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ORIGINAL ARTICLE

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

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The use of selective inhibitors targeting Bcr-Abl kinase is now established as a standard protocol in the treatment of chronic myelogenous leukemia; however, the acquisition of drug resistance is a major obstacle limiting the treatment efficacy. To elucidate the molecular mechanism of drug resistance, we established K562 cell line models resistant to nilotinib and imatinib. Microarray-based transcriptome profiling of resistant cells revealed that nilotinib- and imatinib-resistant cells showed the upregulation of kinase-encoding genes (*AURKC*, *FYN*, *SYK*, *BTK* and *YES1*). Among them, the upregulation of *AURKC* and *FYN* was observed both in nilotinib- and imatinib-resistant cells irrespective of exposure doses, while *SYK*, *BTK* and *YES1* showed dose-dependent upregulation of expression. Upregulation of *EGF* and *JAG1* oncogenes as well as genes encoding ATP-dependent drug efflux pump proteins such as *ABCB1* was also observed in the resistant cells, which may confer alternative survival benefits. Functional gene set analysis revealed that molecular categories of 'ATPase activity', 'cell adhesion' or 'tyrosine kinase activity' were commonly activated in the resistant clones. Taken together, the transcriptome analysis of tyrosine kinase inhibitors (TKI)-resistant clones provides the insights into the mechanism of drug resistance, which can facilitate the development of an effective screening method as well as therapeutic intervention to deal with TKI resistance.

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Introduction

Chronic myelogenous leukemia (CML) comprises ~15% of adult-onset leukemia cases.[1] The most obvious karyotypic change of the disease is a reciprocal translocation t(9;22)(q34;q11) that gives rise to the Philadelphia chromosome (Ph) and the chimeric *BCR-ABL* gene. This fusion gene encodes Bcr-Abl oncoprotein with constitutively activated tyrosine kinase activity, which is responsible for uncontrolled cellular proliferation and development of CML and Ph+ ALL.[2] As the first commercially available inhibitor of Bcr-Abl tyrosine kinase, imatinib mesylate (Gleevec, STI571) has been used as a frontline therapeutic choice for newly diagnosed CML cases.[3] The remarkable rate of cytological remission has been shown in initial clinical surveys and recent follow-up studies.[4,5]

One major concern in the first-line imatinib treatment is the drug resistance, the patients often fail to acquire complete cytogenetic response at initial treatment (intrinsic resistance) or fail to maintain the responses during treatment (acquired resistance). Previous studies showed that somatic point mutations involving the kinase domain of Bcr-Abl protein seem to be the primary cause of resistance in clinical cases.[6] Genomic amplification and transcriptional activation of the *BCR-ABL* loci have been also suspected as possible cause of the resistance.[7] Other putative mechanisms independent of Bcr-Abl kinase pathway have been also reported, for example, the activation of Src family kinases such as Lyn or Hck,[8] transporters involved in drug efflux[9] and the antiapoptotic roles conferred by extracellular matrix.[10]

Increasing the dose of imatinib is one alternative to deal with resistant patients, but it is still controversial whether the resistance can be overcome with the dose escalation.[11,12] More potent second-line tyrosine kinase inhibitors (TKI) such as nilotinib (Tasigna, AMN107) and dasatinib (Sprycel) offer a treatment option for CML patients showing failure or suboptimal response to first-line imatinib treatment.[13–15] However, the patients treated with the second-line TKI also often experience intolerance[16] or resistance, which may require the modulation of drug regiments.[17,18]

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, and thereby lead to 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 can advance the understanding

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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.[19] 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.[20] 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.[6] 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.[20]

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 28 000 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′-AATGTGTACCTGGCT CGGCTCAAG-3′
		Anti-sense	5′-CCGGCGTGCATCAT GGAAATAGTT-3′
BTK	146	Sense	5′-AAAGCAGTTCCTTCA CCGAGACCT-3′
		Anti-sense	5′-ACCGGACTGGAAA TTTGGAGCCTA-3′
FYN	128	Sense	5′-AGCAAGACAAGGTGC AAAGTTCCC-3′
		Anti-sense	5′-TTCCTTTGGTGA CCAGCTCTGTGA-3′
SYK	147	Sense	5′-ATGGAAAGTTCCTGAT CCGAGCCA-3′
		Anti-sense	5′-AGAGCGTGTCGAACT TCTTTCCCT-3′
YES1	157	Sense	5′-AAGCTGCACTGTATG GTCGGTTTA-3′
		Anti-sense	5′-GGGCACGGCATCCT GTATCCTC-3′
GAPDH	301	Sense	5′-GCGGGGCTCTCCAG AACATCA-3′
		Anti-sense	5′-CCAGCCCCAGCGT CAAAGGTG-3′

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

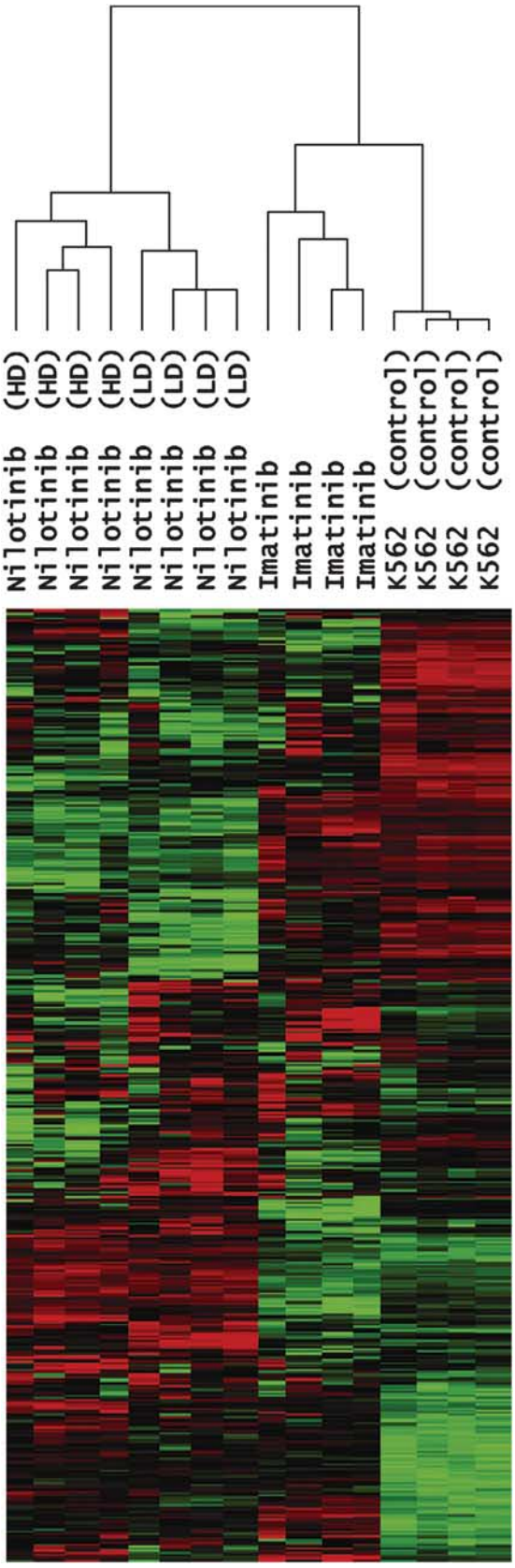

Figure 1 Unsupervised hierarchical clustering of the 455 genes, which showed differential expression between TKI-resistant K562 sublines and TKI-susceptible parental K562.

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

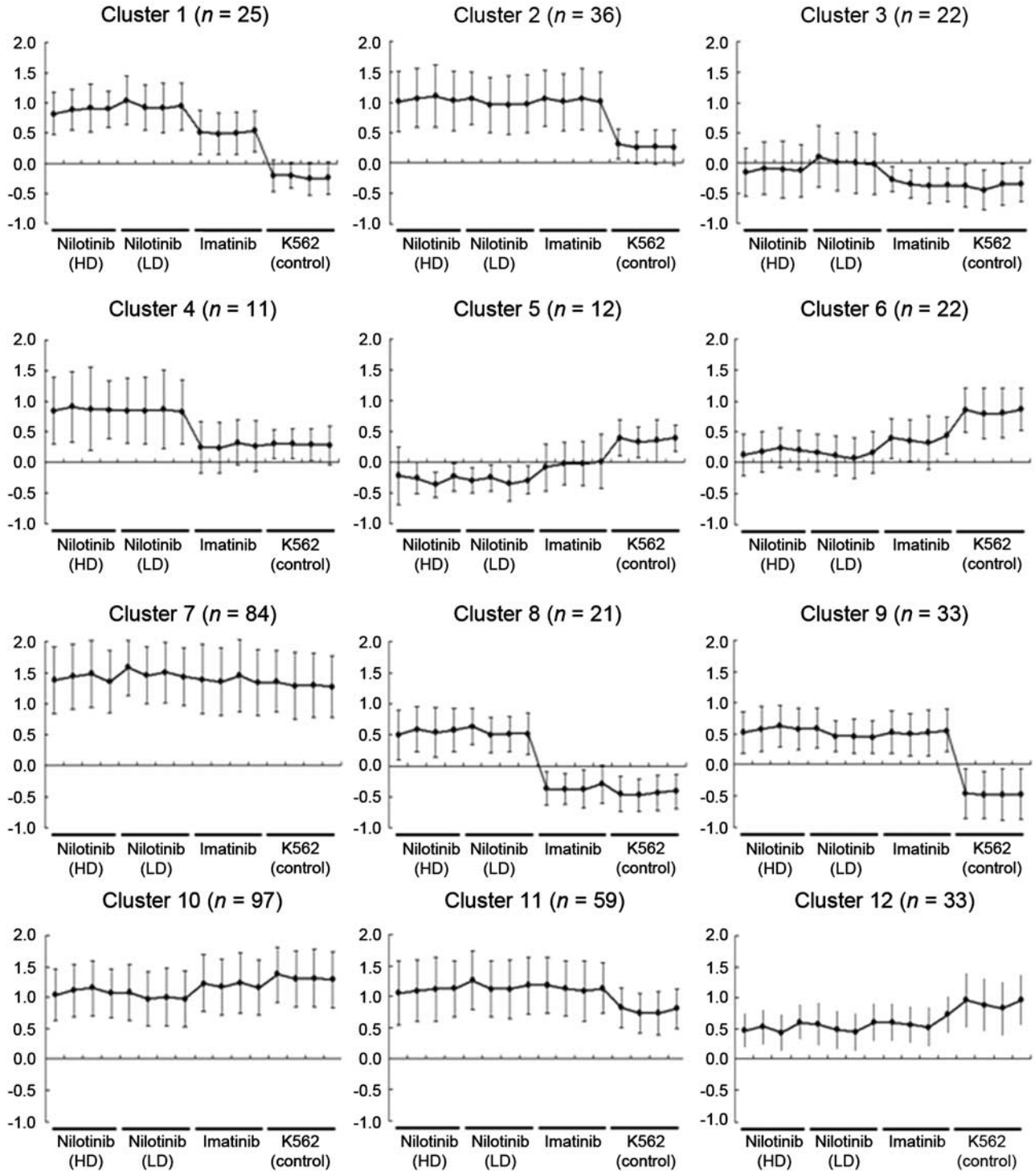

Figure 2 Gene expression patterns of 12 gene clusters categorized from the 455 differentially expressed genes.

P-value <0.10. According to the expression similarities, 12 K-means gene clusters were grouped using the software of Cluster and Treeview.[21] For pathway analysis, we collected functional gene sets from three public databases, GO, KEGG and GenMAPP.[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.[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).[26] The drug perturbation-related gene sets were obtained from Connectivity Map as described previously.[27] In brief, expression $\log_2$ 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 × 3000P system, and analysis was done using the software of M × Pro 3.00 (Stratagene, La Jolla, CA, USA). The reaction mixture of 20 μl contains 10 ng of cDNA, 1 × SYBR Green Tbr polymerase mixture (Finnzymes, Vantaa, Finland), 0.5 × 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 $\Delta\Delta C_T$ 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

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

Upregulation	*Cluster*	*Gene symbols*
Nilotinib	1	*TMEM51, PLA2G4A, KIF1A, C2orf14, OSBPL6, LOC152078, ENTPD3, ENC1, MAP7, LAMB1, CD36, ABCB1, C9orf125, CASP4, HMGA2, NTS, TRHDE, LOC144766, TEX9, ZNF447, AURKC, FAM9B, NXF5, NXF2, SSX3*
	4	*IL1R1, COLEC11, ZNF354A, C6orf85, SPTAN1, KCNMB4, ARHGDIB, MGC16044, FAM9A, VCY, VCY1B*
	8	*ARHGAP15, COBLL1, ANTXR1, ITGA4, FMNL2, TNFSF10, FLJ31033, APIN, ZDHHC11, SLCO4C1, CD109, ZNF462, CHST3, MRPS16, MS4A4A, PAK1, DACH1, DCT, STRA6, BC036928, ASB9*
Nilotinib and Imatinib	2	*SERINC2, KYNU, GAD1, ANKRD20B, CTDSPL, FLJ20647, SNCA, PPP3CA, ELOVL7, PANK3, SLC12A7, TAP1, HEY1, RGS20, ZFHX4, LOC441395, C10orf38, AKR1CL2, C10orf71, CD44, IFITM3, PTPRR, SPRY2, MGAT2, PRKCH, C14orf39, COTL1, PMP22, ZNF420, ZNF85, ZNF567, LILRB1, ZNF626, SYTL4, SSX6, TCEAL4*
	9	*AIM2, PTPRC, FAM5C, TNFRSF9, NR5A2, CD1D, CDC42EP3, MEIS1, IFIH1, GCA, PTX3, BOMB, EGF, DAB2, LOC493869, LAMA4, TPBG, SYK, TLE4, SFXN3, MCAM, ADM, MLSTD1, LRMP, NIN, CHES1, ABCA8, ZNF527, HKR1, JAG1, CPXM, SLC35E4, COVA1*

Abbreviation: TKI, tyrosine kinase inhibitors.
The five gene clusters are selected among 12 K-means clusters and illustrated with genes. Three clusters (Cluster 1, 4 and 8; upper) 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.

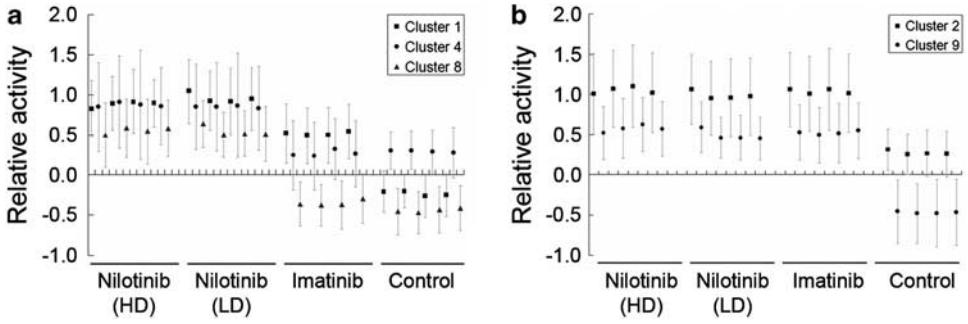

Figure 3 Gene clusters with transcriptional upregulation in TKI-resistant K562 sublines. (**a**) Three gene clusters show the upregulation in two nilotinib-resistant sublines as compared with imatinb-resistant or parental K562 sublines. (**b**) The transcriptional upregulation in both nilotinib- and Imatinib-treated sublines is observed in two gene clusters (Cluster 2 and 9).

Table 3 Molecular functionalities associated with TKI-resistant expression profiles

Type[a]	*Functional annotation*[b]	*Gene size*	P-*value*[c]	*Genes*[d]
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	*IFITM3, TAP1, IFIH1, TNFRSF9, AIM2, PSMB9, IL18RAP, IFITM2, PSME1, IFI16, IFIT3, CD97, DAF, CNIH, CCL5, ISGF3G, IL18R1, PSME2, MR1, EBI2, TNFSF10, LIF, IKBKE, 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, TLN1, AKT1, ITGA4, CAV2, VCL, PAK1*
	GO/cell adhesion	189	0.0006	*CD44, TPBG, MCAM, LAMA4, LAMB1, CD36, ITGB5, CPXM, CD9, TNXB, PTPRF, CD97, SELPLG, CD47, PCDH10, CCL5, IGSF1, PCDH17, FAT, URP2, ITGA4, NELL2, FEZ1, C16orf9, VCL, PKP2, CASK*
	GenMAPP/ Smooth_muscle_contraction	81	0.0007	*RGS20, PRKCH, IGFBP4, TNXB, ADM, ITPR2, PRKCZ, GSTO1, CREB3, ACTA2, GJA1, NOS3, ATF5, ATP2A3, NFKB1*
	GO/non-membrane spanning protein tyrosine kinase activity	11	0.0009	*SYK, FYN*
Downregulated	GO/sterol biosynthesis	19	0.0002	*FDFT1, SC4MOL, NSDHL, MVK, HMGCR, IDI1, CYP51A1, DHCR24, SC5DL*
	GenMAPP/ Cholesterol_Biosynthesis	14	0.0003	*FDFT1, SC4MOL, NSDHL, MVK, HMGCR, IDI1, LSS, CYP51A1, SC5DL*
	GO/steroid biosynthesis	30	0.0003	*HSD17B7, HSD17B8, FDFT1, SC4MOL, HSD17B1, NSDHL, MVK, HMGCR, IDI1, LSS, CYP51A1, DHCR24, SC5DL*
	GO/cholesterol biosynthesis	16	0.0006	*FDFT1, NSDHL, MVK, HMGCR, IDI1, CYP51A1, DHCR24*

Abbreviation: TKI, tyrosine kinase inhibitors.
[a]The signatures were distinguished for upregulated and downregulated gene sets in TKI-resistant sublines.
[b]Three databases (GO, KEGG and GenMAPP) used to collect the gene sets are denoted in the respective gene sets.
[c]The significance for enrichment is calculated using parametric gene set enrichment analysis algorithm based on z-statistics, and unadjusted $P<0.10$ was considered significance.
[d]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).

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 molecules whose altered cellular activities may lead to increased cell survival are also observed. For example, we observed that a number of cancer-related genes such as *EGF, JAG1* and *CDC32EP3* are upregulated in the resistant clones, which can provide additional survival advantages. The upregulation of apoptosis-related genes such as *CASP4* (in Cluster 1), *TNFSF10* (in Cluster 8) and *TNFRSF9* (in Cluster 9) suggests that apoptosis is one of prevailing cellular events in TKI-treated cell lines, which may increase higher cellular turnover rates and also facilitate the selection of clones with survival benefits. Some of the upregulated transcripts in TKI-resistant cell lines are known to involve in cell adhesion (*ITGA4* and *MCAM*) and cytoskeleton (*LAMB1, KIF1A, MAP7* and *LAMA4*). Of interests, *ABCB1* gene (in Cluster 1) encodes ATP-dependent drug efflux pump. The overexpression of this transporter molecule may inhibit the intracellular accumulation of drug, which is one of the potential mechanism 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 functions of significantly enriched ($P<0.001$) functional gene sets and their leading edge gene subsets. The top upregulated molecular function is 'ATPase activity' that includes a number of ATP-binding cassette transporters such as *ABCB1* and *TAP1*. This indicates that TKI-resistant sublines have globally activated transporter activities; their coordinated upregulation suggests that 'ATPase activity' might have a key role in the acquisition of the resistance to TKI. The upregulation of genes belonging to solute carrier (*SLC*) gene family is responsible for the enrichment of 'amino acid transport' category. The roles of the 'amino acid transport' genes in TKI resistance have not been appreciated except for some examples of organic cation transporter.[29] However, these findings suggest that the acquisition of TKI resistance accompanies the transcriptional activation of diverse

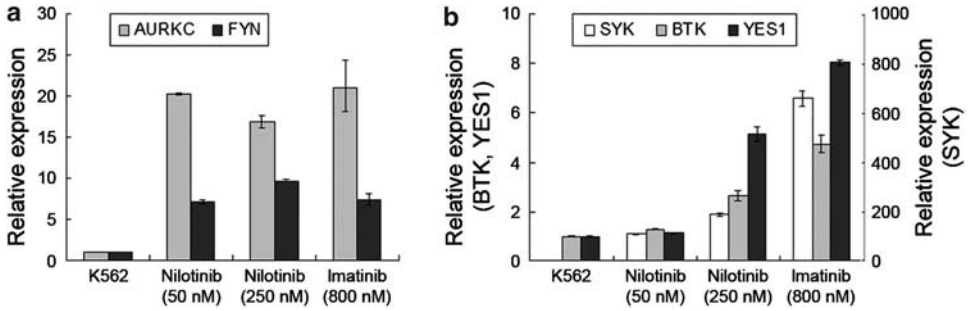

Figure 4 Expression levels of five kinases in TKI-resistant K562 sublines. (**a**) The expression levels of five kinases in four K562 sublines are measured using real-time quantitative PCR. The relative expression levels of *AURKC* and *FYN* for three TKI-resistant sublines are illustrated as compared with those of TKI-susceptible parental cell lines. (**b**) The expression levels of *SYK, BTK* and *YES1* are demonstrated in the same manner.

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

Gene set	*Condition*	*Gene set annotation*	*Gene size*	P-*value*
Regulatory motif gene set	Resistance-up	V$ICSBP_Q6	122	5.2E−07
		STTTCRNTTT_V$IRF_Q6	99	1.8E−05
		V$EVI1_02	67	3.0E−05
		YAATNRNNNYNATT_UNKNOWN	38	6.6E−05
		TTANWNANTGGM_UNKNOWN	24	7.7E−05
		V$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_Q5	111	0.0005
		V$CART1_01	95	0.0006
	Resistance-down	V$ETF_Q6	61	2.1E−06
		V$E2F_Q2	96	7.6E−05
Connectivity map gene set	Resistance-up	Tamoxifen (1.0E−06M)_Down	49	4.8E−09
		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−05
		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.

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[30] 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 'chrolesterol 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

Table 5 Comparison of expression profiles between TKIs

Condition	*Geneset ID*	*Gene size*	P-*value*
Nilotinib versus imatinib	GenMAPP/Prostaglandin_synthesis_regulation	13	0.0006
	KEGG/Glycerolipid metabolism	23	0.0007
Imatinib versus nilotinib	GO/steroid biosynthesis	30	2.4E−07
	GenMAPP/Cholesterol_Biosynthesis	14	5.1E−07
	GO/sterol biosynthesis	19	1.3E−06
	GO/cholesterol biosynthesis	16	6.5E−05
	KEGG/Biosynthesis of steroids	11	0.0003
	GenMAPP/Glycolysis_and_Gluconeogenesis	26	0.0004
	GO/lipid biosynthesis	47	0.0006
	GO/glycolysis	36	0.0007
High versus low dose of nilotinib	GO/amino acid biosynthesis	19	6.9E−20
	KEGG/"Glycine, serine and threonine metabolism	11	9.7E−09
	KEGG/Alanine and aspartate metabolism	13	1.1E−07
	GO/transaminase activity	12	1.2E−06
	GO/growth factor activity	53	2.7E−05
	GO/tRNA binding	11	5.3E−05
	GO/serine-type endopeptidase inhibitor activity	20	0.0001
	GO/endopeptidase inhibitor activity	27	0.0001
	GO/hormone activity	30	0.0003
	GO/cell-cell signaling	102	0.0003
	GO/integrin complex	15	0.0004
	GO/soluble fraction	107	0.0004
	GO/steroid metabolism	33	0.0005
	GO/NADH dehydrogenase (ubiquinone) activity	38	0.0006
	GO/NADH dehydrogenase activity	37	0.0007
	GO/cell motility	65	0.0008
	GenMAPP/Electron_Transport_Chain	88	0.0009
Low versus high dose of nilotinib	GO/rRNA processing	42	1.7E−10
	GO/translation initiation factor activity	61	1.1E−08
	GO/nucleolus	54	1.6E−07
	GenMAPP/Translation_Factors	44	1.4E−06
	GO/helicase activity	109	4.0E−06
	GO/regulation of translational initiation	26	5.0E−06
	GO/ATP-dependent helicase activity	70	5.8E−06
	GO/ribosome biogenesis	30	6.8E−06
	GO/translation factor activity, nucleic acid binding	22	8.8E−06
	GenMAPP/G1_to_S_cell_cycle_Reactome	57	9.2E−06
	GO/mRNA processing	174	2.4E−05
	GO/sterol biosynthesis	19	5.3E−05
	GenMAPP/mRNA_processing_Reactome	109	7.3E−05
	GO/translational initiation	25	0.0002
	GO/cholesterol biosynthesis	16	0.0002
	GO/RNA splicing	58	0.0002
	GenMAPP/Cholesterol_Biosynthesis	14	0.0003
	GO/nuclear pore	41	0.0005
	GO/nucleoside-triphosphatase activity	93	0.0005
	GO/DNA replication	98	0.0006
	GO/mRNA export from nucleus	31	0.0006
	GO/DNA repair	171	0.0006
	GO/tRNA processing	38	0.0009

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[8,31] by mimicking the oncogenic effects of Bcr-Abl kinase.[32] 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.[33,34] 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

to infer 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' (1.9×10^{-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 ISCBP-mediated transcriptional control.[35] The upregulation of potential transcription targets of ISCBP 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 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).[37] 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 suppressed with widely used anticancer or antidiabetic drugs such as tamoxifen and rosiglitazone are upregulated in resistance-related expression profiles, which will require further effort to investigate the potential drug interactions or the possibility that these agents can affect the adverse effects or resistance acquisition of TKI.

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

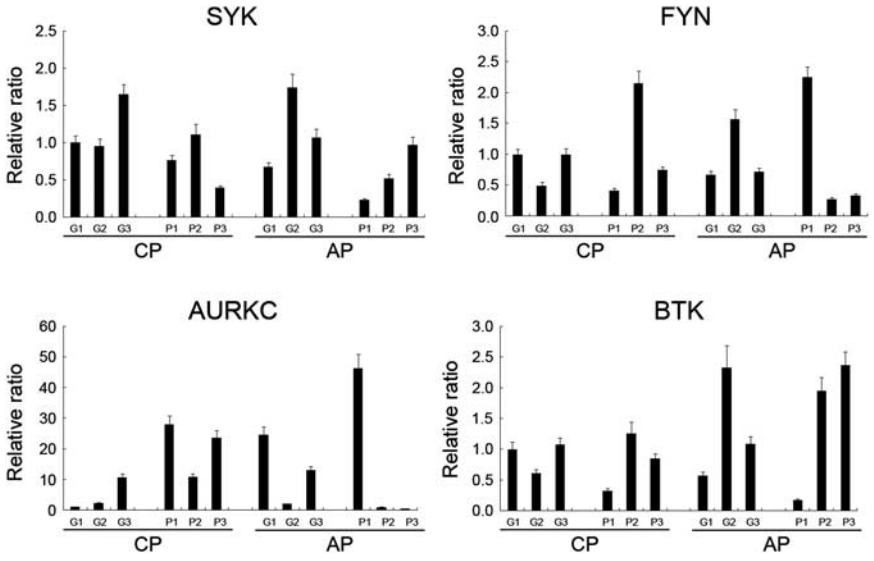

Figure 5 Expression profiles of the four kinase genes in imatinib-resistant patients and good responders. Twelve CML samples were collected, six poor responders (P) containing three chronic phase (CP) and three acute phase (AP); six good responders (G) containing three CPs and three APs. qRT-PCR was 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 oncogenes) 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 (*AURKC*, *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.[40] 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.[17] 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 (*AURKC, FYN, SYK* and *BTK*) in imatinib-resistant patients and good responders (Figure 5). 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. 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.

Acknowledgements

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