Leukemia (2006) 20, 1400–1407

www.nature.com/leu

ORIGINAL ARTICLE

Gene expression signature of primary imatinib-resistant chronic myeloid leukemia patients

O Frank[1], B Brors[2], A Fabarius[1], L Li[3], M Haak[3], S Merk[4], U Schwindel[1], C Zheng[1], MC Müller[1], N Gretz[3], R Hehlmann[1], A Hochhaus[1] and W Seifarth[1]

[1]III. Medizinische Klinik, Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Universitätsklinikum Mannheim der Ruprecht-Karls-Universität Heidelberg, Mannheim, Germany; [2]Abt. Theoret. Bioinformatik, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany; [3]Zentrum für Medizinische Forschung (ZMF), Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Mannheim, Germany and [4]Department of Medical Informatics and Biomathematics, University of Muenster, Münster, Germany

Although the selective tyrosine kinase inhibitor imatinib is successfully used in the treatment of chronic myeloid leukemia (CML), inherent mechanisms confer primary resistance to leukemic patients. In order to search for potentially useful genes in predicting cytogenetic response, a retrospective gene expression study was performed. Leukocyte RNA isolated before imatinib from interferon-alpha-pretreated chronic phase CML patients ($n=34$) with or without major cytogenetic remission ($\leqslant$35% Philadelphia (Ph)$^+$ metaphases) during the first year of treatment was comparatively analyzed using Affymetrix U133A chips. Using support vector machines for gene classification, an outcome-specific gene expression signature consisting of 128 genes was identified. Comparative expression data of specific genes point to changes in apoptosis (e.g. *casp9*, tumor necrosis factor receptor-associated protein 1, *hras*), DNA repair (*msh3*, *ddb2*), oxidative stress protection (glutathione synthetase, paraoxonase 2, vanin 1) and centrosomes (inhibitor of differentiation-1) within primary resistant patients. Independent statistical approaches and quantitative real-time reverse transcriptase-polymerase chain reaction studies support the clinical relevance of gene profiling. In conclusion, this study establishes a candidate predictor of imatinib resistance in interferon-alpha-pretreated CML patients to be subjected to future investigation in a larger independent patient cohort. The resulting expression signature point to involvement of BCR-ABL-independent mechanisms of resistance.
Leukemia (2006) **20,** 1400–1407. doi:10.1038/sj.leu.2404270; published online 25 May 2006
Keywords: CML; gene expression profiling; microarray; imatinib; primary resistance; cytogenetic response

Introduction

Chronic myeloid leukemia (CML) is consistently associated with an acquired cytogenetic abnormality, the Philadelphia (Ph) chromosome, a shortened chromosome 22 resulting from a reciprocal translocation of the long arms of chromosomes 9 and 22. This translocation generates the *bcr-abl* fusion gene, which is translated in an oncoprotein with constitutive tyrosine kinase activity (BCR-ABL) that has important roles in proliferation and survival of myeloid progenitor cells.[1]

Conventional therapeutic options for the treatment of CML are allogeneic hematopoietic stem cell transplantation as the only potential cure, and administration of interferon-alpha (IFN-α). The mean rate of complete cytogenetic remission (CCR) in IFN-α-treated patients is 13% (range 5–33%). The 10-year survival rate from first CCR is 72% and is related to the risk profile.[2] The advent of imatinib (Glivec®, formerly STI571), a potent and selective inhibitor of BCR-ABL, represents an important advance in the management of CML and has led the way to targeted therapy of cancer in general. In a phase III study of newly diagnosed chronic phase (CP) CML, imatinib was superior to IFN-α plus cytarabine in cytogenetic response, freedom-from-disease-progression and tolerability.[3] Even after IFN-α failure, imatinib induced major cytogenetic response rates of 60% in CP CML, CCR rates of 40%, and 18-month estimated freedom-from-progression and survival rates of 89% and 95%, respectively.[4] In a more recent study, imatinib-treated patients demonstrated a better 4-year survival rate than patients receiving non-imatinib therapies.[5] Despite the achievable remission rates, resistance to imatinib is an important issue for therapy, as a minority of CML patients in CP and a substantial proportion in advanced disease phases display either refractoriness to imatinib treatment or loose imatinib sensitivity over time and experience relapse.[4] Whereas acquired resistance could be mainly attributed to an impaired drug-binding capability of BCR-ABL,[6] the nature of intrinsic resistance still needs clarification.[7–9]

Gene expression analysis by microarrays is a powerful technology, which has been used to gain valuable information for tumor diagnosis, prognosis and therapy development on various human cancers including acute and chronic leukemias.[10,11] Robust gene expression profiles had been demonstrated to discriminate between cytogenetically defined acute myeloid leukemia subtypes for diagnostic purposes.[12] In the case of Ph$^+$ acute lymphoblastic leukemia, genetic factors had been identified that can predict the development of resistance to imatinib[13] and a longer survival in CML.[14] In this study, we set out to identify predictive genes for discriminating primary imatinib responders from non-responders in CML. Such a predictor may serve as a prognostic marker and might support therapeutic decisions. Furthermore, the gene expression signature could hint to molecular mechanisms involved in primary cytogenetic imatinib resistance.

Correspondence: Dr O Frank, III. Medizinische Klinik, Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Universitätsklinikum Mannheim der Ruprecht-Karls-Universität Heidelberg, Wiesbadener Strasse 7-11, D-68305 Mannheim, Germany.
E-mail: oliver.frank@med3.ma.uni-heidelberg.de
Received 14 December 2005; revised 10 April 2006; accepted 13 April 2006; published online 25 May 2006

Materials and methods

Patient characteristics

Imatinib-treated patients were evaluated for hematologic, cytogenetic and molecular responses for a minimal observation time of 12 months. Patients who remain in a major (complete or

partial) cytogenetic remission (MCR; $\leqslant$35% Ph^+ metaphases) after initial response were defined as responders ($n=23$; CCR: $n=17$), whereas those with a continuous minor (minimal or no) cytogenetic remission (lack of MCR; >35% Ph^+ metaphases) were considered non-responders ($n=11$; no cytogenetic remission = NoCR: $n=7$). Informed consent was obtained from all patients included in this study. Patients were considered for the gene profiling study if they met the following criteria: (i) CML in CP, (ii) failure of IFN-α treatment owing to intolerance, refractoriness or resistance before imatinib therapy and (iii) definite clinical outcome based on cytogenetics measured within the first 12 months of treatment to discriminate responders from non-responders. Diagnostic groups were matched according to demographic and hematologic parameters as shown in Supplementary Table 1.

Cytogenetic analysis
Cytogenetic studies were performed on bone marrow (BM) aspirates according to standard protocols.[15]

Determination of bcr-abl *variants and transcription levels in patients*
Before the start of imatinib therapy, patients were screened for the expression of *bcr-abl* transcripts by multiplex polymerase chain reaction.[16] Concomitantly, *bcr-abl* transcript levels were determined by quantitative real-time reverse transcriptase-PCR (QRT-PCR).[17,18] In the follow-up, patients were monitored for their molecular response to therapy in three-monthly intervals.

Selection of high-quality leukemic RNA extracts
From leukemic samples (BM, peripheral blood (PB)) that were collected before imatinib therapy, total white blood cells were isolated by hypotonic red cell lysis, washed and transferred into a guanidinium isothiocyanate-containing buffer. Total RNA was extracted by commercially available kits (RNeasy, Qiagen, Hilden, Germany) or by CsCl gradient centrifugation[19] and stored at $-80^{\circ}C$. RNA quality was assayed by Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) and degradometer software (version 1.4).[20]

Oligonucleotide microarray hybridization
Hybridization probe generation (starting from 3.3 to 8 μg of total RNA) and HG-U133A microarray processing were performed according to the standard protocols available from Affymetrix (Santa Clara, CA, USA). Detailed information about experimental settings and raw data have been submitted to ArrayExpress, a public repository for microarray data (http://www.ebi.ac.uk/arrayexpress/) and are available under accession number E-MEXP-433.

Microarray analysis and statistical procedures
Raw data from Affymetrix CEL files were normalized using the method described by Huber *et al.*,[21] for which a brief description can be found in the Supplementary data. For supervised learning, support vector machines (SVM) with a linear kernel function[22] were used. Classification accuracies were determined by 10-fold cross-validation. A discriminative gene expression pattern was obtained by using recursive feature elimination (RFE)[23] together with SVM (for details see Supplements).

Detailed descriptions of further normalization procedures (robust multiarray average (RMA)), statistics and algorithms for data evaluation such as analysis of variance (ANOVA), significance analysis of microarrays (SAM) and prediction analysis of microarrays (PAM) are available from the journals' website in the Supplementary file.

cDNA synthesis
Three micrograms of the same total RNA preparation used for microarray analysis were reverse transcribed for subsequent QRT-PCR using random hexamer priming and moloney murine leukemia virus reverse transcriptase as described previously.[19] This amount of starting material was available for 26 patients in total (18 responders; eight non-responders).

QRT-PCR analysis
For corroboration of microarray data, QRT-PCR (LightCycler technology, Roche, Mannheim, Germany) was performed for a panel of representative genes (cochlin precursor (*coch*), vanin 1 (*vnn1*), anaphase-promoting complex subunit 5 (*anapc5*), tryptase alpha/beta1/beta 2 (*tpsab1/b2*) and rabphilin 3A homolog (mouse) (*rph3a*)). Beta-glucoronidase (*gus*) served as reference gene that has been demonstrated to be the best suitable housekeeping gene for testing leukemic tumor samples.[24,25] Primer pairs, hybridization probes and QRT-PCR conditions are given in the Supplementary file. The resulting

Table 1 Selection of SVM-classified genes from pathways related to mechanisms of primary cytogenetic resistance

Pathway	*Symbol*	*Description*	*Fold change*	*Affymetrix ID*
Apoptosis	*casp9*	Caspase-9, apoptosis-related cysteine protease	0.937	210775_x_at
Apoptosis	*blk*	B lymphoid tyrosine kinase	0.898	206255_at
Apoptosis	*trap1*	TNF receptor-associated protein 1	1.128	201391_at
Apoptosis	*za20d1/cezanne*	Zinc-finger, A20 domain containing 1	0.946	220031_at
Apoptosis	*foxj1*	Forkhead box J1	0.932	205906_at
Apoptosis	*hras*	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	1.087	212983_at
Progression	*enpp2/atx*	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	1.096	210839_s_at
Progression	*gpr132/g2a*	G-protein-coupled receptor 132	0.885	221140_s_at
Oxidative stress	*gss*	Glutathione synthetase	1.106	201415_at
Oxidative stress	*vnn1*	Vanin 1	0.547	205844_at
Oxidative stress	*pon2*	Paraoxonase 2	1.152	201876_at
DNA repair	*msh3*	mutS homolog 3 (*Escherichia coli*)	1.128	205887_x_at
DNA repair	*ddb2*	Damage-specific DNA-binding protein 2, 48 kDa	1.100	203409_at
Centrosomes	*id1*	Inhibitor of DNA-binding protein 1, dominant-negative helix–loop–helix protein	1.216	208937_s_at

Abbreviations: SVM, support vector machines; TNF, tumor necrosis factor.

expression levels of target genes were normalized by division through the mean expression value of the reference gene (*gus*).

Results

Patient characteristics

In order to identify genes associated with primary cytogenetic resistance to imatinib, patient groups corresponding to responders ($n=23$) and non-responders ($n=11$), and matched for demographic and hematologic parameters, were used for all experiments (for details see Supplementary file: Results and Table 1).

Comparing the gene expression in BM versus PB

The aim of this study was to develop a set of genes suitable to predict the outcome of CML patients regarding primary imatinib resistance. As this predictor should be valid irrespective to the cells used (PB or BM), differences in gene expression between both sample types were analyzed first. BM and PB samples simultaneously taken just before the start of an imatinib-based regimen from seven imatinib responders were analyzed by Affymetrix gene chip hybridization. Microarray data were statistically analyzed based on mixed log-linear models (for details see Supplements). Results are depicted in Supplementary Figure 1 as a volcano plot. The red vertical line represents a cutoff value based on Bonferroni correction and therefore serves to define differentially regulated genes between both sample groups. In total, 184 genes discriminating between PB and BM samples were identified.

Gene expression profile of cytogenetic response after IFN-α failure

Analysis of gene profiles corresponding to 23 known cytogenetic responders (29 samples; one BM sample of a responder was kept for test set analysis; see below) and 11 non-responders (12 samples) by SVM-RFE revealed 128 genes (Supplementary Table 2) of highest discriminatory power for optimal separation of both diagnostic groups ('candidate predictor'). Importantly, no intersection between these 128 classification genes was found when compared to the differentially expressed genes ($n=184$ above Bonferroni correction) between BM and PB samples in Supplementary Figure 1. Based on hierarchical cluster analysis, SVM data are shown as heatmap in Figure 1. Specific genes associated with molecular pathways potentially related to mechanisms of primary resistance (inhibition of apoptosis, disease progression, oxidative stress protection, DNA repair, centrosomes) are shown in Table 1.

Comparison of results from independent statistical methods

In order to check for validity of microarray-based gene expression data, lists of differentially transcribed genes obtained from independent statistical analyses (SVM-RFE: $n=128$; ANOVA: $n=295$; PAM: $n=97$ genes; for detailed selection criteria see Supplementary file) were compared. Results are illustrated as a Venn diagram (Supplementary Figure 2) showing a good general comparability between the methods used and 17 commonly found genes. Results of a SAM analysis revealed 10 genes (100%) that are listed in both ANOVA and SVM-RFE output files (data not shown). Six of them were found in the list from PAM analysis.

Confirmation of microarray data by QRT-PCR

Of the 128 SVM-selected and differentially expressed genes (Figure 1, Supplementary Table 2), five with the highest fold changes were chosen for QRT-PCR analysis for verification (Table 2). Four of these are contained within the list of the 17 commonly found genes. Of the selected genes, three were overexpressed in imatinib non-responders compared to responders: *coch*, *tpsab1/b2* and *anapc5*. Downregulation was observed for: *vnn1* and *rph3a*. For all but one gene (*anapc5*), the results demonstrate a good concordance between microarray and QRT-PCR. Consistency was found for *vnn1* and *rph3a* transcript levels ($P<0.0134$), correlation for *tpsab1/b2* and *coch* (Figure 2).

Predicting the cytogenetic outcome in independent patient samples (test set)

The potential predictor consisting of 128 genes was tested on two independent patients with primary resistance that became accessible after having completed the study. Of those, one had been pretreated with IFN-α, whereas the other one had not. From the latter, both PB and BM samples were available. All three samples were used together with a BM sample kept from an IFN-α-pretreated responder patient (see above) for microarray assessment to apply the identified profile based on the 128 SVM-selected genes. The responder served as an internal control, as a PB sample of this patient was used to create the outcome-specific signature based on the 41 training set samples. All three patients were correctly assigned to their respective diagnostic group (Figure 1), confirming the validity of our candidate gene predictor in this small test set.

Discussion

Imatinib has revolutionized drug therapy of CML. However, a subset of patients displays resistance against this selective tyrosine kinase inhibitor. For early risk stratification, it would be highly desirable to predict a patients' individual drug response in order to consider alternative treatment options (e.g. a potentially curative stem cell transplantation). Therefore, the primary aim of this study was to identify prognostic genes by means of gene expression profiling that are capable of predicting clinical outcome (i.e. whether or not an MCR within the first year of treatment will be achieved).

Whereas mechanisms underlying an acquired resistance are well described,[26] uncovering of those responsible for primary inherent resistance has just started.[7,9] Therefore, a secondary aim was to search for genes underlying primary resistance using a genome-wide gene expression approach (Affymetrix HG-U133A DNA chip).

The resulting outcome-specific gene expression signature, identified by the use of SVM, comprises 128 genes. Independent statistical approaches using PAM and ANOVA analyses support the data. In order to confirm the microarray data, those five genes with the highest fold change in SVM classification discriminating both diagnostic groups were selected for QRT-PCR. The observed small differences in fold change found by our microarray analysis could be an explanation for the ability of confirming only a few of the tested genes as differentially expressed. This is in line with observations of Dallas *et al.*,[27] who noticed a trend toward less correlation between microarray and QRT-PCR for genes that exhibited fold change differences of <1.5. Nevertheless, four of the five analyzed genes (*vnn1*, *rph3a*, *tpsab1/b2*, *coch*) could be supported by QRT-PCR.

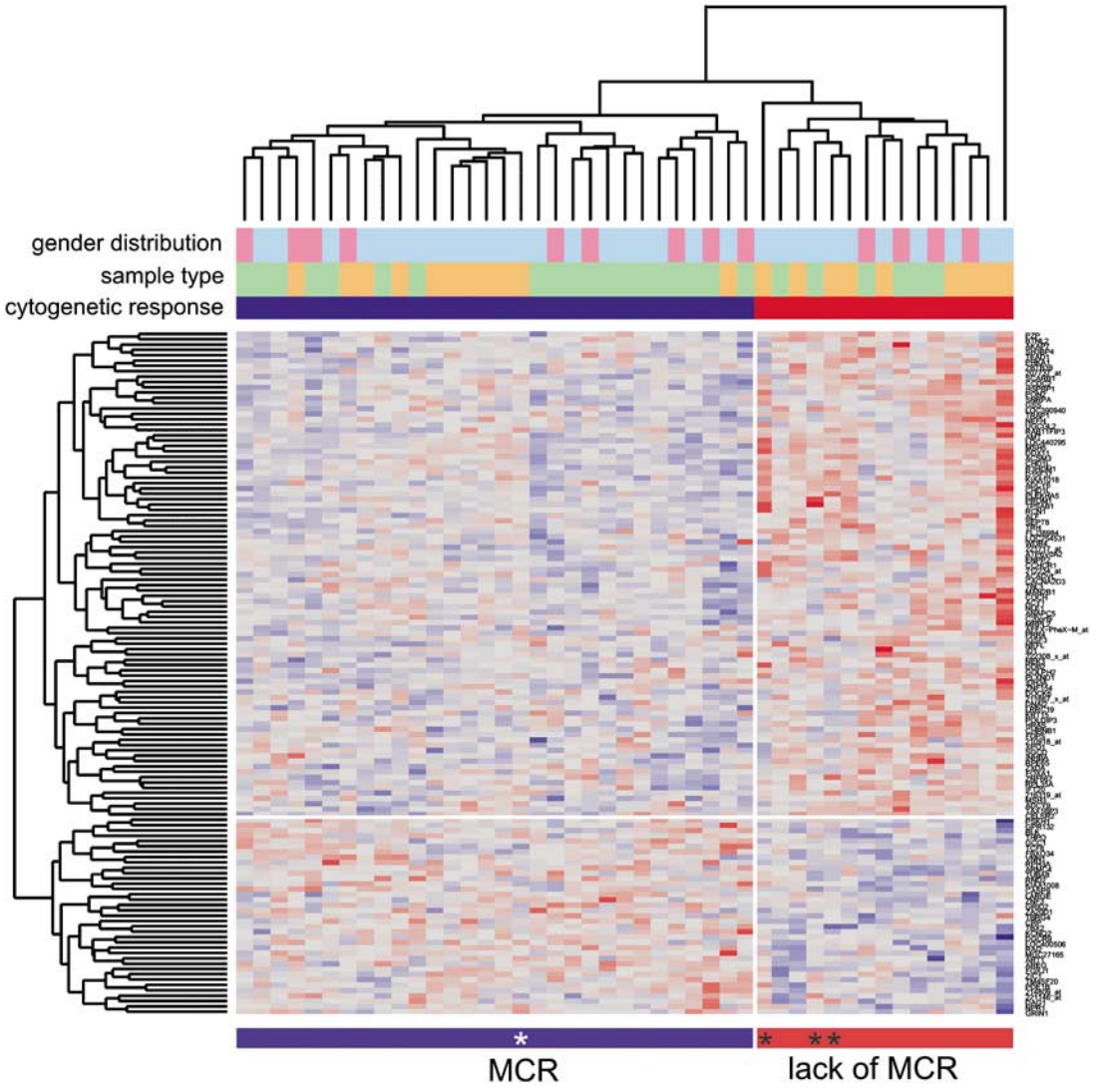

Figure 1 Outcome-specific gene expression signature in CML. Hierarchical cluster analysis of CML samples of 36 patients with different cytogenetic responses to imatinib treatment within the first 12 months of therapy. The heatmap combines training set ($n = 41$) and test set samples ($n = 4$), the latter being marked by asterisks. Using SVM-RFE, 128 genes that were required to discriminate between both diagnostic patient groups according to their cytogenetic response profile were identified. Heatmaps were constructed using z-transformed gene expression values, that is, measurements were scaled to have gene-wise zero mean and gene-wise unit variance. Columns (samples) and rows (probe sets) of the gene expression matrix were reordered by hierarchical clustering using an Euclidean distance metric and the complete linkage algorithm. Colored bars above the heatmap illustrate (i) gender distribution (pink = female, light blue = male), (ii) sample type (green = PB; orange = BM) and (iii) cytogenetic response (blue = MCR, red = lack of MCR).

Table 2 Genes with the highest fold change in SVM classification were used for corroboration of microarray data by QRT-PCR

Pathway	*Symbol*	*Description*	*Fold change*	*Affymetrix ID*
Perception of sound	*coch*	Coagulation factor C homolog, cochlin (*Limulus polyphemus*)	1.362	205229_s_at
Oxidative stress	*vnn1*	Vanin 1	0.547	205844_at
Intracellular protein transport	*rph3a*	Rabphilin 3A homolog (mouse)	0.661	205230_at
Proteolysis and peptidolysis	*tpsab1/b2*	Tryptase alpha/beta 1; tryptase beta 2	1.487	216474_x_at
Ubiquitin-mediated proteolysis	*anapc5*	Anaphase-promoting complex subunit 5	1.279	208722_s_at

Abbreviations: QRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; SVM, support vector machines.

Genes and pathways related to mechanisms of primary resistance

Detailed analysis of the 128 SVM-selected genes revealed several candidates linked to relevant signaling pathways in CML, which may represent pathogenetically relevant genes. One known pathomechanism of BCR-ABL-induced transformation of hematopoietic cells involves inhibition of apoptosis.[28] Albeit displaying minor transcriptional changes, several genes

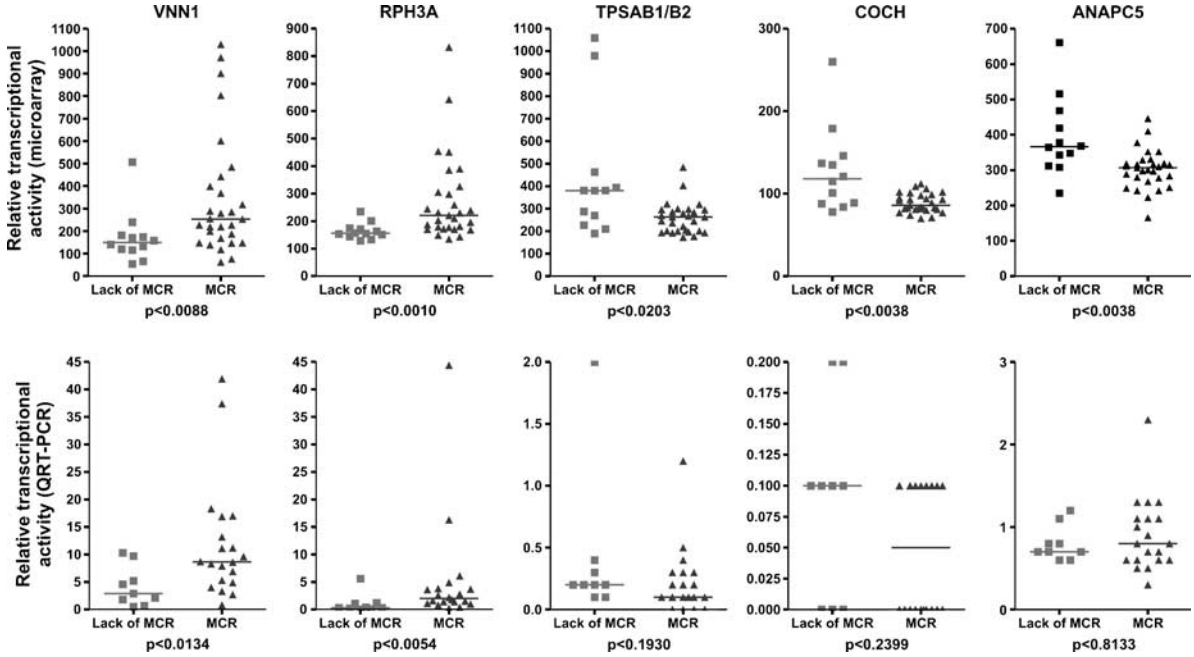

Figure 2 Comparison of gene expression data from selected genes of the 128-gene candidate predictor measured either by microarray analysis or QRT-PCR. Five candidate genes (*vnn1*, *rph3a*, *coch*, *anapc5*, *tpsab1/b2*), for which a differential transcriptional activity was found in microarray analysis (upper panel), were investigated by QRT-PCR (lower panel). Relative gene expression values (*y* axis) are given as gene/*gus* ratio.

may synergistically act on apoptotic pathways. This may hold true for *casp9* that is downregulated in imatinib non-responders. Previous publications have shown that the inhibition of CASP9 activity represents a mechanism of acquired cisplatin resistance in a certain type of head and neck squamous cell carcinoma.[29] This also holds true for *blk*, a gene encoding an SRC (sarcoma virus) kinase found to be downregulated in B-cell chronic lymphocytic leukemia cells that were resistant to DNA damage-induced apoptosis.[30] Of importance, in Ph chromosome-positive leukemia a role of tyrosine kinases has been stated for reducing BCR-ABL dependence and for reducing imatinib efficacy.[13] In addition, it has been suggested that downregulation of tumor necrosis factor receptor-associated protein 1 (TRAP1), a member of the heat-shock family of proteins, might play an important role in the induction of apoptosis potentially caused by the formation of reactive oxygen species (ROS).[31] Therefore, owing to the observed upregulated *trap1* expression, sustained apoptosis-inhibiting effects should be expected as its expression level remained unaffected by imatinib in a previous study.[31] A known pathway associated with antiapoptotic responses includes the nuclear factor-κB (NF-κB) signaling cascade. The transcription factor NF-κB itself has been identified as an inhibitor of apoptosis and as a potential regulator of cellular transformation mediated by BCR-ABL.[1,32] Within the non-responder group, the transcriptional downregulation of *cezanne/za20d1* and *foxj1* corresponding to known suppressors of NF-κB[33,34] was observed. Collectively, these findings point toward an overall impairment of apoptotic pathways in primary imatinib-resistant CP CML patients. Further support to this concept is the finding of *hras* overexpression observed in non-responders. Constitutive activation of this mitogenic molecule leads to an antiapoptotic pathway that is insufficient but required for transformation in CML.[35] These genes could represent a potential interplay in a combinatorial manner, and thus, despite small fold changes, may cause biologically important effects according to a principle suggested by Wurmbach *et al*.[36]

Disease progression genes

Interestingly, overexpression of *atx/enpp2*, another gene that appears to augment the invasive and metastatic potential of ras-transformed cells,[37] was identified. Taking into account that this gene has been shown to be upregulated within CML blast crisis patients when compared to those in CML-CP,[38] it could be hypothesized that the imatinib non-responders, although not in accelerated phase (according to the definition criteria by Talpaz *et al*.[39]), are in a more advanced stage of CP than the responders. This hypothesis is supported by the occurrence of chromosomal aberrations in addition to the Ph chromosome in some (four of 11) of the non-responders compared to the responders (Supplementary Table 1). This may account for a BCR-ABL-independent disease phase resulting in the observed lack of a primary cytogenetic response. Of importance, the latter patient group showed overexpression of *g2a/gpr132* that is known to attenuate the transformation potential of BCR-ABL.[40]

Genes associated with oxidative stress

Some genes were uncovered with functions associated with oxidative stress. Recently, a model has been proposed claiming that overexpression of H-ras increases ROS and glutathione (GSH) levels, thereby triggering ras-transformed cell growth.[41] Expression data of glutathione synthetase (*gss*) within non-responders are in line with this model pointing to the importance of genes involved in oxidative stress protection. In addition, a major downregulation of *vnn1*, a negative regulator of cellular GSH storage, was identified. Elevated GSH levels correlate with resistance to oxidative injury and with reduced apoptosis when *vnn1* is knocked out.[42] Another gene protecting

against cellular oxidative stress that was found to be upregulated is *pon2*. It has been argued that during monocytic maturation into macrophages, PON2 stimulation may modulate cellular superoxide anion production.[43]

Genes with functions in DNA repair

Genes of the DNA repair system were found to be upregulated within the group of non-responders. These comprise *msh3* and a target gene of p53, *ddb2*, the gene product of which is a known interaction partner of the tyrosine kinase c-ABL.[44] This overexpression might compensate for genetic lesions accumulating within malignant cells resistant to apoptotic signals.

Centrosomal genes

Centrosomal aberrations are hallmarks of various cancers. Their occurrence in CML correlates with disease stage and chromosomal instability.[45] The helix–loop–helix protein inhibitor of differentiation-1 (ID1) localizes to centrosomes and rapidly induces abnormal centrosome numbers.[46] As high levels of ID1 expression are frequently detected in various cancer types and may correlate with poor clinical prognosis,[47,48] it is tempting to speculate if the ID1 upregulation as observed in the non-responders exerts a pathogenetic effect by karyotype destabilization. This could be accomplished by modulation of centrosomal fidelity, which is known to be a major parameter in aneuploidy accompanying disease progression.[45]

Comparison with previous microarray studies on CML patients

There were only few overlaps of this gene list resulting from expression profiling of CML and those published by other groups.[8,38,49–52] Specifically, these genes comprise those with functions in DNA repair (*msh5*) and tumor progression (*dag1*, *enpp2*). With respect to the observed deviations, several explanations may be considered. Obviously, major differences in gene expression are warranted owing to the use of samples prepared from diverse cell fractions/cellular origin (i.e. whole blood vs mononuclear cells, AC133$^+$ blasts). Also, variations in the composition of patient populations (i.e. sample number, ethnical and gender aspects, stage of disease, definition criteria of imatinib response, type and duration of pretreatments with other drugs, occurrence of chromosomal aberrations in addition to the Ph chromosome), the quality of RNA samples, the microarray platforms (Affymetrix technology, custom cDNA arrays) and the statistical algorithms used could have contributed to the differences in gene expression. We generally agree that in contrast to acute leukemias, the low amount of blast cell populations within CML samples still represents a challenging task for DNA microarray investigations.[8,52] Nevertheless, in order to apply a clinically feasible approach for predicting patients drug response, whole leukocyte RNA samples that were carefully selected for equal quality were used. State-of-the-art algorithms such as SVM have been employed that has been demonstrated to outperform all other methods for building predictive models.[53] Although the differences detected by microarray analysis were small (<2-fold), it has been reported that changes in gene expression can significantly be underestimated by DNA chip platforms.[54] Thereby, using a high cutoff in order to exclude false-positive results will miss important biological changes. Definitely, these had to be verified by additional tests (QRT-PCR; Northern blot) as has been performed in previous microarray studies on data displaying low fold changes (<2).[55,56] Prospectively, our candidate predictor will be further explored on samples from CML patients currently treated in clinical studies.

Conclusion

Based on SVM classification, a potential 128-gene predictor for early assessment of primary cytogenetic response of CML patients to imatinib was established. The data suggest that transcriptional regulation of apoptotic and antiapoptotic genes, disease progression genes, oxidative stress genes, genes for DNA repair and centrosomal genes are associated with imatinib resistance in CP CML. These findings are in line with the concept of a multifactorial development of resistance against imatinib pointing to the involvement of several BCR-ABL-independent pathways.

Acknowledgements

This work was supported by the Competence Network 'Acute and chronic leukemias' (Grant 01 GI9980/6 from the German Ministry of Education and Research, BMBF and by research funds of the Fakultät für Klinische Medizin Mannheim). We are very grateful for statistical advice by Dr Christel Weiss (Medizinische Statistik, Biomathematik und Informationsverarbeitung, University of Heidelberg, Mannheim, Germany). We thank Maike Haas, Christine Philipps and Christine Folz for excellent technical assistance. Benedikt Brors received funding from the BMBF through the National Genome Research Network (Grant 01 GS 0450).

References

1 Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood* 2004; **103**: 4010–4022.
2 Bonifazi F, de Vivo A, Rosti G, Guilhot F, Guilhot J, Trabacchi E *et al*. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood* 2001; **98**: 3074–3081.
3 O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F *et al*. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2003; **348**: 994–1004.
4 Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C *et al*. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 2002; **346**: 645–652.
5 Kantarjian HM, Cortes JE, O'Brien S, Luthra R, Giles F, Verstovsek S *et al*. Long-term survival benefit and improved complete cytogenetic and molecular response rates with imatinib mesylate in Philadelphia chromosome-positive chronic-phase chronic myeloid leukemia after failure of interferon-alpha. *Blood* 2004; **104**: 1979–1988.
6 Hochhaus A, La Rosée P. Imatinib therapy in chronic myelogenous leukemia: strategies to avoid and overcome resistance. *Leukemia* 2004; **18**: 1321–1331.
7 Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. *Blood* 2004; **104**: 3739–3745.
8 McLean LA, Gathmann I, Capdeville R, Polymeropoulos MH, Dressman M. Pharmacogenomic analysis of cytogenetic response in chronic myeloid leukemia patients treated with imatinib. *Clin Cancer Res* 2004; **10** (Part 1): 155–165.
9 Crossman LC, Druker BJ, Deininger MW, Pirmohamed M, Wang L, Clark RE. hOCT 1 and resistance to imatinib. *Blood* 2005; **106**: 1133–1134.
10 Wadlow R, Ramaswamy S. DNA microarrays in clinical cancer research. *Curr Mol Med* 2005; **5**: 111–120.

11 Haferlach T, Kohlmann A, Schnittger S, Dugas M, Hiddemann W, Kern W *et al*. Global approach to the diagnosis of leukemia using gene expression profiling. *Blood* 2005; **106**: 1189–1198.
12 Kohlmann A, Schoch C, Dugas M, Rauhut S, Weninger F, Schnittger S *et al*. Pattern robustness of diagnostic gene expression signatures in leukemia. *Genes Chromosomes Cancer* 2005; **42**: 299–307.
13 Hofmann WK, de Vos S, Elashoff D, Gschaidmeier H, Hoelzer D, Koeffler HP *et al*. Relation between resistance of Philadelphia-chromosome-positive acute lymphoblastic leukaemia to the tyrosine kinase inhibitor STI571 and gene-expression profiles: a gene-expression study. *Lancet* 2002; **359**: 481–486.
14 Yong AS, Szydlo RM, Goldman JM, Apperley JF, Melo JV. Molecular profiling of CD34+ cells identifies low expression of CD7, along with high expression of proteinase 3 or elastase, as predictors of longer survival in patients with CML. *Blood* 2006; **107**: 205–212.
15 Fonatsch C, Schaadt M, Kirchner H, Diehl V. A possible correlation between the degree of karyotype aberrations and the rate of sister chromatid exchanges in lymphoma lines. *Int J Cancer* 1980; **26**: 749–756.
16 Cross NC, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. *Leukemia* 1994; **8**: 186–189.
17 Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A *et al*. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. *Leukemia* 1999; **13**: 1825–1832.
18 Hochhaus A, Kreil S, Corbin AS, La Rosée P, Müller MC, Lahaye T *et al*. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 2002; **16**: 2190–2196.
19 Cross NC, Feng L, Bungey J, Goldman JM. Minimal residual disease after bone marrow transplant for chronic myeloid leukaemia detected by the polymerase chain reaction. *Leuk Lymphoma* 1993; **11** (Suppl 1): 39–43.
20 Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K. Chipping away at the chip bias: RNA degradation in microarray analysis. *Nat Genet* 2003; **35**: 292–293.
21 Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 2002; **18** (Suppl 1): S96–S104.
22 Vapnik VN. *Statistical Learning Theory*. John Wiley & Sons Inc.: New York, NY, 1998.
23 Guyon I, Weston J, Barnhill S, Vapnik V. Gene selection for cancer classification using support vector machines. *Mach Learn* 2002; **46**: 389–422.
24 Beillard E, Pallisgaard N, van der Valden VH, Bi W, Dee R, van der Schoot E *et al*. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program. *Leukemia* 2003; **17**: 2474–2486.
25 Aerts JL, Gonzales MI, Topalian SL. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. *Biotechniques* 2004; **36**: 84–86, 88, 90–91.
26 Hochhaus A, Hughes T. Clinical resistance to imatinib: mechanisms and implications. *Hematol Oncol Clin N Am* 2004; **18**: 641–656, ix.
27 Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA *et al*. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate? *BMC Genom* 2005; **6**: 59.
28 Melo JV, Deininger MW. Biology of chronic myelogenous leukemia – signaling pathways of initiation and transformation. *Hematol Oncol Clin N Am* 2004; **18**: 545, viii.
29 Kuwahara D, Tsutsumi K, Oyake D, Ohta T, Nishikawa H, Koizuka I. Inhibition of caspase-9 activity and Apaf-1 expression in cisplatin-resistant head and neck squamous cell carcinoma cells. *Auris Nasus Larynx* 2003; **30** (Suppl): S85–S88.
30 Vallat L, Magdelenat H, Merle-Beral H, Masdehors P, Potocki dM, Davi F *et al*. The resistance of B-CLL cells to DNA damage-induced apoptosis defined by DNA microarrays. *Blood* 2003; **101**: 4598–4606.
31 Masuda Y, Shima G, Aiuchi T, Horie M, Hori K, Nakajo S *et al*. Involvement of tumor necrosis factor receptor-associated protein 1 (TRAP1) in apoptosis induced by beta-hydroxyisovalerylshikonin. *J Biol Chem* 2004; **279**: 42503–42515.
32 Shet AS, Jahagirdar BN, Verfaillie CM. Chronic myelogenous leukemia: mechanisms underlying disease progression. *Leukemia* 2002; **16**: 1402–1411.
33 Evans PC, Smith TS, Lai MJ, Williams MG, Burke DF, Heyninck K *et al*. A novel type of deubiquitinating enzyme. *J Biol Chem* 2003; **278**: 23180–23186.
34 Chen F. Endogenous inhibitors of nuclear factor-kappaB, an opportunity for cancer control. *Cancer Res* 2004; **64**: 8135–8138.
35 Sattler M, Griffin JD. Molecular mechanisms of transformation by the BCR-ABL oncogene. *Semin Hematol* 2003; **40** (Suppl 2): 4–10.
36 Wurmbach E, Yuen T, Ebersole BJ, Sealfon SC. Gonadotropin-releasing hormone receptor-coupled gene network organization. *J Biol Chem* 2001; **276**: 47195–47201.
37 Nam SW, Clair T, Campo CK, Lee HY, Liotta LA, Stracke ML. Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells. *Oncogene* 2000; **19**: 241–247.
38 Nowicki MO, Pawlowski P, Fischer T, Hess G, Pawlowski T, Skorski T. Chronic myelogenous leukemia molecular signature. *Oncogene* 2003; **22**: 3952–3963.
39 Talpaz M, Silver RT, Druker BJ, Goldman JM, Gambacorti-Passerini C, Guilhot F *et al*. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* 2002; **99**: 1928–1937.
40 Weng Z, Fluckiger AC, Nisitani S, Wahl MI, Le LQ, Hunter CA *et al*. A DNA damage and stress inducible G protein-coupled receptor blocks cells in G2/M. *Proc Natl Acad Sci USA* 1998; **95**: 12334–12339.
41 Chuang JI, Chang TY, Liu HS. Glutathione depletion-induced apoptosis of Ha-ras-transformed NIH3T3 cells can be prevented by melatonin. *Oncogene* 2003; **22**: 1349–1357.
42 Berruyer C, Martin FM, Castellano R, Macone A, Malergue F, Garrido-Urbani S *et al*. Vanin-1−/− mice exhibit a glutathione-mediated tissue resistance to oxidative stress. *Mol Cell Biol* 2004; **24**: 7214–7224.
43 Shiner M, Fuhrman B, Aviram M. Paraoxonase 2 (PON2) expression is upregulated via a reduced-nicotinamide-adenine-dinucleotide-phosphate (NADPH)-oxidase-dependent mechanism during monocytes differentiation into macrophages. *Free Radic Biol Med* 2004; **37**: 2052–2063.
44 Cong F, Tang J, Hwang BJ, Vuong BQ, Chu G, Goff SP. Interaction between UV-damaged DNA binding activity proteins and the c-Abl tyrosine kinase. *J Biol Chem* 2002; **277**: 34870–34878.
45 Giehl M, Fabarius A, Frank O, Hochhaus A, Hafner M, Hehlmann R *et al*. Centrosome aberrations in chronic myeloid leukemia correlate with stage of disease and chromosomal instability. *Leukemia* 2005; **19**: 1192–1197.
46 Hasskarl J, Duensing S, Manuel E, Munger K. The helix–loop–helix protein ID1 localizes to centrosomes and rapidly induces abnormal centrosome numbers. *Oncogene* 2004; **23**: 1930–1938.
47 Takai N, Miyazaki T, Fujisawa K, Nasu K, Miyakawa I. Id1 expression is associated with histological grade and invasive behavior in endometrial carcinoma. *Cancer Lett* 2001; **165**: 185–193.
48 Polsky D, Young AZ, Busam KJ, Alani RM. The transcriptional repressor of p16/Ink4a, Id1, is up-regulated in early melanomas. *Cancer Res* 2001; **61**: 6008–6011.
49 Cohen N, Rozenfeld-Granot G, Hardan I, Brok-Simoni F, Amariglio N, Rechavi G *et al*. Subgroup of patients with Philadelphia-positive chronic myelogenous leukemia characterized by a deletion of 9q proximal to ABL gene: expression profiling, resistance to interferon therapy, and poor prognosis. *Cancer Genet Cytogenet* 2001; **128**: 114–119.
50 Ohmine K, Ota J, Ueda M, Ueno S, Yoshida K, Yamashita Y *et al*. Characterization of stage progression in chronic myeloid leukemia by DNA microarray with purified hematopoietic stem cells. *Oncogene* 2001; **20**: 8249–8257.
51 Ohno R, Nakamura Y. Prediction of response to imatinib by cDNA microarray analysis. *Semin Hematol* 2003; **40** (Suppl 2): 42–49.

52 Crossman LC, Mori M, Hsieh YC, Lange T, Paschka P, Harrington CA *et al*. In chronic myeloid leukemia white cells from cytogenetic responders and non-responders to imatinib have very similar gene expression signatures. *Haematologica* 2005; **90**: 459–464.
53 Man MZ, Dyson G, Johnson K, Liao B. Evaluating methods for classifying expression data. *J Biopharm Stat* 2004; **14**: 1065–1084.
54 Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC. Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. *Nucleic Acids Res* 2002; **30**: e48.
55 Yang IV, Chen E, Hasseman JP, Liang W, Frank BC, Wang S *et al*. Within the fold: assessing differential expression measures and reproducibility in microarray assays. *Genome Biol* 2002; **3**: research 0062.1–13.
56 Yao B, Rakhade SN, Li Q, Ahmed S, Krauss R, Draghici S *et al*. Accuracy of cDNA microarray methods to detect small gene expression changes induced by neuregulin on breast epithelial cells. *BMC Bioinformatics* 2004; **5**: 99.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)