



## Brief communication

## A gene expression signature of primary resistance to imatinib in chronic myeloid leukemia

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## ABSTRACT

Using gene expression profiling we show that the expression of 105-probe sets in mononuclear cells collected from chronic myeloid leukemia (CML) chronic phase (CP) patients with raised leukocyte counts who subsequently achieved complete cytogenetic response after 12 months on imatinib, differed substantially from that of patients who failed to achieve any degree of cytogenetic response. In the non-responder cohort, 9 of the 50 overexpressed genes were involved in DNA repair by homologous recombination, whereas 36 genes, including *PTEN*, were downregulated. This pattern of altered gene expression in responders and non-responders was validated in another independent dataset. These findings may prove useful for identifying at the time of diagnosis a subset of CP-CML patients who are likely to be resistant to imatinib and require an alternative treatment.

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## 1. Introduction

Imatinib induces durable cytogenetic responses in the majority of chronic myeloid leukemia (CML) patients who receive the drug early in chronic phase (CP) [1,2] but an appreciable minority of patients fail to achieve any degree of cytogenetic response to imatinib. This is commonly referred as up-front or primary cytogenetic resistance and its incidence seems to be consistent in different patient cohorts [1,2]. The clinical heterogeneity of CML at diagnosis has been recognized for more than 20 years [3], and such heterogeneity could in part explain primary resistance to imatinib. However, because this type of resistance is rare, little is known of its biological basis.

To gain understanding on the molecular mechanisms associated with primary resistance to imatinib, we used microarray technology to compare the gene expression profiles of blood samples from

patients who achieved complete cytogenetic responses on imatinib with those of patients who failed to achieve any degree of cytogenetic response (i.e.  $\geq 95\%$  Ph-positive at 12 months). We identified a set of genes whose expression was differentially regulated in patients resistant to imatinib. This set of genes was then tested in publicly available datasets.

## 2. Materials and methods

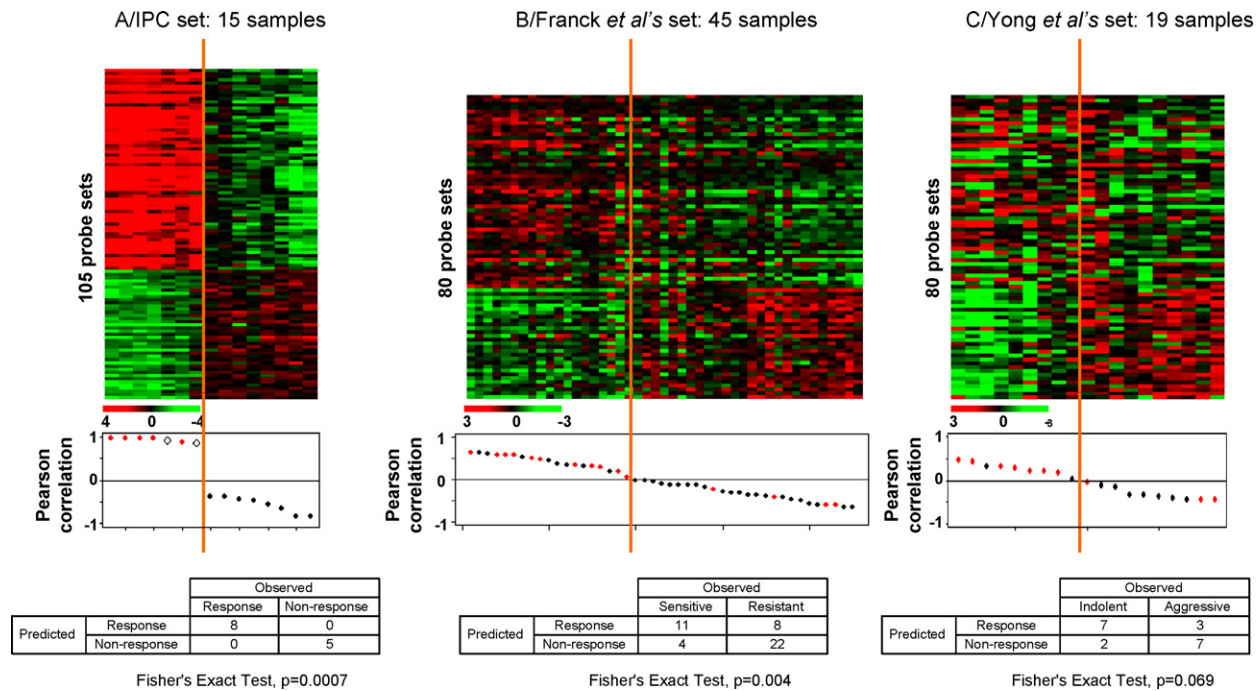
## 2.1. Patient characteristics and response assessment

We selected a total of 125 CP-CML patients treated at our institution with a minimal follow-up of 24 months on imatinib. Among them 12 failed to achieve any degree of cytogenetic response (i.e.  $\geq 95\%$  Ph-positive) during follow-up, of which 5 could eventually be included in the study (Supplementary Table 1). We matched this "non-responder group" with a "responder group" of 8 patients who achieved complete cytogenetic response (CCyR) within the first year of treatment and were still in CCyR at latest follow-up. Two CP patients who could best be classified as having 'acquired resistance' were added. Both had achieved CCyR within 1 year but then lost their responses at 18 and 24 months, respectively.

Cytogenetics was performed on bone marrow (BM) aspirates according to standard protocols. CCyR was defined by the failure to detect any Philadelphia chromosome in 20 metaphases. BCR-ABL transcripts were measured in the blood at 6–12 week intervals using real-time quantitative PCR. All patients signed an informed consent for the use of samples in accordance with the Declaration of Helsinki and with the approval from our Institutional Review Board.

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**Fig. 1.** Supervised classification of CP-CML samples based on gene expression profiling. (A/) Classification of 15 CP-CML samples using the 105-probe sets identified as differentially expressed between the 8 “responder” samples and the 5 “non-responder” samples. (Top panel) Expression data are depicted as a data matrix where each row represents a gene and each column represents a sample. Expression levels are depicted according to the color scale shown at the bottom. Red and green indicate expression levels, respectively, above and below the median. The magnitude of deviation from the median is represented by the color saturation. Probe sets are ordered from top to bottom by their decreasing signal-to-noise ratio. Samples are ordered from left to right according to the decreasing correlation coefficient of their expression profile with the median profile of the “non-responder” samples (middle panel). The solid orange line indicates the threshold 0 that separates the two classes of samples, predicted “non-responder class” (left to the line) and predicted “responder class” (right to the line). The response to imatinib is indicated by colored dots: red, “non-responder”; black, “responder”; open, early secondary resistance. The bottom panel displays the cross-table between the observed imatinib response and the response predicted by the probe set signature. (B/) Similar to (A/), but applied to expression data of the 45 Frank et al.’s samples (30 imatinib-sensitive and 15 imatinib-resistant) and the 80 probe sets common with our list. (C/) Similar to (A/), but applied to expression data of the 19 Yong et al.’s samples (9 “indolent” and 10 “aggressive”) and the 80 probe sets common with our list. The status of samples is indicated by colored dots: red, “indolent”; black, “aggressive”.

## 2.2. Expression data analyses

For each of the 15 patients, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Nycoprep, Oslo, Norway) before starting imatinib. Total RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany). RNA quality was assayed by Bioanalyser 2100 (Agilent, Palo Alto, CA, USA).

Gene expression profiling was done using Affymetrix (Santa Clara, CA, USA) U133 Plus 2.0 human oligonucleotide microarrays. Experiments were done according to the standard protocols available from the manufacturer. Synthesis of first-strand cDNA was done using 2 µg of total RNA by T7-oligo(dT) priming followed by second-strand cDNA synthesis. After purification, *in vitro* transcription associated with amplification generated cRNA containing biotinylated pseudouridine. Biotinylated cRNA was purified, quantified and chemically fragmented (95 °C for 35 min), then hybridized to microarrays in 200 µl hybridization buffer at 45 °C for 16 h. Automated washes and staining with streptavidin–phycoerythrin were done as recommended. Double signal amplification was done by biotinylated antistreptavidin antibody with goat-IgG blocking antibody.

Data were analyzed by the Robust Multichip Average method in R using Bioconductor® and associated packages. Robust multichip average (RMA) did background adjustment, quantile normalization, and summarization of 11 oligonucleotides per gene. Before analysis, a filtering process removed from the data set the genes with low and poorly measured expression as defined by an expression value inferior to 100 units in all 15 CP-CML samples, retaining 24,863 probe sets with expression values ranging from 5 to 30,700 (mean, 156). To identify and rank genes discriminating samples from responder and non-responder patients, supervised analysis [4] was applied to the 24,863 probe sets. The statistical test was the signal-to-noise ratio (SNR), calculated for each gene as  $SNR = (M1 - M2) / (S1 + S2)$ , where M1 and S1, respectively, represent mean and SD of expression levels of the gene in group 1, and M2 and S2 in group 2. Confidence levels, and, because of multiple hypotheses testing, false discovery rates ( $q$ -values) were estimated by 1000 random permutations of samples as previously described, with a false positive rate of 1/10,000. The list of discriminator genes was interrogated by Ingenuity Pathway Analysis (version 5.5.1-1002; Ingenuity Systems, Redwood City, CA).

Once identified, the classification power of the gene profiling was illustrated by classifying samples according to the correlation coefficient of their expression

profile with the median profile of the “non-responders” samples. A “leave-one-out” (LOO) procedure was applied as cross-validation (CV) of the generated gene expression signature.

## 2.3. Test of the gene expression signature on two independent datasets

The predictive performance of our signature was tested on two independent gene expression datasets collected from the EBI public repository (<http://www.ebi.ac.uk/arrayexpress/>), which included pretreatment CP-CML samples profiled using Affymetrix U133A microarrays. These two data sets corresponded to two series treated differently, and permitted us to test the treatment-specificity of our signature. In the first dataset [5] (accession number E-MEXP-433), gene expression profiles from 30 imatinib-responding patients (defined as major cytogenetic response – MCyR – at 12 months) and 15 non-responding patients (defined as lack of MCyR at 12 months) were analyzed. In the second dataset [6] were analyzed 10 patients with an ‘aggressive disease’ (blastic transformation – BT – within 3 years of diagnosis) and 9 patients with an ‘indolent disease’ (BT after 7 or more years from diagnosis), all of whom had been treated with interferon but not with imatinib.

## 3. Results

### 3.1. Establishment of a gene expression signature of primary resistance to imatinib

We established the gene expression profiles of 15 RNA samples prior imatinib treatment (Fig. 1) Supervised analysis identified 105-probe sets, representing 95 unique sequences (9 ESTs and 86 genes) as significantly differentially expressed ( $q$ -value < 0.05) between the 8 responder and 5 non-responder samples (theoretical number of false positives < 5). A total of 64 probe sets (representing 5 ESTs and 50 genes) were overexpressed in the non-responder samples and 41 were underexpressed (4 ESTs and 36 genes) (Table 1 and Supplementary Table 2).

**Table 1**

Top 20 probe sets overexpressed in non-responder CML samples.

| Probe set ID <sup>a</sup> | SNR <sup>b</sup> | q-Value  | Gene symbol     | Gene name                                       | RefSeq transcript ID | Chromosome band |
|---------------------------|------------------|----------|-----------------|---|----------------------|-----------------|
| 244427.at                 | 4.33             | 1.00E–03 | <i>KIF23</i>    | Kinesin family member 23                        | AW192521             | 15q23           |
| 208149.x.at               | 2.83             | 2.33E–03 | <i>DDX11</i>    | DEAD/H (Asp–Glu–Ala–Asp/His) box polypeptide 11 | NM.030653            | 12p11.21        |
| 1556347.at                | 2.72             | 5.25E–03 | <i>CENPP</i>    | Centromere protein P                            | W72151               |                 |
| 1558217.at                | 2.50             | 6.40E–03 | <i>SLFN13</i>   | Schlafen family member 13                       | AK074465             | 17q12           |
| 1570166.a.at              | 2.48             | 6.40E–03 | <i>RAD51L1</i>  | RAD51-like 1                                    | BC020846             | 14q24.1         |
| 233445.at                 | 2.37             | 6.40E–03 | <i>BUB1</i>     | BUB1 budding uninhibited by benzimidazoles 1    | AK022040             | 2q13            |
| 219105.x.at               | 2.36             | 6.40E–03 | <i>ORC6L</i>    | Origin recognition complex, subunit 6 like      | NM.014321            | 16q11.2         |
| 208159.x.at               | 2.34             | 6.40E–03 | <i>DDX11</i>    | DEAD/H (Asp–Glu–Ala–Asp/His) box polypeptide 11 | NM.004399            | 12p11.21        |
| 1556589.at                | 2.32             | 7.10E–03 | –               | CDNA FLJ25645 fis, clone SYN00113               | T83966               | 10q22.2         |
| 237246.at                 | 2.29             | 7.10E–03 | <i>SMC4</i>     | Structural maintenance of chromosomes 4         | AI797163             | 3q26.1          |
| 215006.at                 | 2.19             | 7.50E–03 | <i>EZH2</i>     | Enhancer of zeste homolog 2                     | AK023816             | 7q36.1          |
| 218014.at                 | 2.16             | 7.92E–03 | <i>NUP85</i>    | Nucleoporin 85 kDa                              | NM.024844            | 17q25.1         |
| 239219.at                 | 2.13             | 7.92E–03 | <i>AURKB</i>    | Aurora kinase B                                 | N55457               | 17p13.1         |
| 1556590.s.at              | 2.12             | 7.92E–03 | –               | CDNA FLJ25645 fis, clone SYN00113               | T83966               | 10q22.2         |
| 232889.at                 | 2.05             | 7.94E–03 | <i>GUSBP1</i>   | Glucuronidase, beta pseudogene 1                | AU147591             | 5p13.3          |
| 236976.at                 | 2.01             | 7.94E–03 | <i>FANCA</i>    | Fanconi anemia, complementation group A         | AI569792             | 16q24.3         |
| 207746.at                 | 1.99             | 8.45E–03 | <i>POLQ</i>     | Polymerase (DNA directed), theta                | NM.014125            | 3q13.33         |
| 220918.at                 | 1.99             | 9.48E–03 | <i>C21orf96</i> | Chromosome 21 open reading frame 96             | NM.025143            | 21q22.12        |
| 1556331.a.at              | 1.99             | 9.48E–03 | <i>PDE4D</i>    | Phosphodiesterase 4D, cAMP-specific             | BC035063             | 5q11.2          |
| 230892.at                 | 1.95             | 9.48E–03 | <i>DERA</i>     | 2-Deoxyribose-5-phosphate aldolase              | AI912194             | 12p12.3         |

<sup>a</sup> Probe sets are ordered according to decreasing SNR.<sup>b</sup> SNR, signal-to-noise ratio.

Ingenuity pathway analysis identified “cell cycle” (16 genes,  $p = 3.56\text{E}–07$ ), “DNA replication, recombination and repair” (16 genes,  $p = 2.6\text{E}–04$ ) and “cellular growth and proliferation” (13 genes,  $p = 4.86\text{E}–03$ ) as the three most represented biological processes among the genes overexpressed in the non-responders (Supplementary Table 3). Similar analysis applied to the underexpressed genes identified “gene expression” (5 genes,  $p = 7.32\text{E}–04$ ) as the most represented biological process, which included the tumor suppressor gene *PTEN* (Supplementary Table 3).

The resulting classification of 13 samples correlated perfectly with the cytogenetic response to imatinib (Fig. 1A). By leave-one-out cross-validation, 92% of samples were correctly assigned ( $p = 0.0047$ , Fisher’s exact test).

Using the 105-probe set, the two additional CP patients with secondary resistance to imatinib were also classified as non-responders (Fig. 1A).

### 3.2. Performance of the gene expression signature on independent sets treated with and without imatinib

The 105-probe set signature was tested on two independent gene expression datasets in which pretreatment CP-CML samples had similarly been profiled using Affymetrix U133A microarrays: one included patients treated with imatinib (similar to our series) [5], whereas the other included patients treated with interferon without any imatinib [6].

In the first dataset [5], gene expression profiles from 30 imatinib-responding patients (defined as MCyR at 12 months) and 15 non-responding patients (defined as lack of MCyR at 12 months) were analyzed. Although the definition of resistance to imatinib used in this validation set was slightly different from ours, the classification of samples based on the expression of 80 common probe sets (Fig. 1B) tallied closely with our results, with a sensitivity and a specificity of 73% ( $p = 0.004$ , Fisher’s exact test). This result validated the robustness of our signature.

In the second dataset [6], 10 patients with an ‘aggressive disease’ (BT within 3 years of diagnosis) were analyzed with 9 patients with an ‘indolent disease’ (BT after 7 or more years from diagnosis), all of whom had been treated with interferon alone. The sample classification based on the expression of 80 common probe sets (Fig. 1C) was not accurate ( $p$ -value not significant) but nevertheless suggested a predictive potential (70% sensitivity, 78% specificity) of

our signature for identifying patients with an aggressive disease independently of imatinib therapy. In contrast, the Sokal score was unable to identify the patients with an aggressive disease.

## 4. Discussion

In this study we have established a gene expression signature for a subset of CML cases with primary resistance to imatinib. Using stringent criteria, we eliminated obvious causes of primary resistance and selected 5 non-responder patients and 8 responders, and compared the whole-genome expression profile of their respective samples. Despite this limited number of cases, the signature was validated in an independent data set of similarly treated patients. To our knowledge, this is the first study in which such gene expression pattern of imatinib primary resistance is validated in an independent way. When applied to another data set of patients treated with interferon, the signature lost some degree of performance and the correlation became non-significant. However, the prediction remained rather correct with aggressive patients being more frequently predicted as non-responders.

The content of the gene expression signature was highly coherent and many genes overexpressed in the non-responder samples were involved in the same pathways. Such a coherent signature obtained within a small series indicates that the gene expression profiles translate a major biological process. Indeed, cells from non-responders overexpressed genes encoding several proteins, notably RAD51L1, RAD54L, EME1, ORC6L, POLQ, SMC4, DDX11 and TOPBP1, involved in DNA replication and repair by homologous recombination (HR). Complex DNA damage is repaired by HR and by non-homologous end-joining, both of which can be defective in CML [7,8]. Overexpressed DNA repair genes were also included in gene expression signatures of resistant cases in previous studies [5,9]. One might speculate that non-responding patients have accumulated relatively high levels of genotoxic agents, such as reactive oxygen species, in their BCR-ABL-positive cells and that high expression of genes involved in repair mechanisms is a secondary though possibly relatively ineffective response [10–13]. Another possibility is that imatinib enhances DNA damage initiated by the oncogenic kinase [14]; some patients would be resistant to the drug because their DNA repair is intrinsically functioning at high level. However, there might not be a single trivial explanation because the *EZH2* gene, which is overexpressed in resistant cases, is known to impair DNA repair [15]. Resistant cases also overex-

pressed genes coding for proteins involved in cell cycle such as cyclin E, Aurora kinase B and BUB1, indicating a high level of proliferation.

Of the 36 underexpressed genes in the non-responders one was the tumor suppressor gene *PTEN*. Downregulation of *PTEN* may allow activation of the BCR-ABL-independent PI3K/AKT pathway and thereby enhance resistance to imatinib and proliferation of the leukemic clone, as already described in some other types of cancer drug resistance [16,17]. Because nuclear PTEN regulates RAD51 its low expression might also contribute to increased genomic instability [18].

The pattern of gene expression we found associated with primary resistance to imatinib, possibly coupled to miRNA profiles [19], could help develop surrogate markers and assays to identify at diagnosis the patients who may fare badly when treated with imatinib; such patients might benefit from alternative treatments thus saving a precious time in the fight against the disease.

### Conflict of interest

None.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2009.09.026.

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