis. The significance of enrichment was calculated using parametric gene set enrichment analysis (PAGE) based on z-statistics.\textsuperscript{25} To apply PAGE, the signal-to-noise ratio was calculated for all 15 000 genes in the comparison of TKI-resistant versus TKI-susceptible K562 cell lines. For regulatory motif gene sets representing the potential gene targets of known transcription factors, we downloaded 615 gene sets from MSigDB Ver2.5 (c3 symbol set).\textsuperscript{26} The drug perturbation-related gene sets were obtained from Connectivity Map as described previously.\textsuperscript{27} In brief, expression log\textsubscript{2} ratio was calculated for individual genes, and ordered gene rank list was constructed for each of 281 batches (perturbagen versus vehicle pairs). In the rank list, top-ranking 100 genes (upregulated) and 100 genes at the bottom (downregulated) were selected comprising 562 drug perturbation-related gene sets.

**Quantitative RT-PCR**

Total RNA extracted from the three TKI-resistant K562 sublines and parental cell lines were used for RT-quantitative PCR (qPCR) analysis for five kinase genes (AURKC, FYN, SYK, BTK and YES1). GAPDH was used as internal control. The first-strand cDNA was synthesized using oligo-dT primer and superscript II reverse transcriptase (Invitrogen), and used for subsequent amplification reaction. RT-qPCR analysis was performed using M \times 3000P system, and analysis was done using the software of M \times 3.0.0 (Stratagene, La Jolla, CA, USA). The reaction mixture of 20 \mu l contains 10 ng of cDNA, 1 \times SYBR Green Tbr polymerase mixture (Finnzymes, Vantaa, Finland), 0.5 \times ROX and 20 pmol primer pairs. The thermal cycling was as follows: 10 min at 95 °C, followed by 40 cycles of 10 s at 94 °C, 30 s at 55–60 °C and 30 s at 72 °C. To verify the specific amplification, melting curve analysis was performed (55–95 °C, 0.5 °C/s). Relative quantification was performed by the ΔΔC\textsubscript{T} method. The sequence information of primers used for RT-qPCR is available in Table 1.

**Results**

**Identification of genes associated with TKI resistance**

To identify the genes associated with TKI resistance, we compared the gene expression profiles of TKI-resistant K562 sublines versus TKI-susceptible parental K562 control and selected 455 differently expressed genes showing P < 0.10 (one-way ANOVA). Unsupervised hierarchical clustering of the 455 genes is illustrated in Figure 1. To categorize the 455 genes according to the expression changes, we performed K-means clustering (n = 12). The expression patterns and detailed information of 12 gene clusters are available in Figure 2 and Supplementary Table 1, respectively. Among the clusters, we selected five gene clusters showing relative upregulation in TKI-resistant sublines; three clusters with nilotinib-specific upregulation (Cluster 1, 4 and 8; Figure 3a) and the other two clusters with imatinib-specific upregulation (Cluster 2 and 9; Figure 3b) were illustrated with genes. Three clusters (Cluster 1, 4 and 8) contain genes upregulated in nilotinib-resistant cell lines and two other clusters (Cluster 2 and 9; below) contain genes showing upregulation in both nilotinib- and imatinib-resistant cell lines.

### Table 2 Five gene clusters showing transcriptional upregulation in TKI-resistant K562 sublines

| Upregulation | Cluster | Gene symbols |
|--------------|---------|--------------|
| Nilotinib    | 1       | TMEM51, PLA2G4A, KIF1A, C2orf14, OBSPL6, LOC152078, ENTPD3, ENC1, MAP7, LAMB1, CD36, ABCB1, C9orf125, CASP4, HMGAA2, NTS, TRHDE, LOC144768, TX9, ZNF447, AURKC, FAM9B, NXF5, NXF2, SSX3 |
|              | 4       | IL1R1, COLEC11, ZNF354A, C6orf85, SPTAN1, KCNB8, ARHGDIB, MGC16044, FAM9A, VCY, VCY1B |
|              | 8       | ARHGAP15, COBLL1, ANTXR1, ITGA4, FMNL2, TNFSF10, FLJ31033, APIN, ZDHHC11, SLC4A4C1, CD109, ZNF462, CHST3, MRPS16, MS4A4A, PAK1, DACH1, DCT, STRA6, BCO36928, ASB9 |
| Nilotinib    | 2       | SERINC2, KYNU, GAD1, ANKRD20B, CTDSPL, FLG20147, SNCA, PPRPCA, ELOVL7, PANK3, SLC2A7, TAP1, HCY1, RGS20, ZFHX4, LOC441395, C10orf38, AKR1CL2, C10orf77, CD44, IFITM3, PTPRR, SPRY2, MGAT2, PKCH, C14orf59, COT1L1, PMP22, ZNF420, ZNF85, ZNF567, LILRB1, ZNF626, SYTL4, SSX6, TCEAL4 |
| Imatinib     | 3       | AIM2, PTPRC, FAM5C, TNFRSF9, NLRNS2, CD1D, CDC42EP3, MEIS1, IFIH1, GCA, PTX3, BOMB, EGF, DAB2, LOC493869, LAM4A, TPBG, SYK, TLE4, SFXN3, MCAAM, ADM, MLSTD1, LRMP, NIN, CHE51, ABCB8, ZNF527, HKR1, JAG1, CXPM, SLC35E4, COVA1 |

**Figure 3** Gene clusters with transcriptional upregulation in TKI-resistant K562 sublines. (a) Three gene clusters show the upregulation in two nilotinib-resistant sublines as compared with imatinib-resistant or parental K562 sublines. (b) The transcriptional upregulation in both nilotinib- and imatinib-treated sublines is observed in two gene clusters (Cluster 2 and 9).
clusters showing the upregulation both in nilotinib- and imatinib-resistant sublines (Cluster 2 and 9; Figure 3b). The genes belonging to these five clusters are listed in Table 2.

We observed a number of kinase-encoding genes that are upregulated in TKI-resistant cell lines, which include AURKC (Cluster 1), PRKCH (Cluster 2), PAK1 (Cluster 8) and SYK (Cluster 9). It is notable that the upregulation of different types of kinases may confer alternative survival benefits in place of the Bcr-Abl oncoprotein, which is suppressed by TKI. The molecular function is ‘ATPase activity’ that includes a number of ATP-binding cassette transporters such as ABCB1 and ABCB9, which may contribute to intracellular accumulation of drug, which is one of the potential mechanisms to obtain drug resistance.9,28

### Pathway analysis of expression profiles associated with TKI resistance

For pathway analysis, we used PAGE method that measures the enrichment of differentially expressed genes to predetermined functional gene sets.25 Table 3 lists the annotated molecular functionalities associated with TKI-resistant expression profiles.

| Type   | Functional annotation                                      | Gene size | P-value | Genes                |
|--------|-----------------------------------------------------------|-----------|---------|----------------------|
| Upregulated | GO/ATPase activity, coupled to transmembrane movement of substances | 22        | 1.7E-06 | ABCA8, ABCB1, TAP1, ABCC1, ABCB9, ABCD4 |
|         | GO/amino-acid transport                                   | 28        | 1.2E-05 | SLC12A7, SLC38A6, XK, SLC38A1, SLC43A1, MGC15523, SLC7A8 |
|         | GO/immune response                                        | 163       | 0.0001  | FRTM3, TAP1, IFI1, TNFRSF9, AIM2, PSMB9, IL18RAP, FRTM2, PSMB1, IFI16, IFIT3, CD97, DAF, CNIH, CCL5, ISG39, IL18R1, PSMB2, MR1, EB2, TNFRSF10, LIF, IFKBKE, FCGR2B, ACSL1 |
|         | GO/amino-acid-polyamine transporter activity              | 23        | 0.0002  | SLC12A7, SLC38A6, SLC38A1, MGC15523, SLC7A8 |
|         | GenMAPP/Integrin-mediated cell adhesion                    | 55        | 0.0005  | FYN, SEPP1, ITGB5, RAP1B, CAPN2, RAC2, CAV1, TLR1, AKT1, ITGA4, CAV2, VCL, PAK1 |
|         | GO/cell adhesion                                           | 189       | 0.0006  | CD44, TPB3, MAMC, LAMA4, LAMB1, CD36, ITGB5, CPXM, CD9, TNXB, PTPRF, CD97, SELPLG, CD47, PCDH10, CCL5, IGF1, PCDH17, FAT, URP2, ITGA4, NELL2, FEZ1, C16orf9, VCL, PKP2, CASK |
|         | GenMAPP/Smooth_muscle_contraction                          | 81        | 0.0007  | FG520, PRKCH, IFGBP4, TNXB, ADM, ITPR2, PRKCCZ, GSTO1, CREB3, ACTA2, G4A1, NOS3, ATF5, ATP2A3, NFXB1 |
|         | GO/non-membrane spanning protein tyrosine kinase activity   | 11        | 0.0009  | SYK, FYN |
| Downregulated | GO/sterol biosynthesis                                    | 19        | 0.0002  | FDTF1, SC4MOL, NSDHL, MVK, HMGR, IDI1, CYP51A1, DHCR24, SC5DL |
|         | GenMAPP/Cholesterol Biosynthesis                           | 14        | 0.0003  | FDTF1, SC4MOL, NSDHL, MVK, HMGR, IDI1, LSS, CYP51A1, SC5DL |
|         | GO/sterol biosynthesis                                     | 30        | 0.0003  | HSD17B7, HSD17B8, FDTF1, SC4MOL, HSD17B1, NSDHL, MVK, HMGR, IDI1, LSS, CYP51A1, DHCR24, SC5DL |
|         | GO/cholesterol biosynthesis                                | 16        | 0.0006  | FDTF1, NSDHL, MVK, HMGR, IDI1, CYP51A1, DHCR24 |

**Table 3** Molecular functionalities associated with TKI-resistant expression profiles

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| Upregulated | GO/ATPase activity, coupled to transmembrane movement of substances | 22        | 1.7E-06 | ABCA8, ABCB1, TAP1, ABCC1, ABCB9, ABCD4 |
|         | GO/amino-acid transport                                   | 28        | 1.2E-05 | SLC12A7, SLC38A6, XK, SLC38A1, SLC43A1, MGC15523, SLC7A8 |
|         | GO/immune response                                        | 163       | 0.0001  | FRTM3, TAP1, IFI1, TNFRSF9, AIM2, PSMB9, IL18RAP, FRTM2, PSMB1, IFI16, IFIT3, CD97, DAF, CNIH, CCL5, ISG39, IL18R1, PSMB2, MR1, EB2, TNFRSF10, LIF, IFKBKE, FCGR2B, ACSL1 |
|         | GO/amino-acid-polyamine transporter activity              | 23        | 0.0002  | SLC12A7, SLC38A6, SLC38A1, MGC15523, SLC7A8 |
|         | GenMAPP/Integrin-mediated cell adhesion                    | 55        | 0.0005  | FYN, SEPP1, ITGB5, RAP1B, CAPN2, RAC2, CAV1, TLR1, AKT1, ITGA4, CAV2, VCL, PAK1 |
|         | GO/cell adhesion                                           | 189       | 0.0006  | CD44, TPB3, MAMC, LAMA4, LAMB1, CD36, ITGB5, CPXM, CD9, TNXB, PTPRF, CD97, SELPLG, CD47, PCDH10, CCL5, IGF1, PCDH17, FAT, URP2, ITGA4, NELL2, FEZ1, C16orf9, VCL, PKP2, CASK |
|         | GenMAPP/Smooth_muscle_contraction                          | 81        | 0.0007  | FG520, PRKCH, IFGBP4, TNXB, ADM, ITPR2, PRKCCZ, GSTO1, CREB3, ACTA2, G4A1, NOS3, ATF5, ATP2A3, NFXB1 |
|         | GO/non-membrane spanning protein tyrosine kinase activity   | 11        | 0.0009  | SYK, FYN |
| Downregulated | GO/sterol biosynthesis                                    | 19        | 0.0002  | FDTF1, SC4MOL, NSDHL, MVK, HMGR, IDI1, CYP51A1, DHCR24, SC5DL |
|         | GenMAPP/Cholesterol Biosynthesis                           | 14        | 0.0003  | FDTF1, SC4MOL, NSDHL, MVK, HMGR, IDI1, LSS, CYP51A1, SC5DL |
|         | GO/sterol biosynthesis                                     | 30        | 0.0003  | HSD17B7, HSD17B8, FDTF1, SC4MOL, HSD17B1, NSDHL, MVK, HMGR, IDI1, LSS, CYP51A1, DHCR24, SC5DL |
|         | GO/cholesterol biosynthesis                                | 16        | 0.0006  | FDTF1, NSDHL, MVK, HMGR, IDI1, CYP51A1, DHCR24 |

Abbreviation: TKI, tyrosine kinase inhibitors.

*The signatures were distinguished for upregulated and downregulated gene sets in TKI-resistant sublines.*

*Three databases (GO, KEGG and GenMAPP) used to collect the gene sets are denoted in the respective gene sets.*

*The significance for enrichment is calculated using parametric gene set enrichment analysis algorithm based on z-statistics, and unadjusted P < 0.10 was considered significant.*

*Among the genes belonging to the gene set, the ‘leading edge subset’ are listed for genes whose corresponding signal-to-noise ratio is above mean + s.d. (upregulated) or below mean – s.d. (downregulated).*
transmembrane transporter molecules, altering the drug efflux/influx and cellular susceptibility in a given dose of drugs.

We also observed that ‘cell adhesion’ and ‘integrin-mediated cell adhesion’ categories are upregulated in TKI-resistant sublines. It has been previously shown that the extracellular signals such as fibronectin-induced integrin signaling can convey antiapoptotic signals to BCR-ABL-positive cells in in vitro settings as potential resistance-acquiring mechanism. The upregulated integrin molecules in our study (ITGB5 and ITGA4) may have similar functional implication. In addition, we observed the relative upregulation of various immune-related genes, as well as downregulation of genes belonging to ‘cholesterol biosynthesis’ functional categories, which constitute unique functional categories associated with TKI resistance.

**Resistance-associated transcriptional upregulation of kinase molecules**

We have already observed the upregulation of various kinase-encoding genes in cluster analysis (Table 2). Pathway analysis also identified the upregulation of two non-receptor tyrosine kinases, SYK and FYN, which is responsible for the enrichment of ‘tyrosine kinase activity’ category (Table 3). Thus, we focused on the transcriptional upregulation of kinase molecules as one of the remarkable expression signatures associated with TKI resistance.

**Table 4** Putative transcriptional regulators and chemicals associated with expression profiles in TKI-resistant sublines

| Gene set set | Condition | Gene set annotation | Gene size | P-value |
|--------------|-----------|---------------------|-----------|---------|
| Regulatory motif | Resistance-up | V$ICSBP_Q6 | 122 | 5.2E−07 |
| gene set | | STTTCNRNTT_V$IRF_Q6 | 99 | 1.8E−05 |
| | | V$EVI1_02 | 67 | 3.0E−06 |
| | | YAATPNINNINNNTT_UNKnown | 38 | 6.6E−06 |
| | | TTANWNANTTGGM_UNKnown | 24 | 7.7E−06 |
| | | V$OCT1_06 | 125 | 0.0001 |
| | | V$IRF1_01 | 119 | 0.0002 |
| | | V$TCF11_01 | 109 | 0.0003 |
| | | V$WHN_B | 128 | 0.0005 |
| | | V$CDX2 Q6 | 111 | 0.0005 |
| | | V$SCART1_01 | 95 | 0.0006 |
| Resistance-down | V$SETF_Q6 | 61 | 2.1E−06 |
| | V$SE2F_Q2 | 96 | 7.6E−06 |
| Connectivity map | Resistance-up | Tamoxifen (1.0E−06M)_Down | 49 | 4.9E−09 |
| gene set | Rosiglitazone_Down | 41 | 4.4E−08 |
| | Sodium phenylbutyrate (1.0E−03M, HL60, medium)_Up | 57 | 2.7E−07 |
| | Cobalt chloride (1.0E−04M)_Up | 44 | 2.3E−06 |
| | Rofecoxib (PC3)_Up | 32 | 4.1E−05 |
| | Butein (PC3)_Down | 64 | 5.5E−06 |
| | Troglitazone_Down | 41 | 5.8E−05 |
| | Pyrvinium (1.3E−06M)_Up | 50 | 9.3E−05 |
| | Gefitinib (HL60)_Up | 42 | 0.0001 |
| | Blebbistatin (1.7E−05M)_Up | 39 | 0.0002 |
| | Sodium phenylbutyrate (1.0E−03M, PC3, medium)_Up | 33 | 0.0002 |
| | SC-58125 (HL60)_Up | 47 | 0.0003 |
| | Rofecoxib_Up | 38 | 0.0004 |
| | Monorden (1.0E−07M, PC3)_Up | 63 | 0.0008 |
| Resistance-down | Imatinib (PC3)_Up | 48 | 0.0002 |
| | Pirinixic acid (1.0E−04M, SKMEL5)_Up | 58 | 0.0003 |

Abbreviation: TKI, tyrosine kinase inhibitors.
resistance. The activation of tyrosine kinase, especially Src family kinases including FYN, has been implicated in the acquisition of TKI resistance in a number of models by mimicking the oncogenic effects of Bcr-Abl kinase. The conformational resemblance between Src family kinase and Abl kinase leads to an assumption that dual kinase inhibitors such as dasatinib can be more potential with off-target effects to Src kinase. Similarly, it was proposed that the inhibitor of Aurora kinase can inhibit T315I mutant Bcr-Abl kinase, which is refractory to conventional TKIs.

Thus, the screening of kinases associated with TKI resistance can advance our understanding to the mechanism of drug resistance and also facilitate to select the appropriate TKI inhibitors in a given clinical context. Our findings suggest that the activation of multiple kinase molecules is a common event in TKI-resistant BCR-ABL(+) cell lines and also highlights the potential utility of using multi-kinase inhibitors to modulate imatinib resistance. We further experimentally verified the expression change of the five kinases (AURKC, FYN, SYK, BTK and YES1) in TKI-resistant and TKI-susceptible K562 cell lines using RT-qPCR. Consistent upregulation of AURKC and FYN was observed in all three TKI-resistant cell lines tested, while the other three kinases, SYK, BTK and YES1, showed dose-dependent upregulation pattern (Figure 4).

The analysis of regulatory motifs and drugs associated with TKI-resistant expression profiles
Regulatory motif gene sets (for example, a set of genes whose cis-regulatory sequences are enriched for a specific regulatory motif; MSigDB C3 category) were also used in pathway analysis.
kinase genes did not show prominent difference of expression between the poor and good responders. The upregulation of potential transcription targets apoptotic genes such as binding protein or ICSBP). This suppression reactivates anti-the Irf8 transcription factors (interferon consensus sequence-TKI-resistant sublines. It is known that Bcr-Abl kinase suppresses also showed substantial enrichment as upregulated genes in TKI-resistant sublines. In the comparison of TKI-resistant versus TKI-susceptible K562 profiles,36 which may increase the cellular proliferation after the acquisition of TKI resistance.

We also used the expression profiles associated with drug perturbation of in vitro cell lines (Connectivity Map) to investigate potential chemical agents that may mimic or modulate the TKI resistance in terms of gene expression (Table 4). Among the drug perturbation-related gene sets, ‘imatinib (PC3)Up’ showed downregulation in TKI-resistant sublines (that is, the upregulated genes in the imatinib-treated in vitro PC3 cell line are relatively downregulated in our TKI-resistant K562 sublines). This may also represent the inhibition effects of TKI, indicating that the expression signatures of TKI-resistant clones are largely composed of those from TKI inhibition as shown in the example of upregulation of apoptosis category. The correlation analysis of the expression profiles with in silico drug-screening results should be interpreted with care. However, our preliminary analysis shows that genes that can be inferred the potential regulator involved in TKI resistance. In the comparison of TKI-resistant versus TKI-susceptible K562 sublines, the potential transcription targets of ‘V$ICSBP_Q6’ was most significantly upregulated in TKI-resistant sublines ($P = 5.2 \times 10^{-7}$) (Table 4). The related regulatory motif gene sets, ‘V$IRF_Q6’ (1.8 $\times 10^{-5}$) and ‘V$IRF_Q6’ (1.9 $\times 10^{-4}$) also showed substantial enrichment as upregulated genes in TKI-resistant sublines. It is known that Bcr-Abl kinase suppresses the Irf8 transcription factors (interferon consensus sequence-binding protein or ICSBP). This suppression reactivates anti-apoptotic genes such as BCL2 or BCLX, whose transcription is normally suppressed by the ICSBP-mediated transcriptional control. The upregulation of potential transcription targets of ICSBP or IRF may represent a treatment effect of TKI, but it is expected that TKI-resistant clones will restore some functions mediated by ICSBP, such as apoptosis. Consistent with this expectation, clustering analysis showed that a number of apoptosis-related genes (CASP4, TNFSF10 and TNFRSF9) were upregulated in resistant clones. In addition, the transcription factors previously assumed to have roles in leukemogenesis such as Evi1 were also enriched in TKI-resistance expression profiles,36 which may increase the cellular proliferation after the acquisition of TKI resistance.

We next performed the pathway analysis by comparing two different TKI-resistant cell lines (nilotinib versus imatinib). Table 5 lists the molecular functions enriched to the genes relatively upregulated in nilotinib- or imatinib-resistant cell lines, respectively. We observed that ‘prostaglandin biosynthesis’ and ‘glycerolipid metabolism’ are relatively upregulated in nilotinib-resistant sublines compared with imatinib-resistant clones. The overexpression of cyclooxygenase-2 and increased prostaglandin E2 production have been observed with imatinib treatment,38 which suggests the inhibitor to cyclooxygenase-2 can modulate the imatinib-resistant cases.39 Our pathway analysis indicates that the differential expression between nilotinib- and imatinib-resistant cases was primarily associated with ‘prostaglandin metabolism’ category, which indicates that the disturbance to eicosanoid metabolism is less severe in nilotinib-resistant cases, or such disturbance accompanies different pathway molecules.

Discussion
The use of imatinib as the first-line chemoagent in treating newly developed CML has achieved a high-profile success achieving 80% of response rate in chronic phase CML cases. However, the development of resistance has been a major obstacle that severely limits the clinical utility of this drug.

In this study, the global transcriptome analysis of K562 in vitro cell line model that acquired drug resistance to 1st and 2nd line TKI of imatinib and nilotinib identified a number of candidate genes and potential molecular functions associated with the TKI resistance. The expression profiles of TKI-resistant cell clones are largely composed of two features; one represents

![Figure 5](image-url) : Expression profiles of the four kinase genes in imatinib-resistant patients and good responders. Twelve CML samples were collected, six poor responders (P) containing three chronic phase (CP) and three acute phase (AP); six good responders (G) containing three CPs and three APs. qRT-PCR was performed for the 12 samples as described in Materials and methods section using the same primers listed in Table 1. All three chronic phase poor responders showed relative upregulation of AURKC expression compared with good responders. However, the other three kinase genes did not show prominent difference of expression between the poor and good responders.
the upregulation of genes that may confer survival benefits (for example, kinases and known oncopgenes) while the other represents the effects of TKI inhibition (for example, apoptosis). It is notable that we observed the upregulation of various kinds of kinase-encoding genes in TKI-resistant clones (AU/RKC, FYN, SYK, BTK and YES1). It is expected that the activated kinase molecules can confer the alternative survival signatures when the endogenous Bcr-Abl oncoprotein becomes inactivated by TKI inhibitors. The use of alternative kinase can be an efficient way to obtain drug resistance for the clones that are already addicted to oncogenic Bcr-Abl protein. This feature has also important clinical implications in that the currently available (or those on clinical evaluation) kinase inhibitors have target selectivity and the effort is ongoing to discover synergistic inhibitor combination.30 It is already reported that inhibitors that can target different kinase molecules can have beneficial effects in controlling the imatinib-resistant cases,33,34 and dual Src/Abl inhibitors such as dasatinib can also help overcome the developed resistance to imatinib.37 We also observed a number of genes whose activation may help the clones to obtain the resistance phenotype such as known cancer-related genes (EGF and JAG1) and transporter-encoding genes (ABCB1 and TAP1). These molecules may represent a set of potential biomarkers, which will facilitate the evaluation and screening of potential resistance cases and targets for therapeutic intervention. We have observed expression profiles of the four kinase genes (AU/RKC, FYN, SYK and BTK) in imatinib-resistant patients and good responders (Figure 5). All three chronic phase poor responders showed relative upregulation of AU/RKC expression compared with good responders. However, the other three kinase genes did not show prominent difference of expression between the poor and good responders. Further study is needed with a larger clinical sample size and we are in the process of conducting it.

Conflict of interest

The authors declare no conflict of interest.

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