

A comparative proteomic study identified LRPPRC and MCM7 as putative actors in imatinib mesylate cross-resistance in Lucena cell line

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# A comparative proteomic study identified LRPPRC and MCM7 as putative actors in imatinib mesylate cross-resistance in Lucena cell line

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## Abstract

**Background:** Although chronic myeloid leukemia (CML) treatment has improved since the introduction of imatinib mesylate (IM), cases of resistance have been reported. This resistance has been associated with the emergence of multidrug resistance (MDR) phenotype, as a BCR-ABL independent mechanism. The classic pathway studied in MDR promotion is ATP-binding cassette (ABC) family transporters expression, but other mechanisms that drive drug resistance are largely unknown. To better understand IM therapy relapse due to the rise of MDR, we compared the proteomic profiles of K562 and Lucena (K562/VCR) cells.

**Results:** The use of 2-DE coupled with a MS approach resulted in the identification of 36 differentially expressed proteins. Differential mRNA levels of *leucine-rich PPR motif-containing (LRPPRC) protein*, *minichromosome maintenance complex component 7 (MCM7)* and *ATP-binding cassette sub-family B (MDR/TAP) member 1 (ABCB1)* were capable of defining samples from CML patients as responsive or resistant to therapy.

**Conclusions:** Through the data presented in this work, we show the relevance of MDR to IM therapy. In addition, our proteomic approach identified candidate actors involved in resistance, which could lead to additional information on BCR-ABL-independent molecular mechanisms.

**Keywords:** Imatinib mesylate, Chronic myeloid leukemia, LRPPRC, MCM7, Proteome, Mass spectrometry

## Background

Chronic myeloid leukemia (CML) is a myeloproliferative disease, and the BCR-ABL constitutive tyrosine kinase (TK), an oncoprotein, serves as a marker of this condition. This oncoprotein is responsible for the pathogenesis of CML and is the primary molecular target for disease therapy [1]. Although CML treatment has improved with the development of imatinib mesylate (IM, Glivec<sup>®</sup>, Gleevec - Novartis), a TK inhibitor, some patients fail to respond to this therapy [2]. Resistance to treatment was first related to BCR-ABL-dependent mechanisms, such as mutations of the BCR-ABL kinase site. However, mutations are not seen in all patients who are resistant to IM treatment. This suggests that other resistance mechanisms occur in

these patients. Among these mechanisms, known as BCR-ABL-independent mechanisms, is the multidrug resistance (MDR) phenotype [3-9]. MDR is known to be the major cause of failure in cancer treatment and has a multifactorial origin. It is related to the expression of ATP-binding cassette (ABC) family transporters such as P-glycoprotein (Pgp-*ABCB1*), breast cancer related protein (BCRP-*ABCG2*) and multiresistance protein (MRP-*ABCC1*) [10,11]. Despite the identification and knowledge of ABC transporters, the resulting pathways involved in MDR rise in IM resistance, causing leukemic cells to become resistant to therapy remain uncharacterized. Proteomic analyses by both 2-DE and MS-based methods have been widely used in comparative studies of protein expression patterns in cells or organisms in attempts to identify proteins involved in diverse maladies, including different types of cancer [12-18]. In an attempt to identify other proteins that could be associated with an IM therapy

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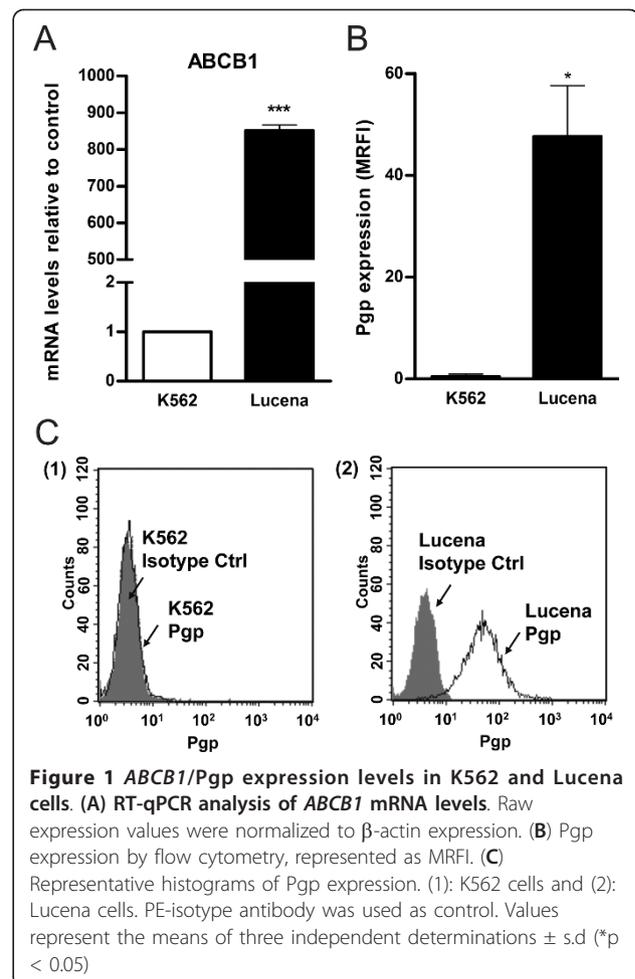
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response through a MDR phenomenon in CML, we compared the proteomic profiles of the K562 (an erythroleukemic) cell line and the Lucena (K562/VCR - vincristine) cell line in our study. Initially, we showed that Lucena cells exhibit cross-resistance to IM. Then, using 2-DE and MALDI-TOF-TOF MS, we identified 36 differentially expressed proteins between these two cell lines. *In silico* characterization of the identified proteins showed that detoxification pathways and cellular maintenance *biofunctions* were increased in the resistant cell line. We selected over-expressed genes in resistant cells for quantitative validation in healthy donors and CML patients with different responses to IM therapy. The *leucine-rich PPR motif-containing (LRPPRC) protein*, *minichromosome maintenance complex component 7 (MCM7)* and *ATP-binding cassette sub-family B (MDR/TAP) member 1 (ABCB1)* genes were able to differentiate CML patients as either resistant or sensitive to IM therapy. Through the data presented in this work, we show that MDR is closely associated with resistance to IM and demonstrate its importance as a prognostic indicator for CML patients. Moreover, this proteomic approach identified LRPPRC and MCM7 as possible new targets associated with IM resistance.

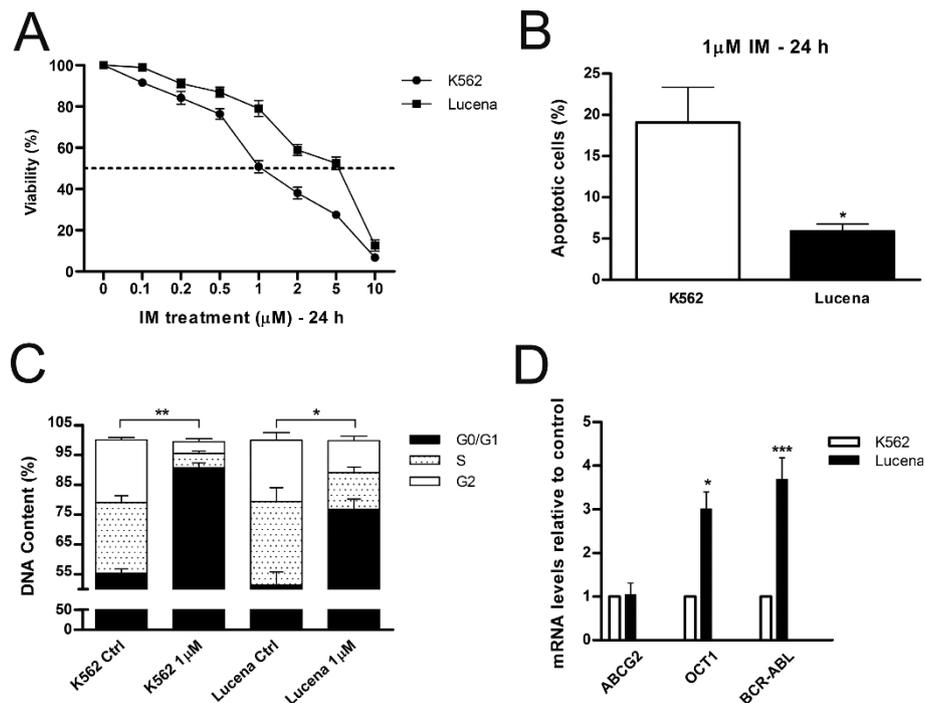
## Results

### Differential IM response in CML cell lines

Lucena cells were established from K562 cells as a multidrug resistance lineage by vincristine (VCR) selection and their pattern of resistance includes a range of chemotherapy drugs [19]. Real time quantitative PCR (RT-qPCR) analysis of K562 and Lucena cell lines showed an 800.0-fold increase of *ABCB1* mRNA levels by Lucena cells compared to K562 cells (Figure 1A). Over-expression of Pgp protein level was examined by flow cytometry and we observed a 45.0-fold increase expression of Pgp in Lucena cells compared to parental cells (Figure 1B-C). As demonstrated by Assef and colleagues, K562 cells managed to exhibit a MDR phenotype through VCR treatment also presented cross-resistance to IM [20]. Because the development of MDR cell lines is not well described in the literature (i.e., different concentrations of chemotherapy drugs are used) and VCR maintenance concentrations differ among these cell lines, we investigated IM effectiveness in Lucena cells. We treated both cell lines with different concentrations of the drug for 24 h and assayed cell viability. A comparative analysis of the viability of K562 and Lucena cells after IM treatment with 0.1  $\mu$ M, 0.2  $\mu$ M, 0.5  $\mu$ M, 1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M doses showed that Lucena cells were more resistant to IM than K562 cells (inhibitory concentration -  $IC_{50}$  5  $\mu$ M and 1  $\mu$ M, respectively; Figure 2A). To prove that the different cell viabilities were due to differential apoptotic activation between the cellular lineages, we conducted Annexin V assays with both cell



lines under IM treatment with 1  $\mu$ M (K562  $IC_{50}$  dose). The results (Figure 2B) revealed that an IM dose of 1  $\mu$ M activates apoptosis in approximately 20% of K562 cells but in only approximately 5% of Lucena cells. This result indicates that a 1  $\mu$ M dose of IM for 24 h is insufficient to induce apoptosis in Lucena cells (Figure 2C) as it does in K562 cells. Moreover, cell cycle assay showed that IM treatment induced arrest in G<sub>0</sub>/G<sub>1</sub> in both cell lines. However, this effect was more pronounced in K562 cells compared to Lucena cells (35.42% and 25.35%, respectively). Furthermore, we investigated the mRNA levels of *BCR-ABL* and the most related drug transporters involved in cancer with the goal of identifying the cause of Lucena cells cross-resistance to IM. In addition to possessing more *ABCB1*/Pgp than K562 cells (Figure 1), Lucena cells also had more *BCR-ABL* and *OCT1* mRNA (Figure 2D), but did not presented significant difference in *ABCG2* mRNA levels, which indicates that the failure of IM to induce apoptosis in Lucena cells may be due to Pgp drug efflux and/or by *BCR-ABL* up-regulation. In other to verify that cross-resistance to IM by Lucena cells might be through



**Figure 2 Panel of Lucena cross-resistance to IM treatment.** (A) K562 and Lucena cell lines were treated with a range of IM doses for 24 h. Cell viability was measured by trypan blue exclusion assay. Apoptotic cells (B) and cell cycle (C) were measured by flow cytometry after 1 μM of IM treatment in both cell lines. (D) *ABCG2*, *OCT1* and *BCR-ABL* mRNA expression levels in K562 and Lucena cell lines were quantified by RT-qPCR. Expression values were normalized to β-actin expression. Values represent the means of three independent determinations ± s.d. (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001)

drug efflux, we performed apoptosis, cell cycle and Pgp activity assays on Lucena cells treated in 3 different conditions: 1 μM IM, 50 μM Verapamil (VP - Pgp blocker) and co-treated with 1 μM IM and 50 μM VP (Figure 3). VP treatment was not toxic and did not induce apoptosis (Figure 3A) and cell cycle arrest (Figure 3B) compared to control (untreated cells). Co-treatment enhanced cell cycle arrest in 10%, compared to IM alone (Figure 3B), which was followed by a 3.0-fold increase (approximately) in apoptosis compared to IM alone (Figure 3A). Rhodamine 123 (Rho 123) retention confirmed that K562 cells did not present functional Pgp efflux pump, even after IM treatment (Figure 3C-D). This accumulation was not verified in Lucena cells in both control (untreated cells) and IM treatment (Figure 3C-D), demonstrating that Pgp pump was functional in these cells. After VP treatment, irrespective of IM presence, Pgp was blocked, showing that Rho 123 was able to accumulate in these cells (Figure 3C-D).

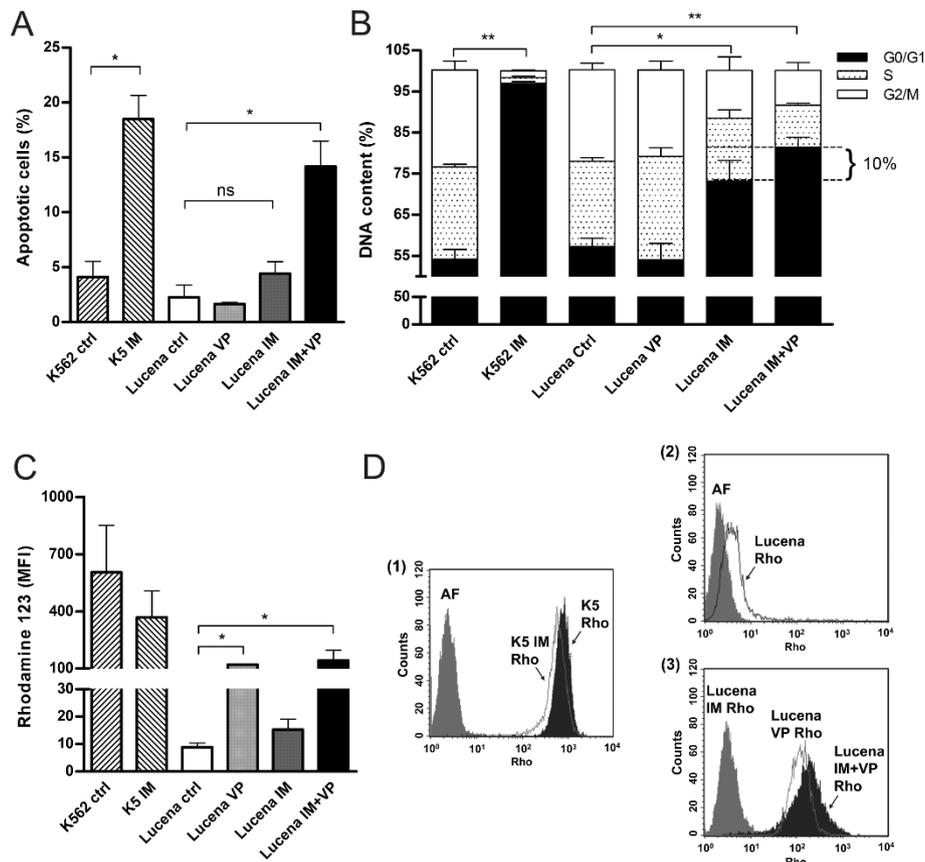
#### Identification of 2-DE differentially expressed proteins

Because the Lucena cell line has a cross-resistance to IM, we sought to generate Lucena and K562 cell lines (without IM treatment) proteomic profiles to understand the differences that contribute to IM cross-resistance. 2-DE was

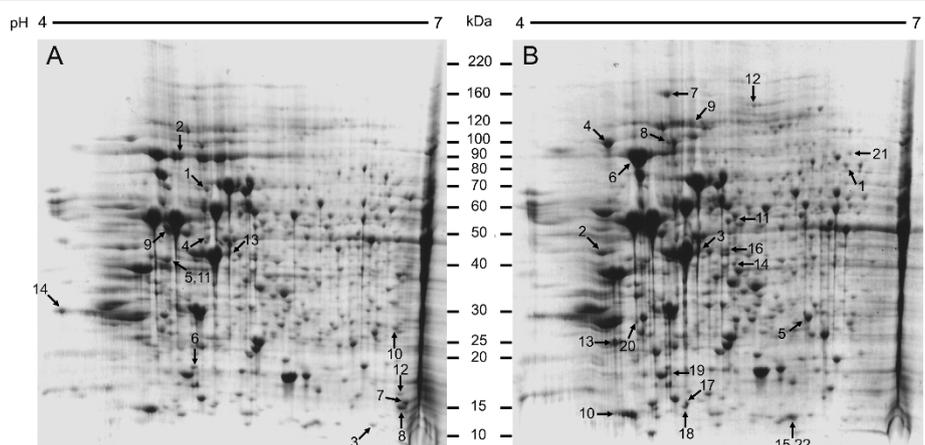
performed using 900 μg of total protein extract from each cell line. The 2-DE gels had more than 80% similarity, and 294 and 295 protein spots were visualized in the K562 and Lucena samples, respectively (Figure 4). All protein spots were excised from the 2-DE gels, digested with trypsin and analyzed by MALDI-TOF-TOF MS. Four hundred seventy-seven proteins were identified using this approach (235 proteins in K562 and 242 proteins in Lucena), which resulted in an identification rate of more than 80%. After an ImageMaster comparative analysis, only spots showing greater than 2.0 fold between samples (resistant versus responsive) were considered differentially expressed. In this analysis 36 proteins were found to be differentially expressed, with 14 proteins down-expressed and 22 proteins over-expressed in Lucena cells. Quantitative differences were observed, and proteins were analyzed and separated into cellular classes according to their potential biological function by gene ontology (GO) analyses <http://www.geneontology.org> (Table 1). Information regarding 2-DE analysis can be found in Additional file 1.

#### In silico analysis of identified proteins

To better understand resistance biology and to select the most promising candidates for further investigation, we assessed proteins using Ingenuity Pathway



**Figure 3** Lucena cells cross-resistance to IM is due Pgp efflux. Apoptotic cells (A), cell cycle (B) and Rho 123 (C-D) were measured by flow cytometry after 3 different treatments conditions: 1  $\mu$ M IM, 50  $\mu$ M VP and co-treatment with 1  $\mu$ M IM and 50  $\mu$ M VP. (D) Representative histograms of Rho 123 extrusion under conditions described above. (1): K562 ctrl and IM treatment; (2): Lucena ctrl and IM treatment; (3) Lucena under: IM, VP and IM + VP treatments. K562 cells were used as positive control for Rho 123 retention, and K562 treated with 1  $\mu$ M IM was used as positive control for apoptosis induction and cell cycle arrest. Values represent the means of three independent determinations  $\pm$  s.d. (\* $p$  < 0.05; \*\* $p$  < 0.01). AF = auto fluorescence; K5 = K562; LU = Lucena.



**Figure 4** Proteome maps of K562 (A) and Lucena (B) cell lines. Nine hundred micrograms of total protein extract were separated by electrophoresis on IPG (pH 4-7) and gradient (8-18%) SDS-PAGE gels. 2-DE gels were stained with coomassie colloidal blue (CBB). The migration of molecular mass markers is represented in the middle. Numbers refer to the spot identity used in table 1. Arrows correspond to the differentially expressed proteins according to ImageMaster 2D Platinum software.

**Table 1 Proteome map of differentially expressed proteins in K562 and Lucena cell lines.**

Spot N°	Identified Protein	Fold Change > 2 (Ratio Lucena vs. K562) <sup>a</sup>
<b>Structural Proteins</b>		
<i>INCREASE</i>		
1 L	MSN Moesin	2.23
2 L	RPSA 33 kDa protein	3.45
3 L	ACTB Actin, cytoplasmic 1	2.75
<i>DECREASE</i>		
1 K	LCP1 Plastin-2	- 3.65
<b>Stress Response/Chaperone</b>		
<i>INCREASE</i>		
4 L	HSP90B1 Endoplasmic precursor	2.22
5 L	HSPB1 Heat shock protein beta-1	4.23
6 L	HSP90AB1 Heat shock protein HSP 90-beta	3.1
7 L	HYOU1 Hypoxia up-regulated protein 1 precursor	3.76
8 L	VCP Transitional endoplasmic reticulum ATPase	2.29
<i>DECREASE</i>		
2 K	HSP90AB1 Heat shock protein HSP 90-beta	- 4.17
<b>Nucleic Acid Binding, Synthesis, Stability</b>		
<i>INCREASE</i>		
9 L	AARS Alanyl-tRNA synthetase, cytoplasmic	3.43
10 L	RPA3 Replication protein A 14 kDa subunit	2.82
11 L	RBM17 Splicing factor 45	2.54
12 L	LRPPRC Leucine-rich PPR motif-containing protein, mitochondrial precursor	3.18
<i>DECREASE</i>		
3 K	LSM2 U6 snRNA-associated Sm-like protein LSM2	- 3.23
4 K	HNRNPF Heterogeneous nuclear ribonucleoprotein F	- 3.01
5 K	HNRNPC Isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2	- 3.22
<b>Protein Binding and Synthesis</b>		
<i>INCREASE</i>		
13 L	EIF3K Eukaryotic translation initiation factor 3 subunit K	2.96
<i>DECREASE</i>		
6 K	EIF1AY Eukaryotic translation initiation factor 1A, Y-chromosomal	- 2.48
7 K	RPS12 ribosomal protein S12	-3.74
8 K	HINT1 Histidine triad nucleotide-binding protein 1	-3.57
<b>Metabolism</b>		
<i>INCREASE</i>		
14 L	ARG2 Arginase-2, mitochondrial precursor	3.83
15 L	COX6B1 Cytochrome c oxidase subunit VIb isoform 1	3.15
16 L	CKB Creatine kinase B-type	2.1
<i>DECREASE</i>		
9 K	ATP5B ATP synthase subunit beta, mitochondrial precursor	- 2.83
10 K	TPI1 Isoform 1 of Triosephosphate isomerase	- 3.96
<b>Signaling Transduction</b>		
<i>INCREASE</i>		
17 L	SH3BGRL SH3 domain-binding glutamic acid-rich-like protein	3.04
18 L	TXNDC17 Thioredoxin domain-containing protein 17	2.68

**Table 1 Proteome map of differentially expressed proteins in K562 and Lucena cell lines. (Continued)**

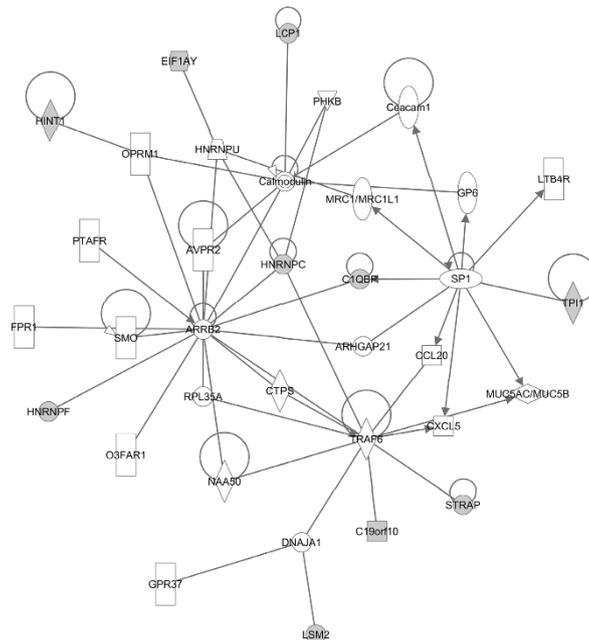
19 L	GMFB GMFB protein	2.89
20 L	CAPNS1 Calpain small subunit 1	2.37
	<i>DECREASE</i>	
11 K	STRAP Serine-threonine kinase receptor-associated protein	- 3.22
	<b>Cell cycle/Proliferation</b>	
	<i>INCREASE</i>	
21 L	MCM7 Isoform 1 of DNA replication licensing factor MCM7	4.02
22 L	S100A11 Protein S100-A11	3.15
	<b>Unknown</b>	
	<i>DECREASE</i>	
12 K	C19orf10 UPF0556 protein C19orf10 precursor	- 3.12
13 K	MTPN Myotrophin	- 2.98
14 K	C1QBP Complement component 1 Q subcomponent-binding protein, mitochondrial	- 2.37

<sup>o</sup> Fold change > 2 in resistance: cell lines have constitutively increased or decreased at least 2.0-fold changes of the given protein. Values expressed by ratio mean: Lucena/K562 (resistance versus responsive)

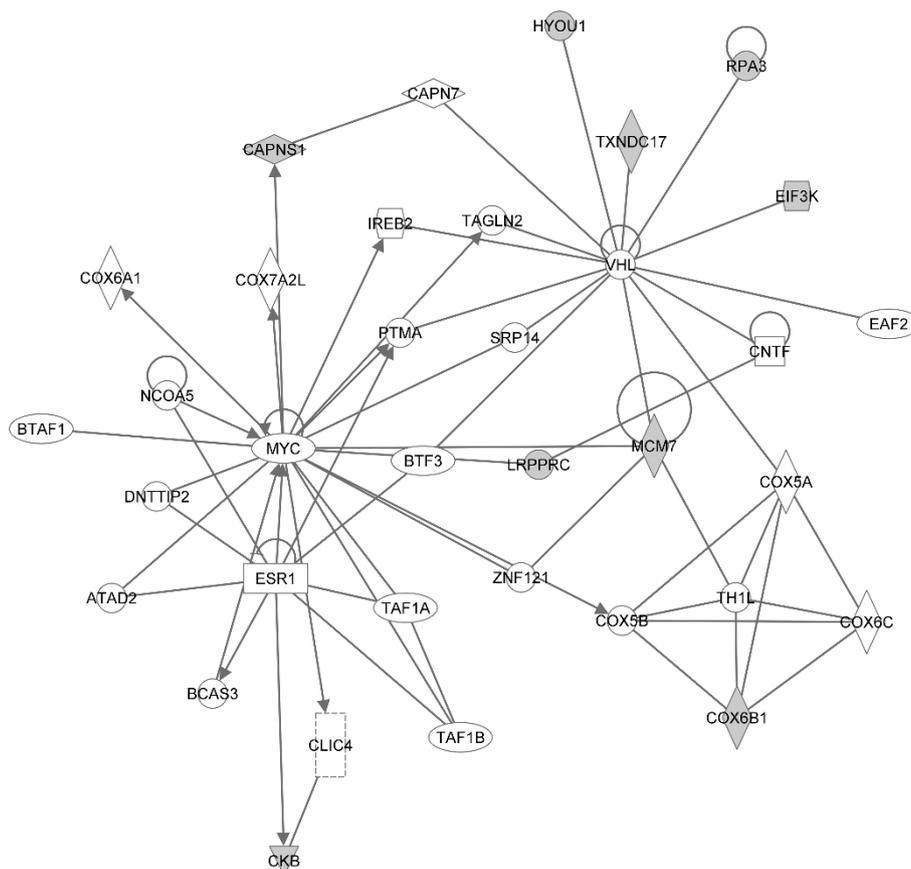
The corresponding spots on 2DE gels were identified with MS/MS

Analysis (IPA) (Ingenuity® Systems, <http://www.ingenuity.com>). We only analyzed direct interactions of increased and decreased proteins (Lucena vs. K562) separately and compared these analyses. The identified proteins were then clustered into two major networks (Figures 5, 6), as well as broader *biofunctional* groups and canonical pathways, by IPA (Figures 7, 8). The created networks indicated *Cellular Function and*

*Maintenance* ( $p = 5.77E-8 - 3.97E-02$ , 8 molecules); *Cell Death* ( $p = 2.86E-06 - 4.55E-02$ , 14 molecules); *DNA Replication, Recombination, and Repair* ( $p = 2.18E-04 - 3.84E-02$ , 5 molecules); *Cell-to-Cell Signaling and Interaction* ( $p = 1.29E-03 - 3.34E-02$ , 3 molecules) and *Small Molecule Biochemistry* ( $p = 1.29E-03 - 3.34E-02$ , 7 molecules) as the most relevant molecular and cellular functions increased in resistance.



**Figure 5 Network analysis of down-expressed proteins involved in resistance.** The biological network was generated after the protein's dataset was uploaded into IPA. Gray nodes denote uploaded proteins, and white nodes denote proteins from the IPA database. Lines between the nodes indicate direct protein-protein interactions. Arrowheads show the direction of interaction. Self-regulation is indicated by lines that begin and end on the same node.

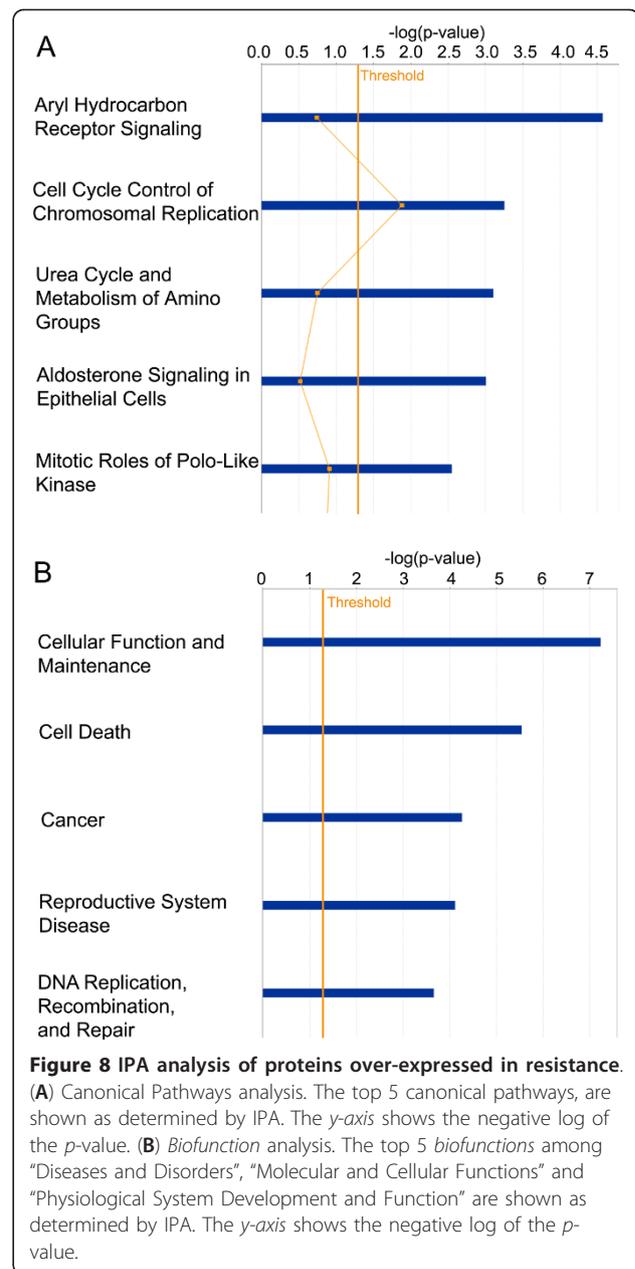
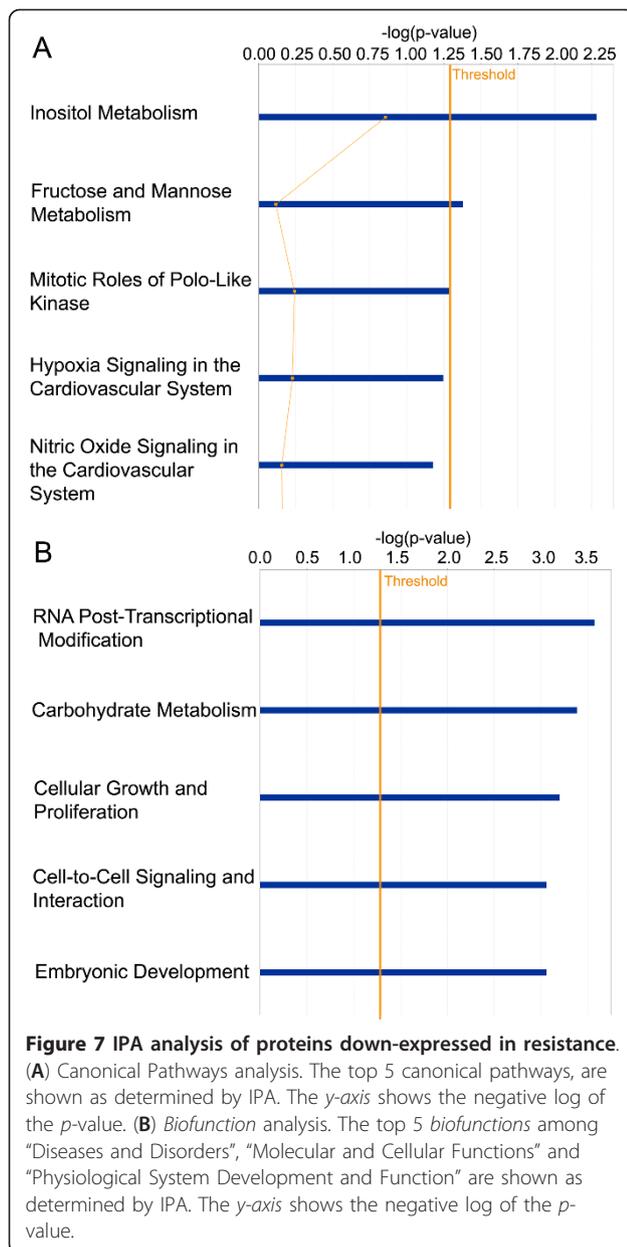


**Figure 6 Network analysis of over-expressed proteins involved in resistance.** The biological network was generated after the protein's dataset was uploaded into IPA. Gray nodes denote uploaded proteins, and white nodes denote proteins from the IPA database. Lines between the nodes indicate direct protein-protein interactions. Arrowheads show the direction of interaction. Self-regulation is indicated by lines that begin and end on the same node.

Proteins identified by our proteomic approach are shown in gray, and they are sometimes present in more than one *biofunctional* group. The down-expressed protein dataset did not provide as many statistical results on predominant canonical pathways (Figure 7A) as the up-expressed protein dataset. However, it is known that the *Fructose and Mannose Metabolism* pathway is down-regulated in the MDR phenotype [21]. *Aryl Hydrocarbon Receptor (AHR) Signaling* ( $p = 2.68E-05$ ), *Cell Cycle Control of Chromosomal Replication* ( $p = 5.57E-04$ ), *Urea Cycle and Metabolism of Amino Groups* ( $p = 7.85E-04$ ), *Aldosterone Signaling in Epithelial Cells* ( $p = 9.86E-04$ ) and *Mitotic Roles of Polo-Like Kinase* ( $p = 2.83E-03$ ), were the top five canonical pathways represented by the over-expressed proteins in resistant cells. *NRF2-mediated Oxidative Stress Response* ( $p = 2.25E-04$ ) and *Aryl Hydrocarbon Receptor (AHR) Signaling* ( $p = 4.57E-05$ ) were considered the top pathways in the Toxicity List, also assessed by IPA analysis.

#### Validation of target genes by real-time quantitative PCR

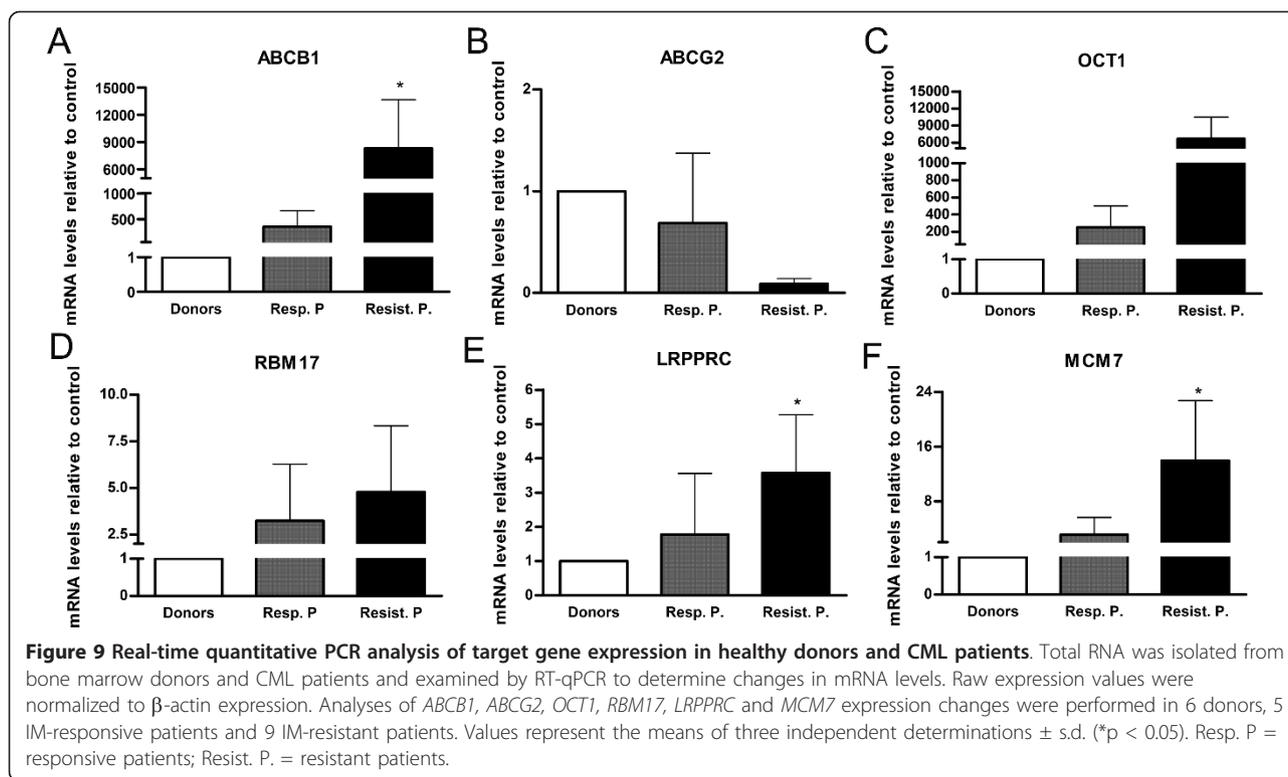
Across a variety of possible candidates for validation, we selected *LRPPRC*, *MCM7* and *RBM17* as representative genes involved in the most representative molecular functions identified by IPA. This validation approach was selected due to limited amounts of patient samples. RT-qPCR methodology is a FDA-approved assay for clinics. RT-qPCR analysis was carried out to evaluate mRNA levels in cell lines (data not shown), healthy donors, IM-responsive patients and IM-resistant patients. Additionally, the expression of drug transporters such as *ABCB1*, *ABCG2* and *OCT1* was analyzed. Figure 9 shows their relative mRNA levels after normalization to  $\beta$ -actin. Analyses of drug transporters showed a significant over-expression of the *ABCB1* in resistant patients. All genes selected from the proteomic approach were transcriptionally over-expressed in CML patients. After statistical analyses, only *RBM17* did not show a significant difference in mRNA expression levels between healthy donors and IM-resistant CML patients.



### Identifying IM resistance targets by multivariate analyses

To determine if the expression of the drug transporters and target genes found by the proteomic approach, along with other variables, could indicate a response to IM therapy, we performed univariate and multivariate analyses with 14 CML patients (5 responsive and 9 resistant to IM therapy). We considered the following variables: target genes verified by RT-qPCR, molecular and cytogenetic response, disease phase (chronic, accelerated and blastic phases are denoted CP, AP and BP, respectively) and duration of disease. We constructed a receiver operating characteristic (ROC) curve to establish the cut-off point for each gene in order to

categorize all mRNA expression levels found by RT-qPCR as either under or above these cut-off points. Using multivariate analysis, we calculated the  $\text{Exp}\beta$  for each variable, which is how much of an increase above basal level is necessary to increase the effect of each gene associated with all the genes studied. Because the increases of *ABCB1*, *LRPPRC* and *MCM7* above their basal levels were statistically significant (Table 2), our analyses suggested these genes as important variables when analyzing IM therapy response. Their ROC curves can be found in the additional files data (see Additional file 2). Taken together, expression of these genes may correlate with response to IM therapy.



Among the evaluated variables in multivariate analyses, only the target genes revealed by 2-DE, showed statistical significance in define CML patient's therapy status

## Discussion

Although the molecular basis of BCR-ABL-dependent mechanisms in IM resistance are well established (such as BCR-ABL mutations and *BCR-ABL* amplification), the same is not true for the BCR-ABL-independent mechanisms. The complexity of BCR-ABL independent resistance has not led to targeted therapy development. Instead, current approaches are focused on overcoming resistance of the T315I mutation, targeting survival pathways, and multi-kinase inhibitors [22]. Various cellular mechanisms may be involved in the nature of cellular resistance. Cells exposed to toxic compounds can develop resistance by a number of mechanisms

including increased amount of drug target, inhibition of apoptosis, changes in gene expression that control cell cycle, enhanced DNA repair, decreased drug uptake, or increased detoxification [23]. Baran and colleagues have pointed some important insights on IM resistance mediated by anti-apoptotic signals. In IM-resistant cells developed by their group, over-expression of Bcl-2 (anti-apoptotic gene) led to mitochondrial membrane potential (MMP) increase [24]. Besides, they examined the role of Sphingosine kinase-1 (SK-1)/sphingosine 1-phosphate (SP1) signaling in IM resistance. SK-1/SP1 activation can promote resistance of IM-induced apoptosis through unbalance between the levels of C<sub>18</sub>-ceramide (pro-apoptotic) and SP1 (anti-apoptotic) [25]. Recently, they determined a novel mechanism in which SK1/SP1 mediates BCR-ABL1 stability and drug resistance by protein phosphatase 2A modulation [26]. Constitutive activation of downstream BCR-ABL signaling molecules such as STAT3, STAT5A, Lyn, NF- $\kappa$ B, ERK1/2, and AKT are also considered as independent mechanisms of IM resistance. These molecules have been studied as potential targets for overcoming resistance [27-29]. Mencialha and colleagues demonstrated that LLL3, a STAT3 inhibitor, led to a decrease in proliferation and viability of BCR-ABL positive cells. As so, LLL3 administered together with IM increased the sub-G1 DNA content by 25% compared to IM treatment alone, demonstrating an additive effect of IM-induced

**Table 2 Multivariate analyses of IM therapy failure.**

Genes	ExpB	95% CI	P <sup>a</sup>	P <sub>2</sub> <sup>b</sup>
ABCB1	18.865	0.83 - 425.88	0.041	
LRPPRC	2.867E-10	1.170E-11- 7.027E-9	0.022	0.013
MCM7	6.897E9	6.897E9- 6.897E9	0.005	

Abbreviation: ExpB, Exponential  $\beta$ ; 95% CI, 95% confidence interval

<sup>a</sup>  $p < 0.05$  was considered to be significant

<sup>b</sup> P<sub>2</sub>: Significance of all 3 genes together

apoptosis [30]. Regarding on CML stem cells, it is a consensus in literature that these cells are intrinsically resistant to TK inhibition [31-35]. Interesting, Hamilton and colleagues have shown recently in transgenic mouse model of CML-like disease and in derived CML stem cells from patients, that CML stem cell survival is BCR-ABL kinase independent. They suggest that curative approaches in CML must focus on kinase-independent mechanisms of resistance [36].

Despite these novel information regarding BCR-ABL independent mechanisms, MDR phenotype still remains poorly understood. Little is known about the mechanisms through which MDR is activated, the molecules that are being induced, the pathways that are altered and exactly which proteins contribute to IM resistance. It is possible that the MDR phenotype functions in the same way for all drugs, but it is also possible that specific accessory proteins play important roles in resistance to distinct and unrelated drugs.

Recently, some groups have demonstrated that K562 cells driven to acquire the MDR phenotype also presented cross-resistance to IM compared to their parental cell lines [37]. This phenomenon is not exclusive by VCR selection in vitro. Illmer and colleagues [38] observed, in a model of K562-VP16 (etoposide) cells, a gradually increasing in Pgp expression with subsequent decline of intracellular IM levels. Decreased IM levels were associated with a retained phosphorylation pattern of the BCR-ABL target CRKL and loss of effect of IM on cellular proliferation and apoptosis. Yamada and colleagues [39] showed similar results related to IM effectiveness in K562-ADM (adriamycin) cells over-expressing Pgp. Pgp over-expression impact on cellular concentration of IM (and others TK inhibitors) was shown by Haouala and colleagues by knocking-down Pgp and measuring intra/extracellular IM ratio [40]. According to Vasconcelos and colleagues [41], an IM treatment at different concentrations for 24 h in K562 and Lucena cells was sufficient to increase *ABCB1* at mRNA levels and Pgp protein levels only in Lucena cells. Based on these data, we ought to investigate IM effect on cellular viability, apoptosis and cell cycle. As showed in Figure 2A, after 24 h of IM treatment, Lucena cells presented an  $IC_{50}$  of 5  $\mu$ M, which is 5.0-fold greater than K562 cells'  $IC_{50}$ . These findings corroborate with Vasconcelos and colleagues' observation that Lucena cells over-express Pgp at 5  $\mu$ M, compared to control (untreated cells). This variation of Pgp could explain Lucena cells' viability under IM treatment and the delay in apoptosis and cell cycle arrest in G0/G1, compared to K562 cells (Figure 2B-C). Moreover, our results summarized in Figure 3 provide evidence that blockage of Pgp pump, due to VP treatment, increased apoptosis and cell cycle arrest induced by IM in Lucena

cells, demonstrating that cross-resistance to IM may be, among others mechanisms, through Pgp drug efflux.

In order to identify proteins involved in BCR-ABL-independent mechanisms through MDR, we used this model in a comparative proteomic study between CML K562 and Lucena (K562/VCR) cell lines. Proteomic approaches such as 2-DE and MS enable the identification of differentially expressed proteins. In addition, these studies also provide pictures of alterations, making possible to better comprehend the biology and mechanisms that lead to a cellular process under investigation.

Our proteomic study of K562 and Lucena cells identified 36 differentially expressed proteins. The diversity of the proteins confirms that several pathways are deregulated and act together to promote the development of resistance. Among the pathways identified by IPA analyses as increased in resistance, we highlight the NF-E2-related factor 2 (NRF2)-mediated oxidative stress response and aryl hydrocarbon receptor (AHR) signaling. The key transcription factors of these pathways are Nrf2 and AHR, respectively. Through their translocation into the nucleus and binding to their respective elements in targets genes, they promote up-regulation of antioxidant and detoxification genes, stress response genes, xenobiotic-metabolizing genes, genes involving the ubiquitin-mediated proteasomal degradation system, intracellular redox-regulating genes, genes controlling cell growth and genes encoding transporters, such as ABC family members [42,43]. The relationship between these pathways and chemoresistance in several types of cancer is well known [44-46]. However, only recently has NRF2 signaling been under investigation for its association with BCR-ABL<sup>+</sup> cell IM resistance. Ozawa and co-workers demonstrated that up-regulation of NRF2 conferred IM resistance to KCL22 cells through transcription of the  $\gamma$ -GCS light subunit ( $\gamma$ -GCSI), a major determinant for glutathione (GSH) homeostasis [47]. The role of GSH in IM resistance was also discussed by Colavita and colleagues in another proteomic study [48]. This same group also showed that NRF2 up-regulation by HEME increased the IM  $IC_{50}$  without changing BCR-ABL kinase activity. NRF2 repression restored IM sensitivity. These results were verified by Bonovolias and Tsiftoglou in a study with CML and acute myeloid leukemia BCR-ABL<sup>+</sup> cells [49,50]. Thus, these findings highlight the importance of investigating not only NRF2 but also the role of AHR signaling in IM resistance with the aim of gaining additional knowledge on the mechanistic basis of IM resistance.

The separate evaluation of networks allowed us to observe that the increased proteins found due to resistance by our approach are localized in the center of the network. This result indicates that these proteins are

either in contact with the cytoskeletal proteins or are involved with cellular proliferation/maintenance proteins as key connecting proteins. It is clear that these interactions can lead to cellular maintenance through cytoskeletal changes that may confer alterations in cellular organization, cell cycle and proliferation. These alterations may give an adaptive advantage to resistant cells. *LRPPRC*, *MCM7* and *RBM17* were chosen as representative genes involved in these cellular processes. Quantitative results permitted the verification of a tendency of increased expression of target gene mRNA levels in IM-resistant patients compared to IM-responsive patients. Multivariate analyses sorted *ABCB1*, *LRPPRC* and *MCM7* as statistically significant genes in IM therapy status.

LRPPRC is an approximately 130 kDa protein that was first identified as over-expressed in the human liver carcinoma cell line HepG2 [51]. This protein seems to be involved in several intracellular processes such as homeostasis and microtubule alteration [52,53]. It seems that LRPPRC may be related to transactivation of MDR genes (*ABCB1* and *LRP*) by invMED sequence in acute lymphoid leukemia [54]. Moreover, an anti-apoptotic role for LRPPRC has been recently determined in hepatocarcinoma cells [55]. In up-regulated network created by IPA, LRPPRC interacts directly with BTF3 protein, which forms a stable complex with RNA polymerase II and is involved with regulation of transcription initiation. BTF3 interacts with MYC protein, which has a well-established role in cell proliferation and CML evolution. Along, these information support the potential role of LRPPRC in cell maintenance, proliferation, regulation of transcription and apoptosis.

MCM7 protein belongs to a highly conserved group of proteins that are essential for the initiation of DNA replication and cell proliferation [56]. This protein can be regulated by MYCN (*v-myc* myelocytomatosis viral related oncogene, neuroblastoma derived - avian) transcription factor in neuroblastoma [57]. Additionally, there is evidence that *ABCB1* can also be regulated by MYCN in neuroblastoma [58,59]. This information is relevant as we found that MCM7 was over-expressed in IM-resistant patients. MYCN could be regulating both genes in CML, so overexpression of MCM7 could be indirectly related to resistance. Alternatively, MCM7 may be directly involved in resistance by altering DNA repair function, as shown previously [60-62]. In IPA network MCM7 was shown to interact with TLH1 and ZNF121 proteins, which are involved in regulation of transcriptional activity. As LRPPRC, MCM7 is also implicated with resistance *biofunctions* found in our study such as cellular maintenance, cell cycle control and cell proliferation.

The involvement of *ABCB1* in IM resistance is controversial, but our results demonstrate that the *ABCB1* gene was significantly over-expressed in IM-resistant patients. The 9 IM-resistant patients in our study did not have mutations at the ABL site, and 5 patients were in the CP or AP stage. This result indicates that CML patients in the BC stage are not the only individuals that over-express this gene, as has been suggested in the literature [63,64]. Ferrao and colleagues have shown that the *ABCB1* gene does not confer IM resistance in vitro [65], but several studies have provided contradictory results. Specifically, studies with patients have shown that over-expression or polymorphisms of the *ABCB1* gene can alter the response to therapy [66-72]. For this reason, a hypothesis was derived that tried to explain the relationship between *ABCB1* over-expression and IM resistance. This hypothesis was based on the fact that *ABCB1* is expressed in early primitive normal hematopoietic stem cells [73,74] and can be over-expressed in leukemic stem cells, which would aid in maintenance of leukemia [75]. However, evidence shows that silencing *ABCB1* in leukemic stem cells does not sensitize them to IM treatment in culture [76,77]. The role of the *ABCB1* gene in IM resistance remains unclear. However, our findings, along with published data, suggest that *ABCB1* could be considered a prognostic factor for CML, as it is in acute myeloid leukemia.

Identification of potentially useful proteomic-based biomarkers must be validated in larger, well-defined retrospective and prospective clinical studies, and these combined efforts should result in identification of biomarkers that will greatly improve early detection, prognosis and prediction of treatment response [78].

In conclusion, our comparative proteomic approach using CML MDR/IM cross-resistant cell line and its parental cell line identified LRPPRC and MCM7 as putative actors in IM resistance. These data were validated in healthy donors and CML patients with different therapy responses. Altogether the expression of these genes and *ABCB1* could discriminate responsive and resistant groups and the therapy state of patients. As we analyzed a small patient cohort, we sought validation by future prospective clinical studies to establish biomarker's application in treatment response prediction or in follow-up monitoring.

## Methods

### Culture conditions

Lucena (a K562 multidrug-resistant cell line induced by VCR) cells over-expressing the *ABCB1* gene were kindly provided by Dr. Vivian Rumjanek (Departamento de Bioquímica Médica, Universidade Federal do Rio de

Janeiro, Brazil). The human myelogenous leukemia cell line (K562) and its VCR-resistant derivative Lucena were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum FBS, 50 units/mL penicillin G, 50 µg/L streptomycin and 2 mM L-glutamine (all from Invitrogen) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Lucena medium was supplemented with 60 nM VCR (Sigma).

#### **Imatinib mesylate treatment**

Cell lines were exposed to different doses of IM dissolved in DMSO (Sigma-Aldrich) with final concentration of 0.5%. DMSO-treated cells were used as vehicle-controls. Treatments were carried out in 12-well culture plates for a period of 24 h with a cell density of  $2.0 \times 10^5$  cells/mL. IM concentrations of 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM and 10 µM were used for cell viability assays. For apoptosis and cell cycle assays, a 1 µM IM dose was used. Cells were treated with 1 µM IM, 50 µM VP [79] and co-treated with 1 µM IM and 50 µM VP for apoptosis, cell cycle and Pgp activity assays. K562 cells were used as positive control for Rho 123 retention and K562 cells treated with 1 µM IM were used as positive control for apoptosis induction and cell cycle arrest. All experiments were performed in triplicate.

#### **Pgp expression assay**

To determine Pgp expression, we analyzed both cell lines with anti-Pgp-PE (phycoerythrin) antibody (Beckman Coulter) according to manufacturer's instructions. Briefly, after treatment,  $5.0 \times 10^5$  cells were harvested, washed twice with cold PBS, resuspended in 1 mL PBS/BSA (0.2% Azide, 1% BSA) and incubated for 15 min. After incubation, cells were harvested and anti-Pgp-PE (5 µL) was added, and the sample was incubated for 30 min in the dark. After incubation, 2 mL of PBS/BSA were added to each sample. Cells were harvested and resuspended in PBS/1%Formol. For every condition, 20,000 events were acquired using a FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed using CellQuest v.3.1 Software (Becton Dickinson, USA). Results are expressed as mean relative fluorescence intensity (MRFI), which was calculated by subtracting the mean fluorescence intensity (MFI) for specific antibody by the MFI of the respective, isotype control. All experiments were performed in triplicate.

#### **Cell viability assay**

Aliquots of IM-treated cells were removed after 24 h of treatment. The number of viable cells was determined using the trypan blue exclusion assay. The concentration of drug necessary to achieve a 50% reduction of viable

cells was denoted as the IC<sub>50</sub>. All experiments were performed in triplicate.

#### **Apoptosis assays**

To determine the percentage of apoptotic cells, we analyzed phosphatidyl serine externalization and membrane integrity by double staining with Annexin V PE and 7-AAD (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen, USA) according to manufacturer's instructions. Briefly, after treatment,  $1.0 \times 10^5$  cells were harvested, washed twice with cold PBS and resuspended in 100 µL of 1× binding buffer. Annexin V PE (5 µL) and 7-AAD (5 µL) were added, and samples were incubated for 15 min in the dark. After incubation, 400 µL of 1× binding buffer was added to each sample. Cells positive for Annexin V PE and 7-AAD were considered apoptotic. For every condition, 20,000 events were acquired using a FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed using CellQuest v.3.1 Software (Becton Dickinson, USA). All experiments were performed in triplicate.

#### **Cell cycle assays**

Cell cycle was evaluated by staining with propidium iodide (PI, Sigma-Aldrich) [80]. Approximately  $3.0 \times 10^5$  cells were resuspended in 400 µL of hypotonic buffer (3.4 mM Tris-HCl (pH 7.6), 10 mM NaCl, 0.1% (v/v) NP-40, 700 U/L RNase, and 0.075 mM PI) and incubated for 30 min at 4°C. For every condition, 5,000 events were acquired in a FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed using Cell Quest v.3.1 Software (Becton Dickinson, USA). All experiments were performed in triplicate.

#### **Pgp activity assay**

Rho 123 (Sigma) was used to measure the activity of Pgp by flow cytometry [81]. For each experiment,  $1.0 \times 10^5$  cells were incubated with 200 ng/mL of Rho 123. After 30 min of incubation at 37°C/5% CO<sub>2</sub> cells were washed with PBS and analyzed in a FACSCalibur Flow Cytometer (Becton Dickinson, USA). For every condition, 20,000 events were acquired using a FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed using CellQuest v.3.1 Software (Becton Dickinson, USA). Results are expressed as MFI. All experiments were performed in triplicate.

#### **Bone marrow samples**

All bone marrow samples were obtained from CML patients in all disease phases (CB, AP, or BP) and donors admitted or registered at the Instituto Nacional de Câncer (Rio de Janeiro, Brazil), according to the guidelines of the local Ethics Committee and the

Helsinki declaration. We selected 6 healthy donors (mean age = 30, range = 20-37, male:female ratio = 4:2), 5 IM-responsive patients (mean age = 45, range = 33-52, male:female ratio = 5:0, CP:AP:BP ratio = 3:2:0) and 9 IM-resistant patients (mean age = 44, range = 26-61, male:female ratio = 5:4, CP:AP:BP ratio = 1:4:4). Diagnoses and follow-ups were based on hematologic, cytogenetic and molecular assays. IM-responsive patients exhibited a major molecular response and complete hematologic and cytogenetic response, whereas IM-resistant patients lacked hematologic, cytogenetic and molecular responses. The inclusion criterion was to investigate CML patients that received IM as a first-line therapy. Marrow aspirates were collected in heparinized tubes and processed on the day they were collected. Bone marrow mononuclear cells were isolated from 2-5 mL of aspirate in a Ficoll-Hypaque density gradient (Ficoll 1.077 g/mL; GE, Sweden) according to manufacturer's protocol. Cells were washed 3 times in PBS and subsequently used for RNA extraction.

## 2-DE

Cells were washed in PBS and resuspended in cold lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM  $\beta$ -glycerophosphate, 250 mM NaCl, 2% NP-40 and protease inhibitors and incubated on ice for 30 min. The lysates were centrifuged at 12,000 *g* for 15 min at 4°C. The supernatants were collected, and the total protein concentrations were determined by the Bradford assay [82]. Nine hundred micrograms of total cell protein were precipitated using a 2D cleanup kit (GE, Sweden) according to manufacturer's instructions and resuspended in buffer containing 6 M Urea, 2 M Thiourea, 15 mM DTT, 2% (w/v) ASB14, 0.5% IPG buffer (pH 3-10; GE, Sweden) and bromophenol blue traces. IEF was carried out in an 11-cm Immobiline DryStrip (pH 4-7; GE, Sweden) on an Ettan IPGphor III electrophoresis unit (GE, Sweden) for a total of 32070 Vh. Subsequently, IPG strips were equilibrated for 15 min in equilibration buffer (6 M Urea, 30% (w/v) Glycerol, and 2% SDS in 0.05 M Tris-HCl (pH 8.8) containing 100 mg DTT per 10 mL SDS equilibration buffer) and then equilibrated for 15 min in buffer containing 250 mg iodoacetamide. IPG strips were run on ExcelGel SDS 8-18% gels according to procedures recommended by the manufacturer (GE, Sweden) and stained with colloidal blue [83]. All gels were scanned with an Image scanner using LabScan v.5.0 software (GE, Sweden), and ImageMaster 2D Platinum v.6.0 software (GE, Sweden) and subjected to visual analysis. *pI* values were determined using a linear 4-7 distribution using a logarithmic curve. Molecular weight values were determined using a Benchmark protein standard (Invitrogen). 2-DE gels were analyzed

separately and averaged. For spots found in all gels, the normalized spot volumes of triplicate samples were averaged. Spot normalization is an internal calibration that makes the data independent of experimental variations between gels caused by conditions such as differences in protein loading or staining. It was performed with the use of relative Volume (%Vol) to quantify and compare the gel spots. The intensity of each spot was quantified by calculating the spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots.

## MS identification

The protein spots of interest were cut out of the gel and processed for MS according to the following protocol. The trimmed gels were washed three times in 50% acetonitrile (AcN) and 25 mM  $\text{NH}_4\text{HCO}_3$  (pH 8.0) for 15 min, soaked in 100% AcN and dried in a Speed-Vac for 30 min. The samples were rehydrated at 4°C in digestion buffer (25 mM  $\text{NH}_4\text{HCO}_3$  (pH 8.0) containing 15 ng/ $\mu\text{L}$  porcine trypsin (Sequencing Grade Modified Trypsin, Promega, USA)) and incubated at 37°C overnight. The peptides were extracted with a solution of 50% AcN and 5% Trifluoroacetic acid (TFA) and dried in a Speed-Vac. Peptides were redissolved in 3  $\mu\text{L}$  of 50% AcN/1% TFA solution. For MALDI MS/MS analysis, 0.5  $\mu\text{L}$  of the redissolved peptide was mixed with fresh cyano hydroxycinnamic acid as a matrix on a MALDI plate. Mass spectra for peptide mass fingerprinting and confirmatory fragmentation analysis were acquired using the MALDI-TOF-TOF instrument 4700 (Applied Biosystems, USA). MS data were acquired in MALDI ion source, in positive ion reflector mode, mass range 900-4000 Da, using a neodymium-doped yttrium aluminum garnet (Nd: YAG) laser with a 200-Hz repetition rate and collision-induced dissociation (CID) mode *off*. Capillary electrophoresis (CE) was not used. Typically, 1,600 shots were accumulated for spectra in MS mode while 2,400 shots were accumulated for spectra in MS/MS mode. Up to eight of the most intense ion signal was selected as precursors for MS/MS acquisition. Spectra were acquired after plate calibration with calibration mixture 1 or 2 (Sequazyme Peptide Mass Standards kit, PerSeptive Biosystems, USA).

## Data processing and bioinformatics analysis

Peak lists were generated by Data Explorer v.4.5 software (Applied Biosystems, USA) using default parameters and searched with Mascot Daemon v.2.1 software (Matrix Science) against the non-redundant International Protein Index (IPI) protein sequence database v.3.6 (80,412 entries, released 17.06.09). Search parameters were as follows: database searches were

restricted to *Homo sapiens*, precursor ion and fragment ion mass tolerance of 0.2 Da, tryptic specificity allowing for one missed cleavage, fixed modification of carbamidomethylation of cysteine residues, and variable modification of oxidation of methionine residues. The criterion for positive protein identification was a minimum of two peptides. The Mascot threshold (relied on a 5% probability that the protein identification is incorrect) is a probability score. This score is described in Supplemental Figure 1 for each protein identified. The data associated with this manuscript may be downloaded from the <http://ProteomeCommons.org> Tranche network using the following hash:

```
F6rVqTaM9oCMmblj4oHeNBhczim01D4sJYEw5AurF-  
juk2n0WgLexO4PUOeJpB7RmFDDaPX + bbIxK-  
Be1Vj7dbHgHjjX0AAAAAACMpQ = =
```

These data include all MS files (.t2d format) from differentially expressed proteins identified. Proteins were investigated according to their GO <http://www.geneontology.com> annotations based on molecular functions. Functional analyses, network constructions and canonical pathway analyses were generated through the use of IPA (Ingenuity® Systems, <http://www.ingenuity.com>). The following parameters were used: reference set (Ingenuity knowledge Base - genes only); relationships to consider (direct relationships); network generation (mode *on*, 35 molecules per network); data sources (all); confidence (consider only relationships that were experimentally observed OR high predicted); species (human); tissues and cell lines (all) and mutations (all).

### Real-time quantitative PCR analysis

Analysis of mRNA levels was carried out by RT-qPCR. Two micrograms of TRIzol- (Invitrogen) extracted RNA from cell lines and healthy donor- and patient-derived mononuclear bone marrow cells were reverse transcribed with Superscript II Reverse Transcriptase® (Invitrogen). cDNA dilutions (1:100) were mixed with SYBR Green PCR Master Mix® (Applied Biosystems, USA) and the following forward (Fow) and reverse (Rev) primers: *ABCB1* NM\_000927.3 Fow 5' CCCATCATTGCAATAGCAGG 3', Rev 5' GTTCAAACCTTCTGCTCTGA 3'; *ABCG2* NM\_004827.2 Fow 5' TGGCTGTCATGGCTTCAGTA 3', Rev 5' GCCACGTGATTCTTCACAA 3'; *OCT1* NM\_003057.2 Fow 5' TCCTCTTCCTGCTCTACTACT 3', Rev 5' ATGAAGGGCTCAGCTTTTTCGG 3'; *LRPPRC* NM\_133259.3 Fow 5' GAGAGATGCCGGAATTGAGC 3', Rev 5' CTCGGACTTCTCCACCTTCT 3'; *MCM7* NM\_005916.3 Fow 5' TCGAGGCATGAAAATCCG GG 3', Rev 5' CGCCATCGATCAATGTATGACA 3'; *RBM17* NM\_032905.4 Fow 5' GTGGGTTTGCAAGGAGACCAG3', Rev 5' AAGTGGGTGGGGCAATGG 3'; *ACTB* NM\_001101.3 Fow 5' ACCTGAGAACTCCACTACCCT 3', Rev 5'

GGTCCCACCCATGTTCCAG 3'. RT-qPCR was performed in a Rotor Gene 6000 thermocycler (Cobertt) with 50 cycles of 20 s at 95°C, 30 s at 60°C and 30 s at 72°C. For each sample, the expression of target genes was normalized to *β-actin* mRNA levels. Changing mRNA levels were evaluated as previously described [84].

### Statistical analysis

Cell viability, apoptosis activation, DNA content/cell cycle and mRNA level differences between K562 and Lucena were compared by a paired *t*-test. These statistical analyses were performed using GraphPad Prism® v.5 software (GraphPad, USA). To determine if increased mRNA levels were associated with IM resistance, cut-off points for each gene were selected. These cut-offs were determined by constructing ROC curves with estimations of sensitivity, specificity and area under the curve. Clinical information on IM resistance was used as the state variable. The cut-off point for each gene was established, and all values were categorized under or above these points. For univariate analyses, Chi-squared and Fisher's exact tests were used to analyze the association between mRNA levels and IM treatment outcome. For multivariate analyses, logistic regression was performed to determine the association between mRNA levels and IM therapy outcome. These statistics were performed using SPSS v.13.0 for Windows® software (SPSS Inc.). *P*-Values less than 0.05 were considered as statistically significant (\**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001).

### Additional material

**Additional file 1: Differentially expressed proteins identified in IM cross-resistance.** Proteins were separated into biological functions according to GO analysis. Information regarding pI/MW, statistical and Mascot score, number of identified peptides, peptide sequence and sequence coverage were described.

**Additional file 2: ROC curve.** ROC curve analysis showed that *ABCB1*, *LRPPRC* and *MCM7* genes, together, are potential candidate biomarkers for IM therapy response for further investigation. Area under curve (AUC): 0.733 (*ABCB1*); 0.550 (*LRPPRC*); 0.767 (*MCM7*).

### List of Abbreviations

CML: chronic myeloid leukemia; TK: tyrosine kinase; IM: imatinib mesylate; MDR: multidrug resistance; ABC: ATP-binding cassette; VCR: vincristine; RT-qPCR: real-time quantitative PCR; IC<sub>50</sub>: inhibitory concentration; VP: verapamil; Rho123: rhodamine 123; GO: Gene Ontology; IPA: Ingenuity Pathway Analysis; FDA: Food and Drug Administration; CP: chronic phase; AP: accelerated phase; BP: blastic phase; ROC: receiver operating characteristic; VP16: etoposide; ADM: Adriamycin; MRFI: mean relative fluorescence intensity; MFI: mean fluorescence intensity; PE: phycoerythrin; PI: propidium iodide; AcN: Acetonitrile; TFA: Trifluoroacetic acid; Nd:YAG: neodymium-doped yttrium aluminum garnet; CID: collision-induced dissociation; CE: capillary electrophoresis; IPI: International Protein Index; Fow: forward; Rev: reverse.

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#### Authors' contributions

SC performed the experiments, bioinformatics analysis and drafted the manuscript. LP participated in the design of the study and mass spectrometry protein identification. BR participated in flow cytometry experiments. AM assisted the real-time quantitative PCR experiments. DP participated in statistical analysis. EA participated in the design of this study. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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