

***RUNX1T1* is overexpressed in imatinib mesylate-resistant cells**

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Abstract. The Philadelphia (Ph) chromosome, which occurs as a result of a translocation between chromosomes 9 and 22, generates a BCR-ABL fusion oncogene in leukaemia cells. The BCR-ABL fusion protein has constitutive tyrosine kinase activity. The development of imatinib mesylate (STI571, Gleevec®), a potent and selective BCR-ABL tyrosine kinase inhibitor, represents an important advance in cancer therapy. However, inherent mechanisms confer resistance to imatinib mesylate in some leukaemia patients. In order to identify the genes potentially related to these resistance mechanisms, we examined genes differentially expressed in BCR-ABL-positive cell lines resistant to imatinib mesylate. A comparison of global gene expression using the HG-U133 2.0 plus Gene Chip array was first performed. Twenty-three genes were shown to be overexpressed in an imatinib-resistant cell line. Among these, *RUNX1T1* was shown to be overexpressed in another resistant cell line and in patients resistant to imatinib mesylate. This suggests that *RUNX1T1* is a putative candidate for the further study of imatinib mesylate resistance.

Introduction

BCR-ABL is a chimeric oncoprotein generated by reciprocal translocation between chromosomes 9 and 22 that is implicated in the pathogenesis of Philadelphia (Ph)-positive human leukaemias (1). The BCR-ABL fusion protein is located in the cytoplasm and has constitutive tyrosine kinase activity (2,3). This protein activates multiple signal transduction pathways, including RAS/Raf/MAPK, JAK/STAT and PI3K/Akt (4,5). These events cause excessive cellular proliferation, prevent apoptosis and decrease cellular adhesion (6-8).

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The enhanced tyrosine kinase activity of BCR-ABL is essential to leukaemia cells. It is present in 95% of patients with chronic myeloid leukaemia (CML), 0.7% of patients with acute myeloid leukaemia (AML) and 20% of patients with acute lymphoblastic leukaemia (ALL). These data demonstrate that BCR-ABL expression plays a key role in leukemogenesis and provides an appropriate and specific target for therapeutic intervention (9).

Imatinib mesylate (IM; STI571, Gleevec) is a 2-phenylamino-pyrimidine-tyrosine kinase inhibitor with specific activity for ABL, PDGFR and the c-Kit receptor (10,11). IM binds to the ATP-binding site of the BCR-ABL tyrosine kinase, preventing tyrosine autophosphorylation and, in turn, phosphorylation of its substrate (12,13). This process results in the deactivation of downstream signalling pathways (14). Although haematological and cytogenetic remissions occur in most patients undergoing IM therapy, a small percentage of patients relapse upon IM treatment. Imatinib resistance mechanisms have been characterised, and resistance is classified as BCR-ABL-dependent or BCR-ABL-independent. BCR-ABL-dependent resistance is attributed to mutations in the BCR-ABL tyrosine kinase domain or to the overexpression of BCR-ABL (15). BCR-ABL-independent resistance is less understood, though it is related to processes independent of BCR-ABL, such as the up-regulation of drug efflux pumps (16), LYN overexpression (17) and NF-κB activation (18). However, these alterations do not account for all cases of IM resistance.

Over the last five years, studies have attempted to identify the genes potentially correlated with IM resistance in CML using microarray assays to compare responsive and resistant cell lines and patients (15,19-21). The results of the analyses present important variations among different cells or gene sets, and suggest that differentially expressed genes correlated with IM resistance are involved in several signalling pathways. Thus, the activation of certain pathways could be another mechanism of resistance (19-21).

In this study, we identified genes differentially expressed between BCR-ABL-positive cell lines resistant to IM and their IM-responsive counterparts. Global gene expression was compared in IM-resistant and IM-responsive MBA cell lines (22) using the HG-U133 2.0 plus Gene Chip array. According to our criteria, genes were overexpressed in MBA IM-resistant cells. One of these genes, *RUNX1T1*, also showed increased expression in K562 IM-resistant cell lines and in imatinib non-responder CML patients. These results suggest that *RUNX1T1* may be related to IM resistance in BCR-ABL-positive cells.

Materials and methods

Cell culture and IM treatment. Mo7e, MBA and K562 cell lines were grown and maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin and 10 mg/ml streptomycin. The Mo7e culture was supplemented with 5 ng/ml Recombinant Human Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF, Invitrogen). Imatinib mesylate (Novartis) was prepared in dimethyl sulfoxide (DMSO, Merck). The AML cell lines, Mo7e human megakaryocytic and Mo7e-p210 BCR/ABL⁺ (MBA), were kindly provided by Dr John E. Dick (University of Toronto, Toronto, Ontario, Canada).

Establishment of IM-resistant cell lines. The K562 and MBA cell lines were cultured and passaged every 72 h, with continuous exposure to IM at concentrations that were increased every week in stepwise increments of 100 nM from 0.1 to 1 μ M. Cellular proliferation was assessed by the trypan blue exclusion assay. IM-resistant cells were maintained under the continuous selection pressure of IM. The morphology and proliferation levels of resistant cell lines were compared to those of the parental cells. The establishment of IM resistance in cells was performed as described by Melo and Chuah (15) and Nimmanapalli *et al* (23). The Mo7e cell line was also cultivated in medium supplemented with 1 μ M IM and used as a BCR-ABL-negative control in the Q-PCR assay.

Bone marrow samples. Bone marrow aspirates were obtained from two male donors (mean age 34.5 years, range 32-37) and 5 CML patients (mean age 32.4 years, range 31-59; male to female ratio, 4:1). Two patients were classified as having a complete cytogenetic response (IM-responsive), and three patients presented cytogenetic and molecular resistance (IM-resistant). Informed consent was obtained according to the local ethics committee guidelines. All samples were obtained at the Instituto Nacional de Câncer (Rio de Janeiro, Brazil). Diagnoses and follow-ups were based on haematologic, cytogenetic and molecular assays. Bone marrow mononuclear cells were isolated from 2 ml aspirates in a Ficoll-Hypaque density gradient. Cells were washed three times in phosphate-buffered saline (PBS) and subsequently used for RNA extraction.

Microarray data analysis. Total RNA from MBA and MBA IM-resistant cell lines was obtained using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed with Superscript III Reverse Transcriptase[®] (Invitrogen), and the synthesised cRNA was biotinylated using the One Cycle Kit (Qiagen, Germantown, MD, USA). The biotinylated cRNA was then hybridised to HG-U133 2.0 plus Gene Chip array (Affymetrix, Santa Clara, CA, USA), washed and stained according to the manufacturer's protocol (Qiagen). The gene chip arrays were scanned using GeneChip[®] Scanner 3000. Analysis was conducted using GeneChip Operating Software (GCOS; Affymetrix). Data were analysed using ArrayStar[®] v2.0 Gene Expression Analysis Software (DNASTAR; www.dnastar.com), with 10-fold changes used as criteria to define overexpression.

Quantitative PCR. All quantitative PCR (Q-PCR) analyses were performed with 1 μ g of mRNA and were reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen). Quantitative determination of mRNA levels was performed using Power SYBR Green PCR Master Mix[®] (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7000 thermocycler (Applied Biosystems) with 45 cycles of 15 sec at 95°C and 1 min at 60°C. Expression levels were estimated in triplicate, and β -actin was used as an internal control. The standard curve, generated by serial dilutions, and the dissociation curve were used to determine the PCR efficiency and specific amplification. The determination of fold-expression was calculated using the $2^{-(\Delta\Delta Ct)}$ method. The following primers were used: *TAOK1* F: 5'-CGGCTCAAGGAGGAACAGACC-3', R: 5'-CCTGTGCTTCATCCAAACGC-3'; *ACSS1* F: 5'-GCTG TGCCTGATGAGATCCTG-3', R: 5'-TAGTGTCTCCCAGCT CCTG-3'; *LRRFP1* F: 5'-GCTGGCTGAATCTAGGCGG-3', R: 5'-GCCTCCTTGACTTCAGCAAAC-3'; *RUNX1T1* F: 5'-CGGAGGACGCACTGGCAG-3', R: 5'-GGTTTCACTCG CTTTACGGCC-3'; *SHANK2* F: 5'-AGCCAAGTCCGCCAG CC-3', R: 5'-GCGAACAGTGAAGGTGACCG-3'; *TP53BP1* F: 5'-GCAGAACTTCCTGGAGCTCTG-3', R: 5'-CTTCAG CACACTTCAGCACCG-3'; *ACTNB* F: 5'-ACCTGAGAAC TCCACTACCCT-3', R: 5'-GGTCCCACCCATGTTCCAG-3'; *BCR-ABL breakpoint* F: 5'-GTGCAGAGTGGAGGGAGA ACATC-3', R: 5'-GGCTCACACCATTCCCCATTGT-3'.

Results

IM-resistant cell lines overexpress BCR-ABL transcripts. K562- and MBA-resistant cell lines (K562-R and MBA-R, respectively) exhibited a similar morphology and proliferation ratio in comparison to their parental cell lines (data not shown). In the presence of 1 μ M imatinib mesylate, the proliferation of K562-R and MBA-R was not inhibited. However, at 24 h the parental cells were significantly inhibited by 1 μ M IM (IC₅₀).

BCR-ABL overexpression has been characterised as a mechanism of IM resistance (5). To verify whether our resistant cell lines overexpressed BCR-ABL, we performed Q-PCR to assess the BCR-ABL mRNA levels. The results showed that BCR-ABL was overexpressed 7-fold in K562-R and 10-fold in MBA-R cells (Fig. 1). This suggests that the K562-R and MBA-R cell lines exhibit a BCR-ABL-dependent mechanism of resistance.

Differential global expression of MBA IM-resistant cells compared to MBA control cells. To determine the global patterns of gene expression in the MBA and MBA IM-resistant cell lines, we used the Affymetrix microarray technique. Total mRNA was extracted from MBA and MBA IM-resistant cell lines and was amplified, labelled and hybridised to the Affymetrix chip HG U133 2.0 plus. After normalisation and analysis of the microarray data using ArrayStar v2.0 Gene Expression Analysis Software, several genes were determined to be under- or overexpressed in the IM-resistant MBA cells. The selected genes were further analysed using the NIH gene annotation software DAVID (<http://david.abcc.ncifcrf.gov/>) to identify their functional classification. Using a cutoff of a 5-fold change in expression, we found 6016 down-regulated and 342 up-regulated genes in IM-resistant MBA

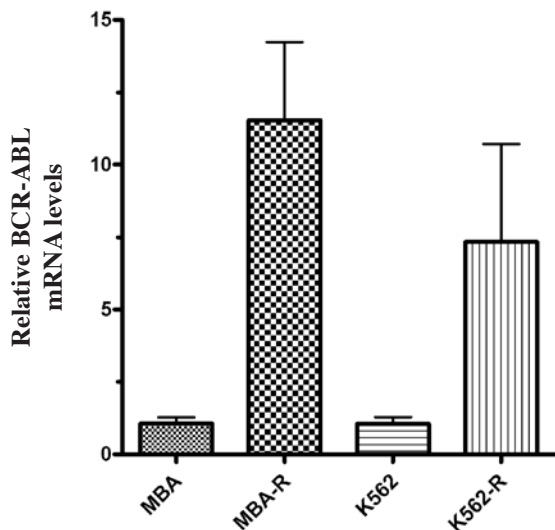


Figure 1. BCR-ABL is overexpressed in MBA-R and K562-R cell lines. Total RNA was isolated from resistant and control (without treatment) cell lines and examined by Q-PCR to determine changes in mRNA expression after normalization to β -actin expression levels. Analyses of BCR-ABL fold changes were performed in the MBA and K562 cell lines. Data are presented as a fold induction relative to the control group. Mean \pm SD (n=3).

cells compared to control MBA cells. This indicated a global down-regulation of gene expression. Among the 342 up-regulated genes, 23 showed at least a 10-fold increase, and were selected for further analysis (Table I).

Overexpressed genes in IM-resistant BCR-ABL-positive cell lines. To further investigate the overexpression of various genes in the BCR-ABL-resistant cell lines, Q-PCR was performed on MBA-R and K562-R cells compared to control MBA and K562 cell lines. The genes selected for analysis were *TAOK1*, *TP53BP1*, *SHANK2*, *RUNX1T1*, *LRRIFP1* and *ACSS1*. Mo7e cells were used as a negative control.

In the MBA-R cells, the genes tested exhibited increased mRNA levels, which confirmed the data from the chip array experiment (Fig. 2). In addition, mRNA levels were not altered in the BCL-ABL negative cell line, Mo7e (data not shown).

However, only one gene, *RUNX1T1*, was overexpressed in the K562-R cell line in comparison to the K562 cells (Fig. 2), suggesting that this gene might be directly affected in relation to a BCR-ABL cellular background. The mRNA levels of *TP53BP1*, *TAOK1*, *SHANK2*, *ACSS1* and *LRRIFP1* were not significantly altered in the K562-R cells (Fig. 2).

RUNX1T1 is overexpressed in IM-resistant patients. Various genes that are differentially expressed between IM-responders and non-responders with CML have already been described (21,23,25,26). To date, none of these genes have been shown to be differentially expressed with 100% consistency. We demonstrated that *RUNX1T1* was >10-fold up-regulated in our IM-resistant cell lines. In order to confirm the possible role of *RUNX1T1* in IM resistance, we performed Q-PCR using samples from IM-responsive and IM-resistant patients. Cells from healthy donors were used as the control. The resistant patients all had high levels of BCR-ABL expression (data not shown). *RUNX1T1*

Table I. Genes up-regulated in an imatinib mesylate-resistant MBA cell line (fold change >10).

Probe ID	Gene Symbol	Molecular function	Fold change
1564227_at	SCL25A13	Carrier activity ^a	10.132 up
232412_at	FBXL20	Ubiquitin-specific protease activity ^a	10.380 up
216465_at	SRPX2	Unknown ^a	10.428 up
1569098_s_at	TP53BP1	Transcription regulator activity ^a	10.625 up
217617_at	PBX1	Transcription regulator activity ^a	10.660 up
223581_at	ZNF577	DNA binding ^a	10.664 up
1570166_a_at	RAD51L1	DNA repair ^a	10.714 up
238534_at	LRRFIP1	Transcription regulator activity ^a	10.718 up
239434_at	-	-	11.052 up
230805_at	-	Similar to sterol regulatory element-binding transcription factor 2 [Bos taurus] ^c	11.156 up
1558515_at	-	CDNA clone IMAGE:4328048 ^c	11.302 up
215123_at	LOC23117	Neuropeptide signalling pathway ^b	11.325 up
1556658_a_at	MBNL	RNA binding ^a	11.677 up
234801_s_at	ACSS1	Metabolic process ^b	11.680 up
1560865_a_at	-	Full length insert cDNA clone YO02C07 ^c	11.761 up
241387_at	PTK2	Protein-tyrosine kinase activity ^a	12.263 up
233728_at	ISG20L2	Exonuclease activity ^a	12.436 up
216832_at	RUNX1T1	Transcription factor binding ^a	12.662 up
1559139_at	NOC2L	Unknown ^a	12.902 up
243454_at	ETV6	Transcription factor activity ^a	15.301 up
213308_at	SHANK2	Structural molecule activity ^a	17.532 up
1557452_at	SSDP2	Metabolism regulation ^b	19.759 up
216310_at	TAOK1	Protein serine/threonine kinase activity ^a	20.853 up

^aHuman Protein Reference Database (www.hprd.org). ^bDAVID Bioinformatics Database. ^cAffimetrix Database.

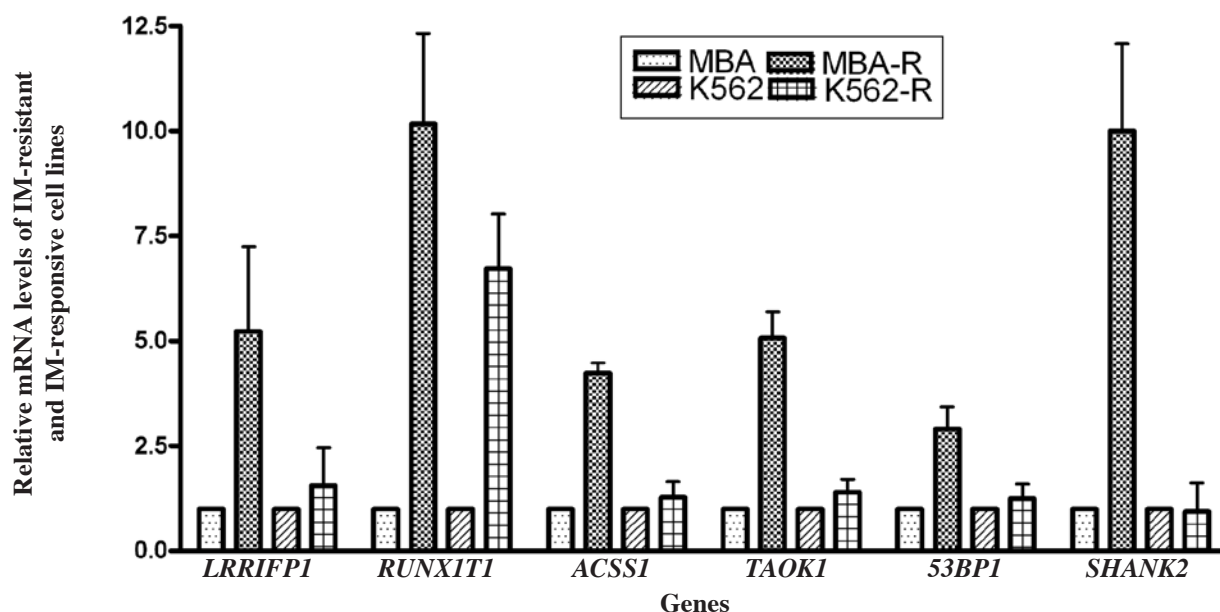


Figure 2. Imatinib mesylate promotes the up-regulation of genes in the MBA-R and K562-R cell lines. Total RNA was isolated from resistant and control (without treatment) cell lines and examined by Q-PCR to determine changes in mRNA expression levels after normalization to β -actin expression. Analyses of LRRIFP1, TP53BP1, RUNX1T1, TAOK1, SHANK and ACSS1 fold changes were performed in the MBA and K562 cell lines. Data are presented as a fold induction relative to the control group. Mean \pm SD (n=3).

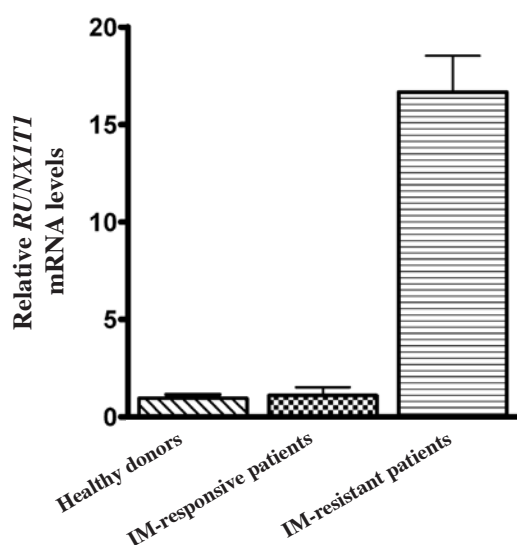


Figure 3. *RUNX1T1* is overexpressed in chronic myeloid leukaemia (CML) imatinib mesylate (IM)-resistant patients. Total RNA was isolated from bone marrow donors and CML patients IM-responsive and IM-resistant). This mRNA was used in quantitative real-time PCR to determine changes in *RUNX1T1* expression levels after normalization to β -actin expression. Data are presented as a fold induction relative to the control group. Mean \pm SD (n=3).

was also overexpressed in IM-resistant patients (Fig. 3). The IM-resistant patients had high levels of *RUNX1T1*, whereas the IM-responsive patients had unaltered levels of this gene.

Discussion

One of the major problems currently confronting IM treatment is the acquisition of resistance. Numerous previous studies have attempted to determine the mechanisms involved in IM

resistance using high throughput methodologies. Proteomic and transcriptomic techniques have been applied to investigate molecular alterations related to IM resistance. Several groups have compared the sensitivity of various IM-resistant cell lines, such as K562, LAMA84 and KCL22, to identify the genes related to resistance. Collectively, these studies have identified 41 genes that were up-regulated and 63 genes that were down-regulated in IM-resistant cell lines (21,23-27). However, none of these findings have been confirmed; no genes identified as being differentially expressed in response to IM resistance have been identified in common in the distinct resistant cell lines. Thus, the identification of molecular differences associated with drug resistance remains a challenge.

The present study used Affymetrix microarray analysis, which allowed for the analysis of more transcripts (~47,000) than previous studies. We compared an MBA cell line with its IM-resistant counterpart and identified several differentially expressed genes, then focused on the overexpressed genes present in the MBA IM-resistant line. Twenty-three genes were overexpressed at least 10-fold. Q-PCR was used to validate the overexpression of *TAOK1*, *TP53BP1*, *SHANK2*, *RUNX1T1*, *LRRIFP1* and *ACSS1*. We demonstrated that all these genes were overexpressed in the MBA-R cell line, and confirmed the results of the chip array experiment.

To verify if these genes were overexpressed in other IM-resistant cell lines, we used a K562 IM-resistant cell line in Q-PCR assays. The results demonstrated that *RUNX1T1* was overexpressed in both resistant cell lines, suggesting that it may be related to IM resistance. To confirm these results, we performed Q-PCR on total RNA from patients who were responsive or resistant to IM treatment. *RUNX1T1* was also shown to be increased in IM non-responder CML patients. We demonstrated that this gene was overexpressed in IM-resistant MBA cell lines, IM-resistant K562 cell lines and IM-resistant

patients. *RUNX1T1* is a co-repressor gene involved in gene regulation, and represents a putative candidate for further study of IM resistance mechanisms.

RUNX1T1, also known as *ETO* and *MTG8*, has frequently been reported as a fusion partner of *AML1* in leukaemias carrying the translocation (8,21), which results in the *AML1-RUNX1T1* hybrid gene (28,29). Although *RUNX1T1* is commonly found fused to *AML1*, neither our cell lines nor our patients showed an *AML1-RUNX1T1* translocation, suggesting that *RUNX1T1* expression occurs independently of *AML1*.

RUNX1T1 belongs to a family of conserved nuclear proteins whose members can be found from *Drosophila* to humans (30). It contains four evolutionarily conserved functional domains called nervy homology regions (NHRs). NHR2 has been described as important for homodimerization and protein-protein interaction with other co-repressors (30). Although *RUNX1T1* does not directly bind to DNA, it does function in transcriptional repression as a member of a multi-protein core repressor complex associated with a promoter. This co-repressor complex, which includes N-CoR, SIN3 and SMRT, recruits histone deacetylases and thus leads to the formation of a deacetylation complex. This complex then catalyses histone deacetylation and produces a repressive chromatin structure (28,31).

In conclusion, we demonstrated that *RUNX1T1* was overexpressed in IM-resistant cell lines and IM-resistant patients. However, further studies need to be performed in order to clarify the association between the overexpression of *RUNX1T1* and IM resistance.

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