Imatinib therapy of chronic myeloid leukemia restores the expression levels of key genes for DNA damage and cell-cycle progression

Rocío Benito[a], Eva Lumbreras[a], María Abáigar[a], Norma C. Gutiérrez[b], Manuel Delgado[b], Cristina Robledo[a], Juan L. García[c], Ana E. Rodríguez-Vicente[a], M. Consuelo Cañizo[b] and Jesús Maria Hernández Rivas[a,b]

Background **Chronic myeloid leukemia (CML) is a malignant clonal disorder of the hematopoietic system caused by the expression of the *BCR/ABL* fusion oncogene. It is well known that CML cells are genetically unstable. However, the mechanisms by which these cells acquire genetic alterations are poorly understood. Imatinib mesylate is the standard therapy for newly diagnosed CML patients. Imatinib mesylate targets the oncogenic kinase activity of BCR-ABL.**

Objective **To study the gene expression profile of bone marrow hematopoietic cells in the same patients with CML before and 1 month after imatinib therapy.**

Methods **Samples from patients with CML were analyzed using Affymetrix GeneChip Expression Arrays.**

Results **A total of 594 differentially expressed genes, most of which (393 genes) were downregulated, as a result of imatinib therapy were observed.**

Conclusion **The blockade of oncoprotein Bcr-Abl by imatinib could cause a decrease in the expression of key DNA repair genes and substantially modify the expression profile of the bone marrow cells in the first days of therapy. *Pharmacogenetics and Genomics* 22:381–388 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]IBSAL(Institute of Biomedicine of Salamanca), IBMCC (Institute of Molecular and Cellular Biology of Cancer), Center of Cancer Research, University of Salamanca-CSIC, [b]Department of Hematology, University Hospital of Salamanca and [c]Institute for the Study of Health Sciences from Castilla y León (IESCYL) and Research Unit, University Hospital of Salamanca, Spain

Correspondence to Jesús María Hernández Rivas, MD, PhD, Department of Hematology, University Hospital of Salamanca, Paseo San Vicente, 58-182, 37007 Salamanca, Spain
Tel: +34 923 291 384; fax: +34 923 294 624; e-mail: jmhr@usal.es

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Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease with distinct biological and clinical features that progresses in several defined stages [1]. CML is associated with an acquired cytogenetic abnormality: the Philadelphia (Ph) chromosome. This translocation generates the *BCR/ABL* fusion gene, which is translated in an oncoprotein Bcr-Abl with highly deregulated, constitutive tyrosine kinase activity. The most commonly occurring form of Bcr-Abl is a 210-kDa protein that plays a critical role in the pathogenesis of CML [2]. This oncogenic protein plays important roles in the proliferation and survival of myeloid progenitor cells [3]. As a result of the increased tyrosine kinase activity, Bcr-Abl activates several signaling pathways, including Ras [4], PI3K-Akt [5,6], Jak [7], and NF-κB [8], pathways leading to proliferation, a reduced growth factor dependence and apoptosis, and an abnormal interaction with extracellular matrix and stroma [9].

Imatinib mesylate (IM) is the standard therapy for newly diagnosed CML patients. In most CMLs, imatinib produces durable hematological and cytogenetic remissions. Complete hematological responses are observed after some weeks of therapy, whereas cytogenetic responses occur later (2–10 months after the treatment) [10]. Therefore, IM increases survival in these patients [10–12].

IM was developed as a specific BCR-ABL tyrosine kinase inhibitor against ABL oncoproteins (c-ABL, BCR-ABL, ETV1-ABL), c-KIT, and platelet-derived growth-factor receptor. Notably, the primary consequence of tyrosine kinase inhibition with imatinib in BCR-ABL-transformed leukemic cells *in vitro* is the induction of apoptosis and the reduction of white blood cell overproduction [13,14].

Gene expression profiling using microarray technologies has increased our understanding of cancer biology [15,16]. For CML, most gene expression profiling studies have focused on the prediction of the response to IM [17], yielding a wide range of results due to the overall variability in CML samples [18–24]. In addition, several studies have analyzed the changes in gene expression in cell lines after imatinib treatment [25,26]. However,

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information about the signaling pathways and the transcriptional response to imatinib in patients with CML is scarce.

The pathogenic role of BCR-ABL in CML in the chronic phase and the therapeutic efficacy of tyrosine kinase inhibitors, such as IM, which targets the oncogenic kinase activity of Bcr-Abl, are both well established. However, fundamental aspects of the biology of CML, such as the mechanisms responsible for the transition from the chronic phase to a blast crisis, the causes of genomic instability and faulty DNA repair, the phenomenon of stem-cell quiescence, the role of tumor suppressors in tyrosine kinase inhibitors' resistance and CML progression, and the cross-talk between BCR-ABL1 and other oncogenic signaling pathways, remain poorly understood [27].

To gain an insight into the molecular mechanisms affected by imatinib, we carried out a study to explore the transcriptional response in the first weeks of imatinib therapy in patients with chronic-phase CML. To our knowledge, this is the first study to analyze *in vivo* the changes in gene expression in CML patients during the first weeks of imatinib therapy.

Materials and methods

Patients

Nine patients diagnosed with Ph-positive CML and nine samples from normal donors were analyzed. In all patients, bone marrow samples were obtained by bone marrow aspiration. All patients received a daily dose of 400 mg IM. In all patients, the bone marrow was sampled before IM administration commenced and 4 weeks after the treatment, by which time all cases were in hematological response. No differences in the composition of the bone marrow were found after imatinib therapy. No blast cells were observed. In addition, all patients showed a complete cytogenetic response 6 months after starting therapy. Patients' characteristics are included in Table 1. The study was approved by the local research ethics committee and written informed consent was obtained from all patients.

RNA isolation, labeling, and microarray hybridization

Total RNA was isolated from the bone marrow in Trizol aliquots, following the manufacturer's instructions, and purified using an RNeasy kit (Qiagen Inc., Valencia, California, USA). RNA integrity and yield were assessed by determining sample absorbance at 260 and 280 nm and by analysis on the Agilent RNA 6000 Nano LabChip (Agilent Technologies Inc., Palo Alto, California, USA).

Microarray assays were performed according to the standard protocol described in the Affymetrix GeneChip Expression Analysis Technical Manual, rev. 3 (*http://www.affymetrix.com/support/index.affx*). Messenger RNA was amplified and labeled from 5 μg samples, which produced a sufficient yield of cRNA. These samples were fragmented and combined with array hybridization controls (Affymetrix, Santa Clara, California, USA) in hybridization buffer. Eleven microgram of the cRNA target was then hybridized with the GeneChip HG_U133 Plus 2.0 array (Affymetrix) and scanned using the GeneArray laser scanner (Affymetrix).

Genome-wide expression data analysis

The expression value for each probe set was calculated using RMA-Express software (*http://rmaexpress.bmbolstad.com/*), which uses the robust multiarray average (RMA) algorithm, as previously described [28]. For an initial analysis, gene-filtering methods were applied following the detection calls calculated using the MAS 5.0 algorithm (Affymetrix). Probe sets with absent calls and those showing minimal variation across all samples (maximum – minimum log 2 variation <2.5) were filtered out.

The significant analysis of microarrays algorithm was used to identify genes with statistically significant differences in expression between classes. All data were permuted over 100 cycles using the two-class (unpaired) and the multiclass response format, with no requirement for equal variances. Significant genes were identified on the basis of the lowest false-discovery ratio, controlling the *q*-value for the gene list. The false-discovery ratio was less than 2% in all class comparisons.

Table 1 Patients' characteristics

			Before imatinib		After imatinib	
Patient	Sex	Age	ID-sample	BCR/ABL (% FISH)	ID-sample	BCR/ABL (% FISH)
12278-FAA	Male	51	cml_2647_A1	86	cml_2860_A2	80
12102-CSA	Male	70	cml_2574_B1	95	cml_2623_B2	90
10669-FCA	Male	74	cml_1802_C1	82	cml_1989_C2	75
12945-JCC	Male	54	cml_3007_D1	82	cml_3071_D2	81
13175-VLS	Male	75	cml_3106_E1	94	cml_3152_E2	90
13659-JGI	Male	73	cml_3285_F1	95	cml_3338_F2	90
13658-FAR	Female	62	cml_3284_G1	95	cml_3364_G2	36
13684-JFV	Male	42	cml_3305_H1	87	cml_3356_H2	85
14415-MMM	Male	60	cml_3593_I1	98	cml_3647_I2	91

FISH, fluorescence in-situ hybridization.

A web-delivered bioinformatics tool set, Ingenuity Pathway Analysis (IPA 5.5; *http://www.ingenuity.com*), was used to identify functional networks. Ingenuity Pathway Analysis is a knowledge database generated from peer-reviewed scientific publications. It enables the discovery, visualization, and exploration of functional biological networks in gene expression data and delineates the most significant functions in those networks.

Gene-specific real-time polymerase chain reaction

To validate the results of the microarray studies, a quantitative real-time reverse transcriptase PCR (RT-PCR) was performed at the time of diagnosis and 4 weeks after imatinib therapy. One microgram of total RNA was subjected to cDNA synthesis, as previously described [29]. Quantification was performed using Fast SYBR-Green Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The expression of the following genes was analyzed: *POLE2*, *FANCD2*, *PTEN*, *FOXO3*, and *ABCB7*. The specific primers were designed using Primer Express 3.0 software (Applied Biosystems) and tested for specificity using NCBI's BLAST software. Gene expression was achieved using relative quantitative real-time RT-PCR and the SYBR Green I. Expression was quantified relative to GAPDH expression using the Pfaffl analysis method. Primer sequences are shown in Table 2.

Results

The gene expression profile was analyzed in each patient before and 1 month after imatinib therapy. Data are available to the public on the NIH GEO (*http://www.ncbi.nlm.nih.gov/geo/*) under accession number GSE33075. Unsupervised hierarchical clustering analysis yielded a dendrogram with two branches: one included all samples at diagnosis and the other included those collected 1 month after the treatment with imatinib. Therefore, specific gene expression patterns were associated with the first stage of treatment. After imatinib therapy, 594 genes were differentially expressed, most of which (393 genes) were downregulated as a result of imatinib therapy.

The functional analysis of the deregulated genes revealed a significant involvement of biological functions such as cell cycle, apoptosis, post-translational modification, protein folding, as well as DNA replication, recombination, and repair (Fig. 1).

Quantitative RT-PCR was carried out to validate the microarray data. We evaluated the expression level of two genes involved in DNA repair (*POLE2* and *FANCD2*) and other genes such as *FOXO3A*, *ABCB7*, and *PTEN*. The results obtained from RT-PCR confirmed the expression data obtained for all the genes in the microarray studies (Supplementary Fig. 1, Supplemental digital content 1, *http://links.lww.com/FPC/A397*).

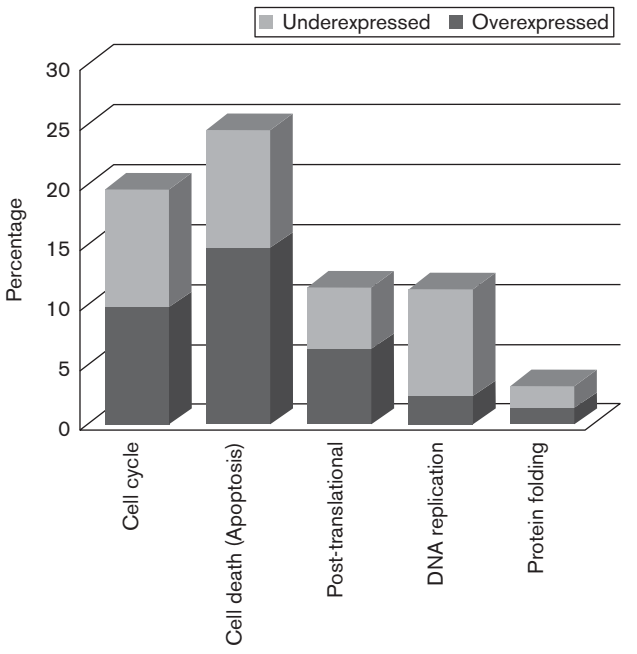
Deregulation of cell-cycle genes after imatinib therapy in chronic-phase chronic myeloid leukemia

IM therapy modulates the expression of genes that play a role in the activation of cyclin-dependent protein kinase (CDK). Thus, significant downregulation was detected after imatinib therapy in genes such as *CDK6*, *CDKN3*, *CKS1B*, *MEN1*, and *CKS2*. By contrast, overexpression of tumor-suppressor genes (*LATS2* and *PTEN*) was also observed (Table 3).

Table 2 Oligonucleotide primers used for real-time polymerase chain reaction

Name	Sequence
GAPDH	
Forward	5′-CAGGGCTGCTTTTAACTCTGGTAA-3′
Reverse	5′-GGGTGGAATCATATTGGAACATGTA-3′
POLE2	
Forward	5′-AGCAGCAATTTGGCTATTCCTAA-3′
Reverse	5′-GCACAGGATACACTCTCAAAGCA-3′
FANCD2	
Forward	5′-AAGCAATGTATGCCGCTCCTA-3′
Reverse	5′-GAATGCCCACACAGGTGATG-3′
PTEN	
Forward	5′-GAGCCCTGTTAAGGAAGGGTACAT-3′
Reverse	5′-GACTTGTATTTGAATGCTGACAGTCA-3′
FOXO3	
Forward	5′-TCAGGTGGCTTCCAAACTTGT-3′
Reverse	5′-ACTGCTTTATTCTTCATGGCCTTT-3′
ABCB7	
Forward	5′-GCTGGACTTCATGATGCAATTC-3′
Reverse	5′-TGGCTCTTGCAATTGCTACTCTT-3′

Fig. 1



Most relevant functions modified in chronic myeloid leukemia patients after 1 month of imatinib therapy. Functional classification was performed according to gene ontology in Ingenuity Pathway Analysis. The percentage of each functional group was derived with respect to the total number of deregulated genes.

Table 3 Gene expression levels of chronic myeloid leukemia patients before and after imatinib therapy as well as gene expression levels of normal controls

Probe set	Genes	Gene title	Mean expression values			Rfold		Function
			Before IM	Normal controls	After IM	Before IM vs. After IM*	Normal controls vs. after IM*	
204510_at	*CDC7*	CDC7 cell division cycle 7 (*S. cerevisiae*)	6.3	6.0	4.8	0.33	0.43*	Cell cycle
209714_s_at	*CDKN3*	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	7.8	7.8	6.7	0.41	0.48*	
208393_s_at	*RAD50*	RAD50 homolog (*S. cerevisiae*)	5.9	5.4	4.8	0.40	0.67	
204641_at	*NEK2*	NIMA (never in mitosis gene a)-related kinase 2	6.4	6.5	5.1	0.41	0.39	
204170_s_at	*CKS2*	CDC28 protein kinase regulatory subunit 2	9.3	8.4	7.9	0.39	0.73*	
200772_x_at	*PTMA*	Prothymosin α (gene sequence 28)	12.2	11.9	11.1	0.44	0.58*	
224847_at	*CDK6*	Cyclin-dependent kinase 6	8.7	8.4	7.5	0.33	0.51*	
242560_at	*FANCD2*	Fanconi anemia, complementation group D2	7.8	7.4	6.5	0.39	0.51	
203625_x_at	*SKP2*	S-phase kinase-associated protein 2 (p45)	7.4	7.2	6.4	0.50	0.57	
203062_s_at	*MDC1*	Mediator of DNA damage checkpoint 1	6.8	6.8	5.9	0.56	0.55*	
201897_s_at	*CKS1B*	CDC28 protein kinase regulatory subunit 1B	9.2	8.8	8.4	0.49	0.74*	
213523_at	*CCNE1*	Cyclin E1	7.3	7.5	6.3	0.45	0.44	
202645_s_at	*MEN1*	Multiple endocrine neoplasia 1	6.8	6.7	5.8	0.42	0.52*	
209421_at	*MSH2*	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)	5.8	5.7	4.9	0.43	0.61*	
1565347_s_at	*TFE3*	Transcription factor binding to IGHM enhancer 3	3.2	3.2	4.9	4.11	3.21	
216205_s_at	*MFN2*	Mitofusin 2	5.6	5.8	7.7	4.51	3.71	
223380_s_at	*LATS2*	LATS, large tumor suppressor, homolog 2 (*Drosophila* spp.)	7.5	7.1	9.4	3.83	4.75	
208200_at	*IL1A*	Interleukin 1 α	2.9	2.9	4.8	4.86	3.84	
226400_at	*CDC42*	Cell division cycle 42 (GTP binding protein, 25 kDa)	9.7	9.5	11.7	3.95	4.88	
233314_at	*PTEN*	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	2.6	2.8	5.5	10.91	6.55	
203628_at	*IGF1R*	Insulin-like growth factor 1 receptor	4.5	4.3	7.3	6.80	7.72	
225116_at	*HIPK2*	Homeodomain interacting protein kinase 2	5.5	5.0	7.7	4.31	6.34	
221923_s_at	*NPM1*	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	10.2	9.9	9.3	0.47	0.65*	Cell proliferation
211605_s_at	*RARA*	Retinoic acid receptor α	5.1	5.4	6.2	2.42	1.85	
235589_s_at	*MDM4*	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)	6.3	6.0	9.6	9.52	11.66	
216396_s_at	*EI24*	Etoposide induced 2.4 mRNA	7.3	7.6	5.5	0.25	0.24	Apoptosis
205176_s_at	*ITGB3BP*	Integrin β3 binding protein (β3-endonexin)	8.0	7.6	6.8	0.38	0.59*	
222985_at	*YWHAG*	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ polypeptide	9.3	9.2	8.5	0.52	0.61	
205692_s_at	*CD38*	CD38 antigen (p45)	7.2	7.2	6.2	0.41	0.50	
208905_at	*CYCS*	Cytochrome c, somatic	9.7	9.7	8.9	0.49	0.57*	
236988_x_at	*ITGB2*	Integrin, β2 [antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) β subunit]	6.3	6.4	7.3	2.25	1.76*	
210564_x_at	*CFLAR*	CASP8 and FADD-like apoptosis regulator	6.9	7.3	8.2	2.27	1.80*	
201392_s_at	*IGF2R*	Insulin-like growth factor 2 receptor	6.6	6.8	8.6	3.39	3.35	
204131_s_at	*FOXO3A*	Forkhead box O3A	8.9	8.6	10.5	3.18	3.69	
209305_s_at	*GADD45B*	Growth arrest and DNA-damage-inducible β	7.4	7.3	9.0	3.95	3.28*	
200796_s_at	*MCL1*	Myeloid cell leukemia sequence 1 (BCL2-related)	5.5	5.5	9.7	17.53	18.73	
218397_at	*FANCL*	Fanconi anemia, complementation group L	6.0	5.1	4.1	0.23	0.50*	DNA repair
218961_s_at	*PNKP*	Polynucleotide kinase 3′-phosphatase	7.0	6.9	6.1	0.47	0.59	
204023_at	*RFC4*	Replication factor C (activator 1) 4, 37 kDa	8.3	8.1	7.1	0.41	0.51*	
205395_s_at	*MRE11A*	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	5.7	5.3	4.8	0.43	0.72*	
1559946_s_at	*RUVBL2*	RuvB-like 2 (*E. coli*)	8.9	8.8	7.9	0.44	0.56*	
205024_s_at	*RAD51*	RAD51 homolog (RecA homolog, *E. coli*) (*S. cerevisiae*)	7.2	7.0	6.1	0.42	0.52*	
202589_at	*TYMS*	Thymidylate synthetase	11.0	10.7	9.9	0.42	0.59*	
209507_at	*RPA3*	Replication protein A3, 14 kDa	8.9	9.1	8.2	0.50	0.54	
206066_s_at	*RAD51C*	RAD51 homolog C (*S. cerevisiae*)	6.9	6.8	6.1	0.54	0.62*	
203720_s_at	*ERCC1*	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	7.7	7.5	6.9	0.51	0.64*	
203209_at	*RFC5*	Replication factor C (activator 1) 5, 36.5 kDa	7.3	6.9	6.2	0.49	0.62*	
205909_at	*POLE2*	Polymerase (DNA directed), ϵ2 (p59 subunit)	6.7	6.8	5.8	0.48	0.49*	
218428_s_at	*REV1L*	REV1-like (yeast)	6.6	6.5	5.6	0.48	0.54*	
209579_s_at	*MBD4*	Methyl-CpG binding domain protein 4	8.2	8.2	9.7	2.66	2.81	
239163_at	*UBE2B*	Ubiquitin-conjugating enzyme E2B (RAD6 homolog)	4.2	4.1	6.5	5.46	5.36	
204835_at	*POLA*	Polymerase (DNA directed) α	6.2	5.9	4.9	0.37	0.50	DNA replication
204127_at	*RFC3*	Replication factor C (activator 1) 3, 38 kDa	7.2	7.0	6.0	0.39	0.49*	
222036_s_at	*MCM4*	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	8.1	8.2	7.0	0.39	0.43	
205053_at	*PRIM1*	Primase, polypeptide 1, 49 kDa	7.8	7.4	6.9	0.45	0.72*	

The table also shows the comparison between the gene expression levels before and after imatinib as well as the differences in the expression levels between normal controls and chronic myeloid leukemia patients after IM therapy. Values above 1 indicate gene upregulation, whereas values below 1 indicate gene downregulation.
E. coli, *Escherichia coli*; IM, imatinib mesylate; *S. cerevisiae*, *Saccharomyces cerevisiae*.
**q*-values above 0.002, and they were considered not significant.

Samples from patients receiving imatinib therapy showed overexpression of the *TFE3*, *PTEN*, *LATS2*, *GADD45B*, and *CDC42* genes, as well as downregulation of *CDC7*, *CDKN3*, *CDK6*, *CCNE1*, and *SKP2* genes, which are related to the regulation of cell-cycle progression and proliferation (Supplementary Fig. 2, Supplemental digital content 2, *http://links.lww.com/FPC/A399*). *CDC7* is involved in G1/S and G2/M transition and its decreased level of gene expression is essential for cell-cycle arrest at an early stage.

Imatinib induces overexpression of apoptosis-related genes in chronic-phase chronic myeloid leukemia patients

Several apoptosis-related genes were overexpressed after imatinib therapy (Table 3). Notably, the expression levels of *FOXO3A*, *IL1A*, *IGF1R*, *IGF2R*, and *CFLAR* were higher 1 month after starting IM therapy than before it commenced.

Changes in the expression of DNA damage and recombination genes after imatinib therapy in chronic-phase chronic myeloid leukemia

Imatinib therapy in CML induced the downregulation of DNA-damage checkpoint genes such as *MDC1* (mediator of DNA damage checkpoint 1) and *YWHAG*, which is required to maintain the DNA replication stress checkpoint and the G2 DNA damage checkpoint. These proteins participate in the DNA damage response pathways, activating DNA damage-dependent cell-cycle checkpoints, leading to cell-cycle arrest (Table 3).

With respect to DNA replication and recombination, the majority of genes involved in these functions were downregulated in patients after IM therapy. Their products include some proteins and enzymes required for DNA replication, such as Mcm4, Pola, Pole2, Prim1, Cdc7, Ccne1, Rpa3, elongation of DNA (Rfc3, Rfc4), as well as the proteins Mre11, Rad51, Rad50, Ruvbl2, Ercc1, and Msh2, which are involved in DNA recombination, and Fancd2 (Fanconi D2 protein), which is a major protein of the Fanconi anemia repair pathway (Table 3). By contrast, the changes observed in the expression profile of most of these genes were not significant on comparing healthy controls and patients after imatinib therapy (Table 3).

Discussion

The present study demonstrated the occurrence of gene deregulation after imatinib therapy in chronic-phase CML patients. Thus, patients treated with imatinib showed increased apoptosis and downregulation of genes related to proliferation and DNA replication, recombination, and repair. Ultimately, this led to decreased cell proliferation, a finding that is consistent with previously published results [30].

Gene-expression profiling using microarray technologies has deepened our understanding of the biology of CML [16]. To define new markers of disease progression or to identify genes that may be involved in this resistance, most studies have paid particular attention to comparisons of cell expression in CML patients and in healthy individuals [20,24]. However, little is known about the cellular mechanism involved in the early phases of imatinib therapy. To the best of our knowledge, this is the first report on the transcriptional response *in vivo* to imatinib during the first few weeks of treatment.

The present study revealed that IM significantly modifies the cell cycle, cell growth, proliferation, cell death, and DNA repair in chronic-phase CML patients (Fig. 1). A number of genes involved in cell-cycle progression, such as CDKs, underwent alterations in their expression levels in CML patients treated with imatinib. Thus, significant downexpression was detected in genes belonging to this group, including *CDK6*, *CDKN3*, *CKS1B*, *MEN1*, and *CKS2* (Table 2). CDKs are key regulatory enzymes in G1/S and G2/M transition. Reduced gene-expression levels are essential for cell-cycle arrest at an early stage. The ability of imatinib to alter the transcription of cell-cycle and proliferation genes has previously been demonstrated in the K562 cell line [31]. Interestingly, *CDK6* expression decreased in cells lines treated with imatinib in the same way as in treated CML patients. Our study also identified changes in other genes involved in cell-cycle progression and G1/S transition, such as *LATS2* and *CCNE1*. After imatinib therapy, *LATS2* was overexpressed, whereas the levels of expression of *CCNE1*, *CDK6*, and *CDKN3* decreased in relations to the time of diagnosis. These modifications led to cell-cycle arrest. *LATS2* is a putative tumor suppressor that inhibits G1/S transition by down-regulating cyclin E/CDK2 kinase activity [32]. Furthermore, overexpression of both *GADD45B*, which induces growth arrest after DNA damage and cell-cycle arrest at G2/M, and *CDC42*, which promotes the blocking of G0/G1 progression to the S phase, was also observed after imatinib therapy. In addition, we observed downexpression of *CCNE1*, *SKP2*, and *CDKN3* that was related to the arrest of cell-cycle progression and reduced proliferation. In summary, imatinib increases cell-cycle arrest by regulating some cell-cycle-related genes.

After 1 month of imatinib therapy, the chronic-phase CML patients showed an increase in *FOXO3A* and *PTEN* expression. Forkhead box class O (FoxO) target genes were induced in response to stress situations such as oxidative stress, DNA damage, and growth-factor depletion [33]. *FoxO3a* is a member of the FoxO transcription factor family and an important apoptosis regulator. FOXO3a is constitutively phosphorylated and therefore inactive in cell lines expressing Bcr-Abl [34,35]. The overexpression of *FOXO3A* may be related to the increased apoptosis in CML patients during imatinib therapy.

Upregulation of the tumor-suppressor gene, *PTEN*, was also observed. *PTEN* is frequently mutated in a large number of cancers [36,37]. The expression of PTEN phosphatase seems to regulate certain apoptotic signals affecting phosphoinositide 3-kinase function. *PTEN* antagonizes the *PI3K-AKT/PKB* signaling pathway by dephosphorylating phosphoinositides and thereby modulating cell-cycle progression and cell survival [38]. In addition, the *PI3K/PKB* signaling pathway plays an important role in proliferation and apoptosis, and mediates these effects through the modulatory activity of FoxO transcription factors [39]. These findings suggest that the response to imatinib involves mechanisms that alter *PTEN* signaling by increasing the expression of FoxO3a and PTEN, leading to increased apoptosis and a low proliferation rate.

A considerable number of genes involved in cellular responses to DNA lesions were found to be downregulated in our study (Table 3). These results are consistent with those of previous reports, showing that imatinib reduced the expression of several DNA-damage repair pathway genes in K562 cell lines, such as *RAD50* [31]. Thus, the patients receiving IM therapy exhibited changes in the expression levels of genes related to the detection of DNA lesions and signaling pathways, leading to the repair of these breaks (*RAD51*, *RAD51C*, *FANCD2*, *RAD50*, and *MRE11*) (Table 4) [31,40–42]. Mre11, Rad50, and Rad51 proteins are components of the MRN protein complex, which is a major player in cellular responses to DNA double-strand breaks (DSBs), which include break-sensing and the activation of signaling pathways that control repair of DNA lesions by homologous recombination repair and nonhomologous end-joining mechanisms. The MRN complex is essential for maintaining genomic integrity.

Rad51 plays a pivotal role in the response of BCR/ABL-positive leukemia cells to DSBs induced by reactive oxygen species (ROS). Thus, the increased cellular levels of RAD51 and other proteins involved in DNA repair might promote genomic instability [43]. In addition, Rad51 overexpression promotes alternative DSB repair pathways and chromosomal instability [44]. Elevated RAD51 protein levels can stimulate intrachromosomal and interchromosomal deletions and cause chromosomal aberrations in mammalian cells [45]. We showed that *RAD51* is transcriptionally downregulated in this study, as it has been previously described in cell lines treated with imatinib [46].

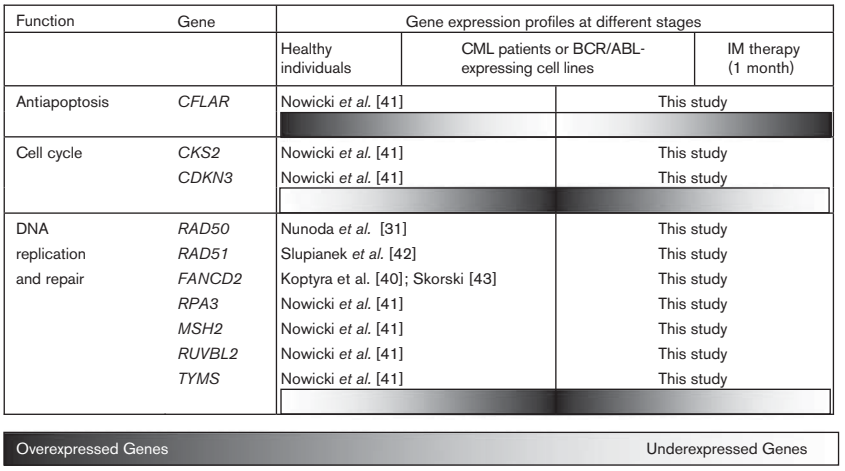
The mechanism by which BCR/ABL promotes DNA damage remains uncertain. It has been suggested that BCR/ABL inhibits DNA repair and promotes genomic instability that results in point mutation and cytogenetic abnormalities. Several studies have suggested that the alterations in the expression of genes such as *RAD51* [42] and *BRCA1* [47], or in the function of the DNA damage sensor, ataxia telangiectasia and Rad 3-related (ATR) protein, or other ATR effector functions, could be responsible for the genomic instability in CML. Furthermore, BCR/ABL has been shown to induce an increase in the relative levels of ROS and to cause chronic oxidative DNA damage, resulting in DSBs during genome duplication and division (S and G2/M cell-cycle phases). These lesions are repaired by BCR/ABL-stimulated homologous recombination repair and nonhomologous end-joining mechanisms, but in the BCR/ABL-positive leukemia cells, the oncogene promotes unfaithful mechanisms of DSB repair and contributes to genomic instability and malignant progression of Ph-positive leukemias [48].

The increase in ROS was directly due to BCR/ABL because it was blocked by the ABL-specific tyrosine kinase inhibitor STI571 [49]. BCR-ABL-mediated ROS generation in combination with aberrant regulation of DNA repair pathways contributes to a mutator phenotype in CML cells and results in genomic instability [49,50]. In this study, we have observed that inactivation of BCR-ABL tyrosine kinase by imatinib plays an important role in the DNA-damage response. The blockade of BCR-ABL tyrosine kinase by imatinib reduces intracellular levels of ROS [49], which may decrease DNA lesions including DNA DSBs and cause a reduction in the stimulus (DNA damage) that triggers the pathways of the DNA-damage response. Gene expression data can only provide insights into functional processes and it is important to note that most processes in cell-cycle regulation and DNA repair are regulated at post-transcriptional or post-translational levels, as for example modifications such as phosphorylation and ubiquitylation.

In this study, we show that imatinib therapy promotes significant changes in the expression profile in the bone marrow of patients treated with this drug. Imatinib inhibits the activity of ABL oncoproteins (c-ABL, BCR-ABL, ETV1-ABL), c-KIT, and platelet-derived growth-factor receptor A/B. This blockade of the molecular target of imatinib reduces the level of expression of key genes. Therefore, cells attempt to restore the expression levels of key genes affecting DNA repair as a result of a tendency to revert to the initial levels of a normal cell. IM may attempt to revert to the 'basal' levels required for cell proliferation and chromosomal integrity. These changes observed in the expression have been described by other authors who detected increased expression of certain genes related to DNA damage in patients with CML or in models that express BCR-ABL compared with healthy cells or cells without oncogenic protein (Table 4).

In agreement with previous reports, an upregulation of genes also overexpressed in cell lines expressing BCR/ABL was observed in CML patients [40–43]. Most of these genes are involved in DNA repair such as *RAD50*, *RAD51*, *FANCD2*, *RPA3*, *MSH2*, *RUVBL2*, and *TYMS* (Table 4). Thus, a slight increase in the expression of

Table 4 Summary of the most relevant genes that are differentially expressed in chronic myeloid leukemia patients and after imatinib therapy



White indicates downregulation and black indicates upregulation.
CML, chronic myeloid leukemia; IM, imatinib mesylate.

these genes was observed; thus, the mean expression values of samples at diagnosis were higher than those in normal controls. However, the present in-vivo study did not detect a marked difference in the expression of these genes, such as *MSH2* (Table 3).

Of note, in the present study, most of the deregulated genes modified their expression in terms of the expression of normal bone marrow (BM). After IM therapy, most of the genes showed altered expression levels in the same way as the normal BM cells, these changes being more relevant after IM therapy than in normal BM. Therefore, imatinib could substantially alter the expression profile of the bone marrow cells in the first days of therapy. First, the change is substantial, but the variation of most of the genes is similar to the expression levels of healthy controls in an attempt to revert to the normal gene expression in the bone marrow.

This in-vivo study demonstrates that IM promotes a tumor-suppressive effect in CML through the inhibition of cell-cycle progression and cell death, followed by an increase in apoptosis. In addition, IM restores genomic stability. Therefore, the present data provide new insights into the mechanism of action of imatinib.

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Conflicts of interest

There are no conflicts of interest.

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