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# Chronic myelogenous leukemia molecular signature

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**To obtain comprehensive information about the genes involved in BCR/ABL-dependent leukemogenesis, samples from 15 chronic myelogenous leukemia (CML) patients and seven normal donors were analysed using a cDNA microarray assay. After subtraction of the artificial, random or cross-hybridization signals, data about 5315 genes have been effectively analysed in all samples. The assay revealed $\geqslant$4-fold difference in the average expression of 263 genes in all CML samples when compared to normal counterparts, with 148 genes being upregulated and 115 being downregulated. Differentially expressed genes include those associated with BCR/ABL-induced abnormalities in signal transduction, gene transactivation, cell cycle, apoptosis, adhesion, DNA repair, differentiation, metabolism and malignant progression. Interestingly, CML-blast crisis cells in peripheral blood differ from those from bone marrow, indicating major changes in gene expression profiles upon entering into the bloodstream. Moreover, BCR/ABL modulates expression of genes, which are involved in regulation of chromosome/chromatin/DNA dynamics during S and M cell cycle phase. Moreover, the ability of CML cells to recognize and respond to a pathogen infection may be compromised. Altogether, this work provides a large body of information regarding gene expression profiles associated with CML and also represents a source of potential targets for CML therapeutics.**
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## Introduction

BCR/ABL is derived from relocation of the portion of c-ABL gene from chromosome 9 to the portion of BCR gene locus on chromosome 22 [t(9;22)], and is present in most of chronic myelogenous leukemia (CML) and a cohort of acute lymphocytic leukemia (ALL) patients (Shtivelman *et al*., 1986; Clark *et al*., 1988; Epner and Koeffler, 1990). BCR/ABL hybrid genes produce p230, p210 and p185 fusion proteins exerting constitutive tyrosine kinase activity, transforming hematopoietic cells *in vitro*, and causing CML- or ALL-like syndromes in mice (McLaughlin *et al*., 1987; Daley and Baltimore, 1988; Daley *et al*., 1990; Heisterkamp *et al*., 1990; Kelliher *et al*., 1990; Gishizky and Witte, 1992). BCR/ABL activates multiple signaling pathways responsible for the protection from apoptosis, induction of growth factor-independent proliferation, modulation of adhesion/invasion ability and induction of resistance to drugs and $\gamma$ radiation (Raitano *et al*., 1997; Sattler and Salgia, 1997; Zou and Calame, 1999). As a cytoplasmic tyrosine kinase, BCR/ABL interacts with multiple downstream effectors to transduce transforming signal to the nucleus (Sattler and Griffin, 2001). Despite major progress in identification of the protein effectors of BCR/ABL kinase, little is known about the kinase-dependent regulation of genes at the mRNA level. Several transcriptional factors are regulated by BCR/ABL, such as MYC (Sawyers *et al*., 1992), MYB (Ratajczak *et al*., 1992), JUN (Raitano *et al*., 1995), FUS (Perrotti *et al*., 1998), NF$\kappa$B (Reuther *et al*., 1998), BACH2 (Vieira *et al*., 2001) and Egr-1 (Fukada and Tonks, 2001), suggesting that the mRNA gene expression profile in BCR/ABL cells may be significantly different from that in normal counterparts. Since these transcription factors play an important role in BCR/ABL leukemogenesis (Raitano *et al*., 1997; Sattler and Griffin, 2001), we speculate that examination of mRNA gene expression profile may be very informative for better understanding the biology of BCR/ABL-positive leukemias and can produce new information about potential targets for therapeutic applications.

The cDNA microarray is a powerful technology, which enables large-scale screening of gene expression. It has already been used to analyse several human cancers including leukemias (Golub *et al*., 1999; Ramaswamy *et al*., 2001; Yeoh *et al*., 2002). Microarray assay was applied to study gene expression profiling in an BCR/ABL-positive cell line (Jena *et al*., 2002) or primary BCR/ABL-positive hematopoietic cells from CML and ALL patients (Ohmine *et al*., 2001; Hofmann *et al*., 2002), but these reports did not present a comprehensive analysis of the links between gene clusters and phenotype/function of the leukemia cells.

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We applied this assay to identify novel genes and gene expression profiles responsible for biological abnormalities in CML cells. The results present in this work give more in depth insight into the biology of CML and reveal some novel aspects associated with BCR/ABL-dependent leukemogenesis, especially about nuclear events linked to chromatin dynamics and the ability of leukemia cells to respond to pathogen infections.

## Results

### *Gene expression profile signature of CML*

Initial analysis of the hybridization signal from 12 530 probes in 15 CML samples revealed that significant signal was consistently recorded only for 5315 sequences: these were further analysed. Clustered display of the data is shown in Figure 1a. It clearly indicates that CML samples exhibit noticeable differences in global gene expression profiles.

To validate the gene expression profiling results presented in Figure 1a, expression of genes previously reported as regulated by BCR/ABL has been reviewed. Results presented in Figure 1b confirm that microarray analysis is accurate. Elevated expression of the genes previously described as upregulated by BCR/ABL was detected in most CML cases; conversely, decreased expression of the genes previously described as downmodulated by BCR/ABL is also shown (Sawyers *et al*., 1992; Afar *et al*., 1995; Raitano *et al*., 1995; Sattler *et al*., 1999; Deininger *et al*., 2000, 2001; Hao and Ren, 2000; Parada *et al*., 2001; Slupianek *et al*., 2001; Tauchi *et al*., 2001; Nieborowska-Skorska *et al*., 2002; Perrotti *et al*., 2002). In addition, gene expression profiles downstream of c-MYC and STAT5, the two transcriptional factors activated by BCR/ABL (Sawyers *et al*., 1992; Nieborowska-Skorska *et al*., 1999), are in accordance with the predicted expression pattern (Figure 1c) (Dang, 1999; Grimley *et al*., 1999).

In CML samples, expression of 263 genes was changed on average $\geqslant$4-fold (148 upregulated: 117 known and 31 unknown; 115 downregulated: 99 known and 16 unknown) when compared to normal counterparts. The most changed known genes ($\geqslant$6-fold average difference in comparison to normal cells) are shown in Figure 1d. Their products are involved in regulation of a variety of cell functions including cell cycle (CDK4, CCND1, CCNB, WEE1), apoptosis (CASP3, PDCD8), cell adhesion (PSTPIP1, NK4), signaling (IL1$\beta$, DOK2, LILRB3), transcription (MYB), DNA replication and repair (POLD2, POLE, MSH5, ORC2L, RAD2, UNG), metabolism (FASN, DHFR, ABCC4, PRMT3, RNASEH1) and chromosome/chromatin dynamics (BUB1, MAD2L1, MCM3, KNSL6). To validate the microarray data, a semiquantitative RT–PCR was applied as described (Huang *et al*., 2000). Using this approach, differential expression of several genes involved in various aspects of CML pathogenesis was confirmed (Figure 2).

### *Gene expression profile depends on the disease stage*

CML-CP cells in comparison to normal cells displayed on average $\geqslant$4-fold changes in the expression of 313 genes (129 upregulated: 102 known and 27 unknown; 184 downregulated: 157 known and 27 unknown). The most changed genes (on average $\geqslant$6-fold difference in comparison to normal cells) are presented in Figure 3a. The highest upregulated genes do not belong to any specific cluster regulating a particular function. However, a group of genes whose products are responsible for antibactericidal response (Fc receptors, receptor for bacterial cell wall component, antibacteria peptides and proteins regulating secretion and permeability) such as FCGR3B, FCAR, CD14, CAMP, DEFA1, DEFA3, DEFA4, BPI, LTF and MS4A3 are downregulated in CML-CP cells in comparison to normal counterparts.

In CML-BC bone marrow samples, 303 genes were changed on average by $\geqslant$4-fold (146 upregulated: 109 known and 37 unknown; 157 downregulated: 135 known and 22 unknown) when compared to normal counterparts. A list of genes exhibiting the highest change in expression (on average $\geqslant$6-fold difference in comparison to normal cells) is shown in Figure 3b. Expression of several of them was also detected in CML-CP samples. For example, karyopherin (importin) beta 3 (KPNB3), a protein involved in nuclear import is consistently overexpressed in CML-CP and CML-BC cells (see Figure 3a and b). Interestingly, genes responsible for anti-pathogen response (CAMP, DEFA1, DEFA3, DEFA4, BPI, MS4A3, S100A12, S100P) continue to be downregulated in CML-BC cells (Figure 3b). In addition, genes such as lipocalin (LCN2), G0/G1 switch gene (G0S2), carbonic anhydrase IV (CA4) and vanin-2 (VNN2) are also downregulated in CML-CP and CML-BC bone marrow samples. As expected, highly altered expression of additional genes appeared in CML-BC versus normal cells array when compared to CML-CP versus normal cells array. Some of the substantially overexpressed genes regulate chromosome/chromatin dynamics (KNSL6, MCM3, TTK), DNA replication (POLD2, POLE), intracellular signaling (IL1B), metabolism (AUH, PCK2, FASN, DHFR) and malignant tumor progression (ENPP2); downregulated genes include these working as signal transducers (RAB27A, DOK2).

CML-BC samples from peripheral blood mononuclear cells were also analysed and compared to normal counterparts. In these samples, 418 genes were changed on average by $\geqslant$4-fold (202 upregulated: 167 known and 35 unknown; 216 downregulated: 166 known and 50 unknown) when compared to normal counterparts. A list of genes exhibiting highest change in expression ($\geqslant$6-fold average difference in comparison to normal cells) is shown in Figure 3c. Surprisingly, almost all highly changed genes are different from these found in CML-BC samples from bone marrow (see Figure 3b and c). These overexpressed in peripheral blood CML-BC samples represent transcriptional factors (ZNF24, KFL1, MYB, ERG11), signaling proteins (TFRC, STATI2, LENG4, GRO2, RAC3, PSPHL), cell cycle

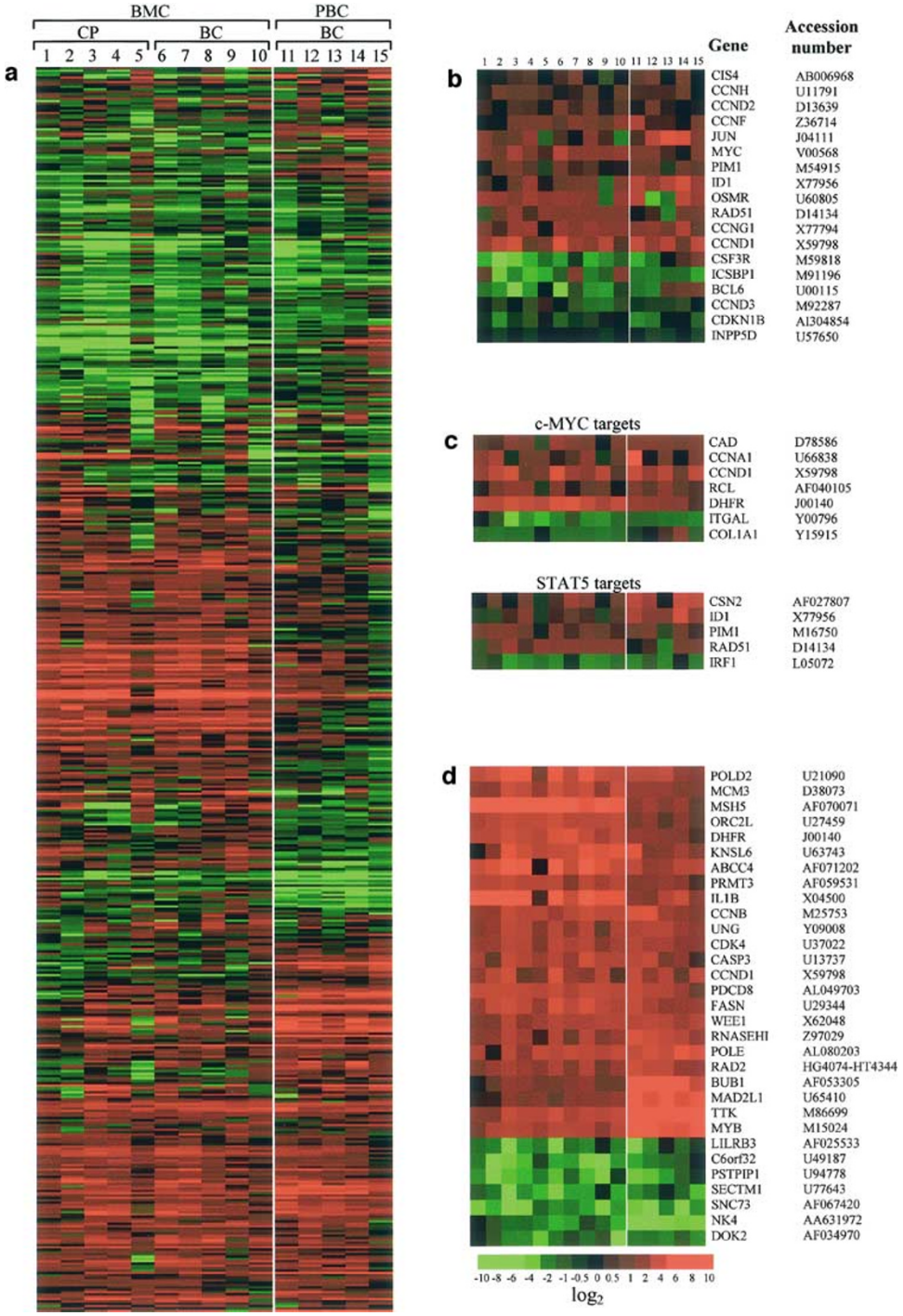


**Figure 1** Gene expression profile signature in CML. (**a**) Hierarchical clustering of 5315 genes based on their expression profiles derived from five bone marrow CML-CP samples (1–5), five bone marrow CML-BC samples (6–10) and five peripheral blood CML-BC samples (11–15) compared to three bone marrow and four peripheral blood samples obtained from healthy donors. Each column represents a separate patient sample, and each row a single gene. (**b**) Validation of the microarray analysis. Expression of the genes, which were known as BCR/ABL downstream transcriptional targets (CIS4→CCND1 upregulated and CSF3R→INPP5D downregulated, see Sawyers *et al*., 1992; Afar *et al*., 1995; Raitana *et al*., 1995; Sattler *et al*., 1999; Deininger *et al*., 2000, 2001; Hao and Ren 2000; Parada *et al*., 2001; Slupianek *et al*., 2001; Tauchi *et al*., 2001; Nieborowska–Skorska *et al*., 2002; and Perrotti *et al*., 2002, is shown for proof that the array analysis is accurate. (**c**) The signature of the gene expression profile according to the known patterns downstream to the c-MYC and STAT5 transcription factors (see Dang, 1999; and Grimley *et al*., 1999), which are constitutively active due to BCR/ABL. (**d**) Most changed (on average $\geqslant$6-fold) genes in CML. The level of color on the bar indicates the magnitude of upregulation (red) or downregulation (green) in comparison to healthy donor

proteins (CDKN3), metabolically active proteins (TPS1, FAH, FACVL1, PDHA1, MPO, CTSG, PRTN3, TK1, NME4) and chromosome dynamics protein (KNSL1). Suppressed expression was detected for the genes, whose products may negatively affect malignant progression of the disease (SNC73, CD3D, TCL1A), inhibit angiogenesis and dissemination (NK4), facilitate recruitment to the inflammatory site and bactericidal response (CCR2, CX3CR1, CD14) and regulate intracellular signaling (ITPR3, FLT3LG, RASGRP1, SCAP1).

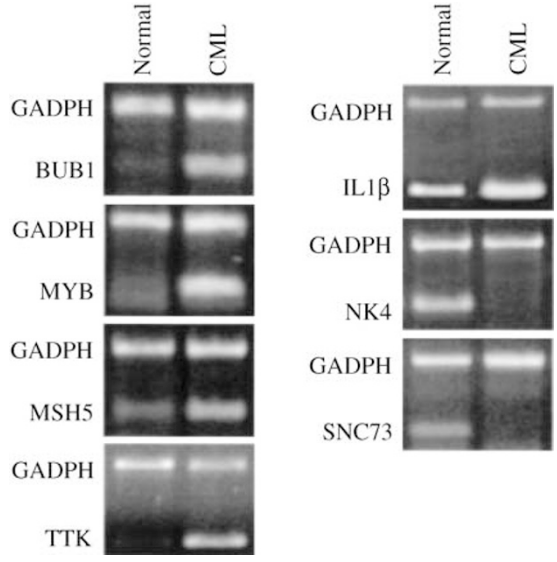


**Figure 2** Semiquantitative RT–PCR analysis of the several most changed genes in CML. RT–PCR reactions were performed from cDNA samples of normal healthy donor (Normal) and CML patients to detect simultaneously the expression level of a gene of interest and GADPH (control of reaction efficiency). Results are representative for two to three pairs of samples for each gene

Malignant evolution of a relatively benign CML-CP to a very aggressive CML-BC is associated with the appearance of a variety of cytogenetic and molecular genetic changes, for example, upregulation of transcriptional factor EVI-1 and mutations of tumor suppressors p53, Rb and CDKN2A (Johansson *et al*., 2002). Even if these are most popular, alterations in their expression and/or mutations are detectable in at most 30% of CML-BC patients (Ahuja *et al*., 1991). This confirms that genetically CML-BC is very heterogeneic and demonstrates that our understanding of the molecular basis of this disorder is rather limited. Despite the differences in genetic background, the leukemia blasts share common phenotypic characteristics: high proliferation rate, differentiation arrest, invasion of nonhematopoietic tissues and strong resistance to genotoxic treatment. Microarray assay offers an excellent opportunity to look for the aberrant gene expression patterns shared by CML-BC patients, which may contribute to the aggressive phenotype of leukemia. We directly

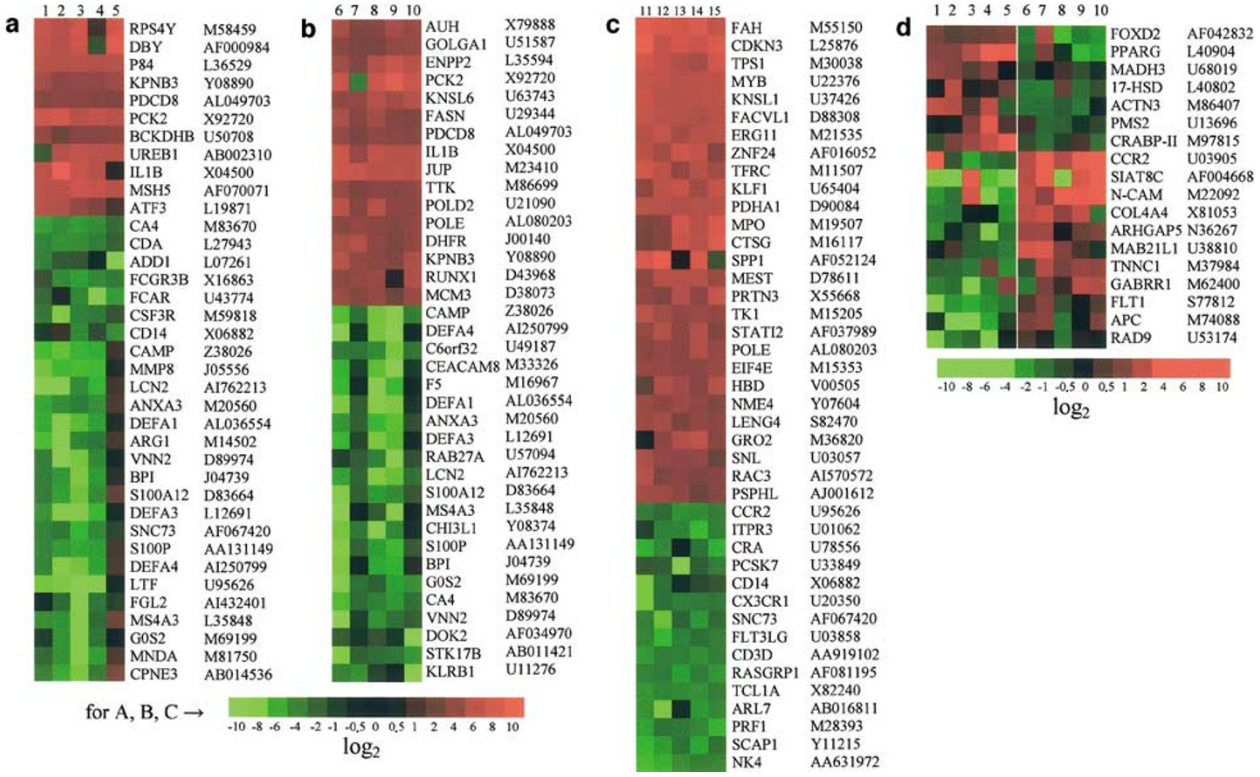


**Figure 3** Genes that distinguish various stages of CML. The highest changed genes in CML-CP bone marrow (**a**), CML-BC bone marrow (**b**) and CML-BC peripheral blood samples (**c**), in comparison to appropriate healthy donor samples (control). Color bar shows the magnitude of upregulation (red) or downregulation (green) in comparison to control. In addition, bone marrow samples of CML-CP were compared to CML-BC and color bar indicates the magnitude of difference in expression of these genes in CML-CP and CML-BC (**d**). Patient sample numbers correspond to those in Figure 1

compared CML-CP and CML-BC samples by analysing the difference between mean gene expression levels. This analysis identified 67 genes, whose expression was changed on average by $\geqslant$4-fold (56 known and 11 unknown). Known genes exhibiting on average $\geqslant$6-fold difference in expression between CML-CP and CML-BC are shown in Figure 3d. Among these are genes involved in malignant progression of the disease (FOXD2, PPARG, MADH3, 17-HSD, APC), differentiation (FOXD2, CRABP-II), chromosome dynamics and DNA repair (MAB21L1, PMS2), checkpoint activation (RAD9), adhesion and angiogenesis (N-CAM, COL4A4, FLT1, ACTN3) and intracellular signaling (MADH3, ACTN3, ARHGAP5, GABRR1).

### *CML-BC from bone marrow and peripheral blood display significant differences in gene expression profile*

The studies presented in Figure 3b and c suggest that different genes are deregulated in bone marrow leukemia cells in comparison to peripheral blood leukemia cells; therefore, gene expression was compared directly in CML-BC samples obtained from bone marrow and peripheral blood. The analysis identified 459 genes, whose mean expression level differed on average by $\geqslant$4-fold (361 known and 98 unknown). Known genes, whose mean expression levels differed on average by $\geqslant$6-fold are shown in Figure 4. Genes exhibiting diminished expression in CML-BC cells obtained from peripheral blood in comparison to leukemia blasts from bone marrow include those affecting cell cycle (CDC25B), intracellular signaling (FZD2, RYK, ACVR1, TC21), DNA metabolism and chromosome assembly (TREX1, GOLGA1, KPNB3), adhesion (JUP), intracellular trafficking (RAB4, KPNB3), glucose metabolism (PCK2) and malignant progression of the disease (DBY). Several genes displayed enhanced expression in peripheral blood CML-BC cells in comparison to bone marrow counterparts, for example, those involved in regulation of adhesion/invasion and malignant progression of the disease (HMMG, LENG4, GSN, ELA2, CTSG, PRTN3, CEACAM8, IL8, MMP9). In addition, enhanced expression of genes affecting cell cycle (CDKN3, CDKL1, UBE2C), mitotic checkpoint and spindle assembly (KNSL1, BUB1, MAD2L1, TTK), DNA repair and synthesis (RAD54L, BRCA1, TYMS), transcription (ZNF24, ERG11), antimicrobial response (BPI, MPO, AZU1) and metabolism (FAH, ALAD) was also observed in peripheral blood CML-BC cells. Interestingly, these cells displayed also enhanced expression of genes, whose products are involved in stress response (TPS1) and complement attack (CD59).

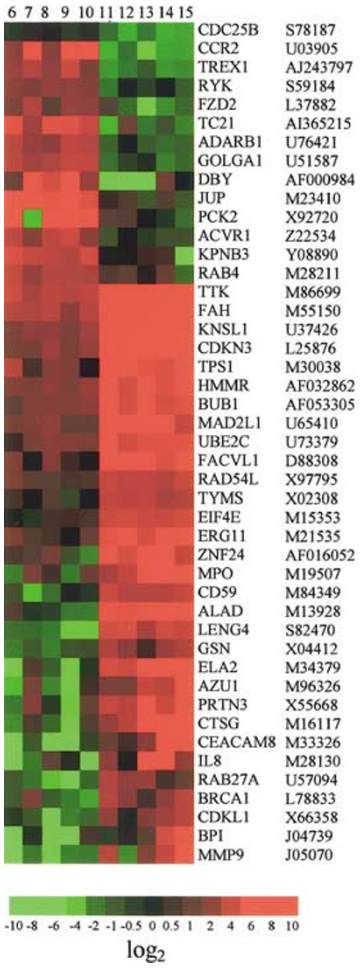


**Figure 4** Genes that distinguish CML-BC in bone marrow and peripheral blood. The highest changed genes, which are differentially expressed in blasts obtained from bone marrow and peripheral blood, are listed. Patient sample numbers correspond to those in Figure 1. The level of color on the bar indicates the magnitude of upregulation (red) or downregulation (green) in comparison to appropriate control samples obtained from healthy donors

### *Functional gene expression profiles in CML*

BCR/ABL induces protection from apoptosis (AP) in growth factor-free environment, stimulates cell cycle (CC), modifies adhesion/invasion (AI), induces drug resistance (DR) and contributes to malignant progression (MP) of the disease. To induce these modifications, BCR/ABL regulates intracellular signaling pathways (SP), metabolism (MT) and transcriptional factors (TF). Information gathered from the microarray analysis significantly expanded our knowledge about the pools of genes regulated by BCR/ABL and associated with these functions (see below). In addition, comprehensive analysis of the differences in expression of genes involved in other cellular functions revealed potentially novel phenotypes/functions affected by BCR/ABL: cell differentiation (DF), capability to fight infections (IF) and chromosome/chromatin/DNA dynamics (CH).

Of the 66 apoptosis-regulating genes represented on the array, only 10 were differentially expressed on average by $\geqslant$4-fold in CML cells in comparison to normal counterparts (Figure 5, panel AP). Paradoxically, two pro-apoptotic genes (PDCD8, CASP3) were upregulated and four anti-apoptotic genes (BIRC3, FOXO1A, CFLAR, MCL1X) were downregulated in

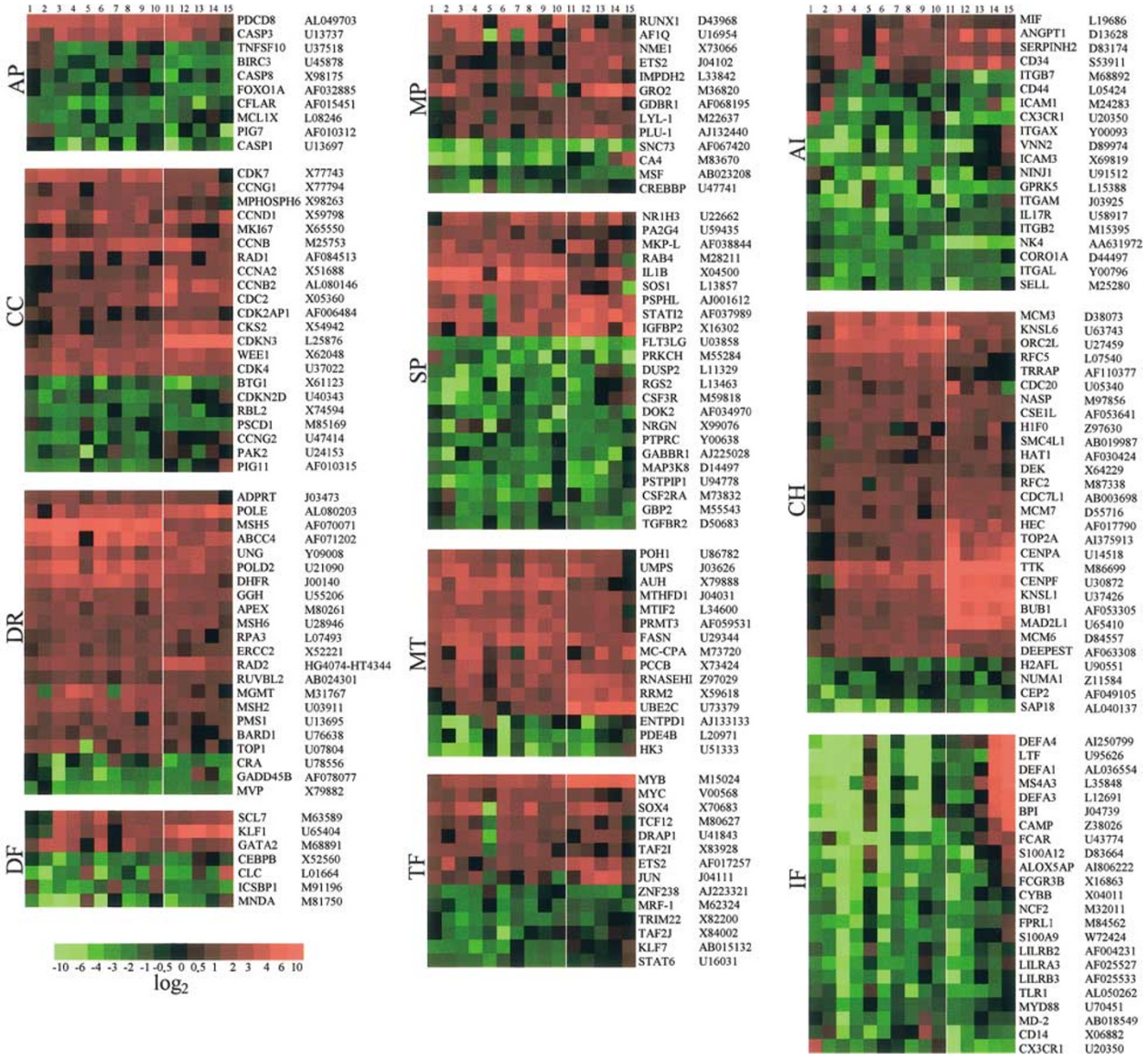


**Figure 5** Gene expression profiling according to the functional categories. Genes were assigned to the functional categories based on published information about their performance (AP = apoptosis, CC = cell cycle, AI = adhesion/invasion, DR = drug resistance, DF = differentiation, MP = malignant progression, SP = signaling pathways, MT = metabolism, TF = transcription factors, CH = chromosome/DNA dynamics, IF = infection). The highest changed genes in each category in CML samples versus healthy samples are shown. Patient samples numbers correspond to those in Figure 1. The level of color on the bar indicates the magnitude of upregulation (red) or downregulation (green) in comparison to appropriate control samples obtained from healthy donors

CML cells; only four pro-apoptotic genes (TNFSF10, PIG7, CASP1, CASP8) were inhibited in CML cells. Thus, microarray analysis did not detect the anti-apoptotic phenotype of CML cells, indicating that this function may be regulated at the post-transcriptional level. This conclusion, however, should be treated with caution, because the study represents a variety of leukemia cell subpopulations (mononuclear cells). It may be inaccurate with respect to a specific progenitor cell type.

Cell cycle in CML cells could be affected by aberrant expression of 148 genes represented in the array: 24 of them were changed on average by $\geqslant$4-fold (Figure 5, panel CC). Upregulated genes encode cyclins and associated proteins (CCNG1, CCND1, CCNA2, CCNB, CCNB2, CDK7, CDC2, CKS2, CDKN3, WEE1, CDK4, CDK2AP1), proliferation antigen Ki-67 (MKI67), checkpoint control (RAD1) and M-phase phosphoprotein (MPHOSPH6). Among negatively regulated genes was cyclin G2 (CCNG2), Arf GTPase (PSCD1), RB-like-2 p130 protein (RBL2) and those inhibiting cell cycle progression (BTG1, CDKN2D, PAK2, PIG11).

Among the 74 genes affecting adhesion/invasion/homing, 20 were changed on average by $\geqslant$4-fold in CML cells (Figure 5, panel AI). Upregulated genes

encode for macrophage-inhibitory factor (MIF), CD34 (putative role in adhesion/homing) and pro-angiogenesis factors (ANGPT1, SERPINH2); interestingly, an inhibitor of angiogenesis (NK4) was down-modulated. In addition, genes encoding integrins (ITGB7, ITGAX, ITGAM, ITGB2, ITGAL), integrin ligands (ICAM1, ICAM3), proteins involved in cell–extracellular matrix interactions and homing (CD44, NINJ1, CORO1 A, SELL), motility (VNN2) and response to chemokines (CX3CR1, GPRK5, IL17R) were also inhibited.

From 186 genes potentially involved in resistance to DNA damage, 22 were aberrantly regulated in CML cells on average by $\geqslant$4-fold (Figure 5, panel DR). The majority of upregulated gene products are involved in DNA repair, for example, recombination (RPA3, BARD1), nucleotide-excision repair (RPA3, ERCC2, RAD2), base-excision repair (UNG, APEX), mismatch recognition (MSH2, MSH6, PMS1), demethylation (MGMT), DNA synthesis and ligation (POLE, POLD2, ADPRT) and DNA relaxation (MSH5, RUVBL2, TOPI). The expression of three genes involved in drug efflux (MVP, ABCC4, GGH, CRA), one in metabolic targeting (DHFR) and one in genotoxic stress-induced apoptosis (GADD45B), was also modified.

Relatively indolent CML-CP inevitably progresses to malignant CML-BC. Among 104 genes present on the chip and potentially important for malignant transformation of CML-CP, only 13 were consistently dysregulated on average by $\geqslant$4-fold in all CML samples (Figure 5, MP panel). The expression of genes encoding transcriptional factors often involved in chromosomal translocations in AML, MLL or TCL (RUNX1, AF1Q, ETS2, LYL-1), chromatin-binding protein specifically upregulated in breast cancer (PLU-1), those associated with glioblastoma, epithelial ovarian carcinoma and squamous cell carcinoma metastatic phenotype (GBDR1, NME1, GRO2) and enzyme inosine-5-monophosphate dehydrogenase (IMPDH2) was enhanced. On the other hand, decreased expression of CA4 (inhibited in renal carcinoma), MSF (a fusion partner gene of MLL in therapy-related AML), SNC73 (inhibited in colon carcinoma) and CREBBP (inactivation causes Rubinstein-Taybi malformation syndrome) genes was detected.

BCR/ABL kinase regulates many signaling proteins by changing their phosphorylation status (Sattler and Griffin, 2001). In addition, it may also influence signaling pathways by regulating the expression pattern of the genes, whose products are involved in intracellular signaling. Among 743 genes encoding signaling molecules, 22 of them are aberrantly regulated in CML cells on average by $\geqslant$4-fold (Figure 5, SP panel). Upregulated genes encode for various proteins, for example, SOS1 (exchange factor in RAS pathway), phosphatases (MKP-L, PSPHL), GTPase involved in endosomes trafficking (RAB4), STAT-induced inhibitor (STATI2), nuclear orphan receptor (NR1H3), interleukin 1B (IL1B), and ErbB-3 and IGF-1-binding proteins (PA2G4, IGFBP2). Several genes, whose products inhibit signaling such as phosphatases (DUSP2, PTPRC, PSTPIP1), GTPases (RGS2, GBP2) and receptors and docking proteins exerting a negative influence on signaling (TGFBR2, DOK2) are downregulated. In addition, the expression of receptors and ligands (CSF3R, CSF2RA, GABBR1, FLT3LG) and other signaling molecules (PRKCH, NRGN, MAP3K8) was also inhibited.

Cellular metabolism may be affected in CML cells by abnormal expression on average by $\geqslant$4-fold of 17 genes among 758 listed on the chip (Figure 5, MT panel). Upregulated genes may enhance proteolytic (MC-CPA) or ubiquitin/proteasome (POH1, UBE2C)-dependent protein degradation, or stimulate protein metabolism (PRMT3) and synthesis (MTHFD1, MTIF2). In addition, genes involved in the metabolism of mRNA and ribonucleotides (AUH, UMPS, RNASEHI, RRM2) and lipids/fatty acids (FASN, PCCB) were also upregulated. A few genes were inhibited, including ENTPD1 (ATP and ADP hydrolysis) and PDE4B and HK3 (fatty acid and carbohydrate metabolism, respectively).

Analysis of the expression profile of 356 genes encoding transcriptional regulators revealed that 14 genes ($+5$ genes in DF panel: GATA2, SCL7, KFL1, ICSBP1, CEBPB) displayed abnormal expression in CML cells on average by $\geqslant$4-fold (Figure 5, panel TF). General transcriptional factors facilitating proliferation (MYB, MYC, ETS2, JUN) and development (SOX4), engaged in chromosomal transformations (TCF 12), formation of TFIID transcriptional complex (TAF21) and even repression of transcription (DRAPI), were stimulated. Those causing repression of transcription (ZNF238, TRIM22, KLF7) and others such as MRF-1 and STAT6, were downregulated.

Differentiation is represented on the chip by 40 genes, seven of them were affected in CML samples on average by $\geqslant$4-fold (Figure 5, panel DF). Genes encoding transcription factors, which are thought to promote and maintain the proliferation of early hematopoietic progenitors (GATA2, KFL1, SCL7) were upregulated; conversely, transcription regulators, which function as positive regulators of hematopoietic cell differentiation (CEBPB, ICSBP1), and also genes associated with maturation of hematopoietic cells (MNDA, CLC), were downregulated.

Gene expression profiling studies revealed a novel phenotype of CML cells: modification of chromosome/chromatin/DNA dynamics. Among 149 genes listed on the chip and potentially involved in these functions, expression of 29 was changed on average by $\geqslant$4-fold (Figure 5, panel CH). Many of the upregulated genes control chromosome/chromatin dynamics, for example, mitotic spindle assembly and checkpoint (CSEIL, KNSL6, KNLS1, CDC20, BUB1, MAD2L1, TTK, DEEPEST), centrosome organization (CENPA, CENPF), resolution of sister chromatids and chromosomes segregation (SMC4L1, HEC) and chromatin topology (DEK, TOP2A). In addition, DNA dynamics may be affected by upregulation of genes encoding histones (H1F0), histone-interacting protein (NASP) and histone acetyltransferases (HAT1, TRRAP). Moreover, DNA replication can be significantly modified by enhanced expression of genes encoding origin recognition complex

protein (ORC2L), replication initiation and maintenance complex proteins (CDC7L1, MCM3, MCM6, MCM7, RFC2, RFC5). A few genes were downregulated, for example, those encoding for H2A family member (H2AFL), spindle assembly (NUMA1), centrosome cohesion (CEP2) and partner for histone deacetylase (SAP18).

In addition, microarray analysis suggested another unknown phenotype of CML cells: a reduced capability to respond to the pathogen infections. Products of about 350 genes present on the chip may be involved in fighting infections, 23 of them were abnormally regulated in CML cells on average by $\geqslant$3-fold (Figure 5, panel IF); interestingly, all of them were downmodulated in most of the patient samples. Genes, whose products encode receptors involved in antipathogenic response (FCAR, FCGR3B, MS4A3, LILRA3, LILRB2, LILRB3, CD14, TLR1, MYD88, MD-2) and chemotactic response (S100A12, S100A9, CX3CR1, FPRL1, ALOX5AP) are included, suggesting that signaling, chemotaxis and phagocytosis may be inhibited in CML cells. In addition, antipathogen peptides (DEFA1, DEFA3, DEFA4, CAMP, BPI, LTF) and enzymes (CYBB, NCF2) produced by myeloid cells were also downregulated.

## Discussion

Significant heterogeneity in gene expression profiles in CML-CP samples has been detected by hierarchical clustering of 5315 genes based on their expression profiles, in accordance with a previous report (Ohmine *et al*., 2001). These observations indicate that although at this stage most of the leukemia cells usually demonstrate the phenotype of fully differentiated granulocyte, a subpopulation of mononuclear leukemia cells display profound differences in genotype (Figure 1a), which may have a substantial impact on their malignant progression toward blast crisis. Heterogeneity of our microarrray patterns probably did not result from individual genetic variations, because each leukemia sample was compared to a control sample representing pooled healthy donors. Further validation of the data was provided by analysis of expression of genes previously identified as BCR/ABL downstream transcriptional effectors (Figure 1b and c). Our analysis revealed that their expression pattern was in accordance with previous reports, thus providing the evidence that our array is faithful. The array of genes, with expression changed on average by $\geqslant$6 fold provides a signature of the gene expression profile in CML cells (Figure 1d). The signature includes aberrant regulation of the genes associated with cell cycle, DNA replication and repair, chromosome/chromatid dynamics and cell adhesion/invasion, thus indicating poor prognosis (van't Veer *et al*., 2002). This observation suggests that even at the chronic stage of the disease, BCR/ABL not only prolongs survival of leukemia cells, but also 'primes' the cells for progression towards a more malignant phenotype upon additional genetic changes.

CML-CP$\rightarrow$CML-BC progression is associated with major changes in phenotype and genotype of leukemia cells, which is reflected by differences in gene expression profile (Figure 3d). Surprisingly, we did not find many genes consistently changed in all CP and BC samples, which supports the idea that CML-BC is a very heterogeneic disorder. Although our samples were not delivered longitudinally from the same patients in CML-CP and after progression to CML-BC, this should not affect the array analysis identifying genes whose abnormal expression is 'universally' associated with the disease progression.

Interestingly, highly dysregulated genes in CML-BC cells obtained from bone marrow were mostly different from those detected in the peripheral blood leukemia blasts (Figure 3b and c, respectively). Direct comparison of the blasts from peripheral blood and bone marrow confirmed substantial differences in their gene expression profiles. Peripheral blood CML blasts seem to represent more aggressive phenotype than bone marrow counterparts. Genes encoding translation initiation factor (EIF4E), interleukin 8 (IL8) and activin receptor (ACVR1) in the former cells displayed expression pattern associated with malignant progression of solid tumors (Dua *et al*., 2001; Wildi *et al*., 2001; Xie, 2001). In addition, these cells demonstrated aberrant regulation of genes involved in cell–cell interactions, digestion of the extracellular matrix components, motility and chemotaxis (GSN = gelsolin; MMP9 = metalloproteinase 9; ELA2 = neutrophil elastase; PRTN3 = proteinase 3; CEACAM8 = CD66c, CEA family member; HMMR = hyaluronan-mediated motility receptor; JUP = plakoglobin; CCR2 = chemokine receptor 2). The perturbations in CC regulation (in particular, the completion of G2 and M phases) might increase if CML-BC cells enter the blood stream. Facilitation of CC completion could be achieved because of upregulation of UBE2C (ubiquitin-dependent proteolysis of the mitotic cyclins A and B, required for the completion of mitosis and entry into the next cell cycle (Aristarkhov *et al*., 1996)) and cdc2-related kinase CDKL1. On the other hand, downregulation of cdc25B may exert a negative influence on activation of cdc2, thus delaying mitotic onset. In addition, genes encoding BUB1 and MAD2L1, whose products inhibit anaphase progression (see the discussion below), are more dramatically upregulated in peripheral blood blasts than bone marrow ones. These perturbations in G2/M phase are associated with upregulation of mitotic checkpoint protein TTK and downregulation of centrosomal protein GOLGA1.

Peripheral blood blasts may be more resistant than bone marrow blasts to a variety of moderate stress signals such as free radical oxidative species, osmostress and dehydration, because of the upregulation of trehalose-6-phosphate synthase (TPS1) (Soto *et al*., 1999). Also, then relative resistance to complement and cytotoxic T lymphocyte attack may develop, because of the potential elevation of expression of protectin (CD59) and downregulation of HLA class II antigen DBY, respectively (Maio *et al*., 1998; Dasari

*et al*., 2001). These findings did not simply represent the differences in gene expression profile between bone marrow and peripheral blood cells, because the arrays were obtained after subtraction of the tissue-specific background. We also believe that these results do not reflect genetic variations between individuals because only genes changed in at least eight of 10 samples are listed. Thus, CML-BC cells residing in the bone marrow and those released into the peripheral blood may represent substantially different populations requiring different therapeutic approaches.

Expression profiling of the genes listed in functional categories may not only provide additional information about the genes potentially involved in the process (e.g. CC regulation), but also predict novel phenotype/function (e.g. chromosome/DNA dynamics). For example, the role of pro-angiogenetic factors in enhanced invasion/homing of CML cells in the nonhematopoietic organs has been implicated by the array (Figure 5, panel AI), in accordance with other studies (Janowska-Wieczorek *et al*., 2002). Except angiogenesis, BCR/ABL regulates adhesion/invasion/homing (Skorski *et al*., 1998; Salgia *et al*., 1999; Peled *et al*., 2002) by down-regulation of several genes, whose products intermediate in cell–cell or cell–extracellular matrix interaction and chemotaxis. Drug resistance in CML cells seems to be stimulated by overexpression of genes involved in various mechanisms of DNA repair, but not in drug metabolism and/or efflux (Figure 5, panel DR), in accordance with previous reports (Slupianek *et al*., 2001; Majsterek *et al*., 2002; Skorski, 2002).

BCR/ABL stimulates several major transcriptional activators such as: c-Myb, c-Myc and c-Jun (Ratajczak *et al*., 1992; Sawyers *et al*., 1992; Raitano *et al*., 1995), which in general promote cell proliferation. Microarray analysis revealed also that, independently of the disease stage, CML cells displayed elevated expression of transcriptional factors maintaining proliferation and reduced expression of those promoting differentiation. This observation suggests that BCR/ABL modulates transcription activity favoring proliferation over differentiation also in CML-CP, when the disease process is accompanied by increased numbers of immature hematopoietic cells retaining their ability to terminally differentiate. This is in agreement with the finding that BCR/ABL caused marked inhibition of cell maturation, but the cells eventually undergo terminal differentiation (Pierce *et al*., 2002). Moreover, CML cells in chronic phase and in blast crisis displayed a common 'malignant expression pattern' of several genes aberrantly regulated in other hematological and nonhematological malignancies (Figure 5, panel MP). Thus, the gene expression profiling indicated that the primary function of BCR/ABL is not only the inhibition of apoptosis, but also the changing of gene expression to favor eventual uncontrolled outgrowth. Thus, a new mutation, while harmless in normal hematopoietic cells, may readily trigger a malignant progression in CML-CP cells.

Gene expression profiling also revealed a novel putative function/phenotype of CML cells: modification of the chromosome/chromatid/DNA dynamics (Figure 5, panel CH). It seems that even if CC progression is facilitated by BCR/ABL (Figure 5, panel CC) (Cortez *et al*., 1997), CML cells may experience serious difficulties in completing mitosis in proper order. Leukemia cells display aberrant expression of a set of genes encoding proteins responsible for proper assembly of mitotic spindle, resolution of sister chromatids and segregation of chromosomes. For example, BCR/ABL promotes overexpression of cdc20, which can associate and activate anaphase-promoting complex (APC) (Fang, 2002). Activated APC assisted by the elevated levels of SMC4 and HEC may facilitate alignment and resolution of sister chromatids and progression through anaphase (Shah and Cleveland, 2000). On the other hand, BCR/ABL enhanced the expression of BUB1 and MAD2L1, two proteins, which bind to cdc20-APC complex and act cooperatively to prevent premature separation of sister chromatids by directly inhibiting APC (Fang, 2002). In addition, genes involved in centrosome splitting and bipolar mitotic spindle assembly (CEP2 and NUMA1) (Mayor *et al*., 2000; Merdes *et al*., 2000) were down-regulated in CML cells that creates even more problems for proper mitotic spindle function. This 'conflict of interest' at the mitotic spindle is probably sensed by the security mechanisms, because two mitotic checkpoint proteins TTK and CSEIL are overexpressed by CML cells (Behrens *et al*., 2001; Olesen *et al*., 2001). These abnormalities during M phase may contribute to the appearance of additional chromosomes, chromosomal translocations, chromosomal deletions, etc. often visible in CML-BC (Johansson *et al*., 2002). The topology of DNA could be also modified by enhanced histone acetylation and topoisomerase II expression, allowing better access for replication, transcription and repair machinery.

Analysis of expression of the genes engaged in anti-pathogen response revealed that the ability of CML myeloid cells to respond to the infection might be compromised (Figure 5, panel IF). CML cells displayed diminished levels of expression of the genes, whose products are involved in the innate immunity recognition (Janeway and Medzhitov, 2002), such as CD14, Toll-like receptor 1 (TLR1), MYD88 and MD-2. These proteins may form a functional complex, which upon recognition of the LPS can induce pathways signaling to anti-bacteria response. Inactivation of the innate immunity mechanisms abrogates the ability of an organism to respond immediately to microbial infection before the development of adoptive immunity. Moreover, a putative dysfunction of the Toll-receptor pathway is accompanied by diminished expression of the genes regulating chemotactic response along with those encoding anti-pathogen peptides and enzymes. Down-regulation of the genes involved in anti-infection responses may reflect delayed myeloid maturation of CML cells and their precocious and uncontrolled release into the blood stream (Dotti *et al*., 1999; Pierce *et al*., 2002). Altogether, CML patients could eventually demonstrate a tendency for suffering of infections (Akiyama *et al*., 1991; Itoh *et al*., 2001). The altered

expression profile of genes in CML cells conferring a reduced ability to respond to pathogen infections should be viewed with caution, since this was not assessed specifically in the cells involved in fighting pathogens.

BCR/ABL is expressed in a variety of cell subpopulations, for example, undifferentiated stem cells and mature granulocytes. Our analysis was performed on mononuclear cells, which include most of the cell types excluding those terminally differentiated. Therefore, the array represents gene expression profile in a wide range of leukemia cell subtypes. Caution should be applied because of the differences in composition of the mononuclear cell populations in normal and CML samples. However, these variations seem not to be a major factor, because the validation tests showed that expression of the reference genes detected by microarrays was in accordance with the published findings (see Figure 1b, c). Nevertheless, the use of more homogenous cell populations may reveal differences between gene expression profiles in the leukemia cell subpopulations.

In conclusion, gene expression profiling confirmed several previous findings about the biology of CML, added more genes to the list of potential effectors regulating known functions/phenotypes and implicated novel properties of CML cells. We believe that this work expands our knowledge regarding the genes associated with CML. However, it should be kept in mind that post-translational modification may counteract the direction of transcriptional regulation.

## Materials and methods

### *Patients and sample preparation*

We analysed 15 samples from $Ph^1$ CML patients, 10 from the bone marrow and five from the peripheral blood. Bone marrow samples were obtained from patients with chronic phase (CML-CP) and diagnosis (five cases) and from blast crisis (CML-BC) (five cases). Peripheral blood samples were harvested from CML-BC patients. All CML-BC samples displayed a myeloid phenotype. Cytogenetically, more than 90% of patient cells were Philadelphia chromosome positive ($Ph^1$), and blast crisis was characterized by $>30\%$ or $>50\%$ of the blasts + promyelocytes in peripheral blood or bone marrow, respectively (Hehlmann *et al*., 1994). Cells were collected for analysis before the patients received cytotoxic treatment, besides Hydrea in the BC cases. We obtained written informed consent and approval was obtained from the IRB at the University of California at Irvine, CA, USA, and from Johannes Gutenberg University at Mainz, Germany. Normal cells (four peripheral blood and three bone marrow samples) were obtained from healthy volunteers. Mononuclear cells were separated by density-gradient centrifugation through Histopaque-1007 (Sigma Diagnostics, Inc., St Louis, MO, USA). CML samples usually consisted of almost 100% of $Ph^1$ cells; normal bone marrow and peripheral blood samples usually contained $>90\%$ and 20–30% of myeloid cells, respectively. Total RNA was extracted by QIAamp® RNA Blood Mini Kit 52304, RNeasy® Mini Kit 74104 and RNase-Free DNase Set 79254 (QIAGEN, Valencia, CA, USA).

### *Oligonucleotide microarray*

The protocol for sample preparation and microarray processing is available from Affymetrix (Santa Clara, CA, USA). In brief, at least 8 $\mu$g of total RNA was reverse transcribed by Superscript II reverse transcriptase (Life Technologies, Grand Island, NY, USA) with T7-$(dT)_{24}$ primer, which contains a T7 RNA polymerase promoter site. We synthesized the second cDNA strand and then used this product in an *in vitro* transcription reaction to generate biotinylated cRNA. Fragmented cRNA (10 $\mu$g) was hybridized to a Human Genome U95Av2 Array (Affymetrix) for 16 h at 45°C with constant rotation at 60 r.p.m. This high-density oligonucleotide-based array targets 12 530 human genes from the US National Center for Biotechnology Information GenBank and The Institute for Genomic Research (TIGR) databases. After hybridization, the microarray was washed, stained on an Affymetrix fluidics station and scanned with an argon-ion confocal laser with 488 nm excitation and 570 nm detection wavelengths. The array images were quantified utilizing MicroArray Suite (MAS) v4.0 software (Affymetrix). The average fluorescence intensity was determined for each microarray and then the output of each experiment was globally scaled to a target intensity of 2500 (Voehringer *et al*., 2000). The data results from all of the arrays were imported into Microsoft Excel XP (Microsoft, Redmond, USA). All microarray assays were performed in the DNA Microarray Core Facility, University of California at Irvine, CA, USA.

### *Data analysis*

Normalized Affymetrix data were converted into a ratio between the mean expression level of CML samples from bone marrow and the mean from three normal bone marrow samples, or the mean expression level CML samples from peripheral blood and the mean from four normal blood samples. Data are presented as logarithmic scale (base = 2). All analyses carried out were based only on genes where the average expression level was significantly present (P) or marginally present (M) (Wodicka *et al*., 1997; Schadt *et al*., 2000); the raw data values had to be at least 500 (Hofmann *et al*., 2002). Hierarchical clustering and self-organizing maps (SOM) analysis were performed in the GENE CLUSTER program. TREEVIEW program was used for graphic presentation of the results (Eisen *et al*., 1998).

### *RT–PCR*

Total RNA was extracted by RNeasy® Mini Kit 74104 (QIAGEN, Valencia, CA, USA). Reverse transcription reactions were performed in the following conditions: total volume 20 $\mu$l, 1 $\mu$g of total RNA, 2 $\mu$g of random primer $p(dN)_6$ (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannnheim, Germany), 10 $\mu$M dNTPs (Eppendorf AG, Barkhausenweg 1, 22331 Hamburg, Germany), 5 mM DTT (Fisher Scientific, Fair Lawn NJ 07410, USA), 150 ng/ml acetylated BSA (Promega, 2800 Wood Hollow Rd., Madison WI 53711, USA) and 24 U of reverse transcriptase AMV (Roche). RT reaction (8 $\mu$l) was used as a template for 30 cycles of PCR (initial denaturation 94°C/5 min, denaturation 94°C/45 s, annealing 54.5°C/45 s, polymerization 72°C/45 s, final polymerization 72°C/5 min) along with 5 U of Taq polymerase (Promega). The following primers were used: BUB1 (gb:AF053305, primers: 2845–2863, 3075–3095, PCR prod: 231 bp), IL1B (gb:X04500, primers: 8313–8333, 8486–8506, PCR product: 174 bp), MSH5 (gb:AF070071, primers: 2617–2637, 2826–2846, PCR product: 210 bp), MYB (gb:M15024, primers: 2728–2748, 2882–2902, PCR product: 155 bp), NK4 (gb:AA631972, primers: 463–483, 703–723, PCR product: 241 bp), SNC73

(gb:AF067420, primers: 997–1017, 1166–1186, PCR product: 170 bp), TTK (gb:M86699, primers: 3408–3428, 3626–3646, PCR product: 219 bp). GADPH (gb:AF261085, primers: 629–649, 1080–1100, PCR product: 452 bp) gene product was used as loading/performance control of the double-product semi-quantitative PCR. The products were resolved in 2% agarose gels containing 0.5 $\mu$g/ml of ethidium bromide.

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