Survivin and P-glycoprotein are associated and highly expressed in late phase chronic myeloid leukemia

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Abstract. Resistance to tyrosine-kinase inhibitors is a serious problem in the treatment of chronic myeloid leukemia (CML). Using Western blot, real-time qRT-PCR and flow cytometry, we investigated the expression of survivin, Smac/DIABLO and P-glycoprotein (Pgp) in patients with CML. Survivin overexpression has been associated with cancer progression, multidrug resistance, poor prognosis and short survival in several types of neoplasms including hematological malignancies. In this work, survivin expression was significantly elevated in late, in contrast to early, chronic phase CML (p=0.044). Patients with high or intermediate prognostic Sokal score presented higher survivin levels (p=0.012), as well as Smac/DIABLO levels (p=0.009) compared to low Sokal score. The strong correlation between survivin and Pgp expression in late (p=0.018), but not in early (p=0.5) chronic phase of CML, suggests that this association may play a biological role in late CML phase and may offer an important target for the development of new therapies.

Introduction

Chronic myeloid leukemia (CML) is a clonal proliferative malignancy that originates from hematopoietic stem cells. This disorder is characterized by the presence of the Philadelphia (Ph) chromosome, which contains the chimeric BCR-ABL fusion gene. This gene encodes a constitutively active tyrosine kinase that confers enhanced proliferative activity and decreased sensitivity to apoptotic cell death (1). Clinically, CML is divided into three stages: chronic phase (CP), accelerated phase (AP) and blastic phase (BP). Using the Sokal score system, prognosis information was classified as low-, intermediate- and high-risk (2-4).

Until recently, the therapeutic options for CML were limited to allogeneic bone marrow transplantation and therapy with busulfan, hydroxyurea and interferon-α (5-7). Nowadays, imatinib mesylate, an inhibitor of Bcr-Abl tyrosine kinase activity, has been used as a major therapeutic approach. Although imatinib is unquestionably effective in the treatment of CML at chronic phase (CML-CP), some patients may become resistant to this drug due to the appearance of an imatinib-resistant clone (8). Resistance to imatinib may result from a variety of cellular mechanisms such as reactivation of Bcr-Abl kinase activity within the leukemic cells by either point mutations or gene amplification, drug detoxification, alterations in DNA repair, activation or overexpression of drug export proteins and deregulation in the apoptosis control (8,9).

Survivin overexpression, an anti-apoptotic protein, is associated with cancer progression, multidrug resistance (MDR), poor prognosis and short survival in several types of neoplasms including hematological malignancies (10). Survivin is a member of the inhibitor of apoptosis protein (IAP) family. It plays an important role not only in inhibiting apoptosis, but also in regulating mitosis (11). Its particular feature is its expression in cancer, but not in normal tissues. Survivin is strongly expressed in embryonic and fetal organs but undetectable in most differentiated normal tissues. However, the gene of survivin is reactivated and several types of tumors display high levels of this protein (10). Due to this peculiar characteristic, survivin may be a promising target for anticancer intervention. Currently, studies that evaluate the potential role of survivin in the CML pathogenesis and drug resistance are incipient. At present, only three studies using quantitative RT-PCR (qRT-PCR) have suggested a possible role for survivin overexpression in the pathogenesis or progression of CML (12-14).

P-glycoprotein (Pgp) overexpression, an ABC transporter family protein, encoded by ABCB1, is the MDR mechanism more frequently present in patients with acute leukemia (15,16). However, in samples from CML patients, only few reports have addressed the relationship between Pgp and MDR (17-19), although imatinib has been shown to be a substrate of Pgp (20). The role of Pgp molecule as a drug efflux pump

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Peripheral blood leukemic cells from 50 K562 cell line (derived from a CML-BP) was used as positive control for survivin expression. Peripheral blood cells (PBC) from healthy donors were used at 37˚C in a humidified atmosphere containing 5% CO₂. Cells were incubated maintained in RPMI-1640 medium (Gibco). Controls.

In this work, we analyzed the survivin expression and in correlation with the Sokal score of CML-CP patients. Smac/DIABLO, a pro-apoptotic protein and an inhibitor of survivin, was also evaluated to analyze whether the balance between anti- and pro-apoptotic proteins could have a different profile in each early or late CML-CP. We investigated the association between survivin and Pgp expression in a group of patients to verify whether the survivin and Pgp co-expression (23), could be detected in a particular stage of the CML. We report in this study that survivin expression is significantly elevated in late, in contrast to early, CML-CP. In addition, there was also a significant correlation between Pgp and survivin in late but not in early CML-CP.

Materials and methods

Patient samples. Peripheral blood leukemic cells from 50 patients (30 males and 20 females) in CML-CP were analyzed. CML diagnosis was based on the World Health Organization (WHO) criteria (1). Patients were categorized according to Sokal score which is calculated using peripheral blood blast number, platelet count, spleen size and age in low, intermediate or high-risk groups (2). Upon diagnosis, all patients were categorized into low, intermediate or high-risk groups. Low Sokal score (<0.8) was observed in 30 patients, intermediate Sokal score (0.8-1.1) in 14, and high Sokal score (>1.1) in 6 patients. Due to the small number of samples in the intermediate and high Sokal score, both groups were gathered and compared to the group expressing low Sokal score to compare survivin and Smac/DIABLO expressions. Twenty patients received interferon-α as front-line therapy and after resistance or intolerance, they received imatinib. These patients were selected for the present study due to the fact that their disease was not in hematological and/or cytogenetic control. The median of 58.5 months (range: 24-157) was the period of time since CML was diagnosed until the disease progressed. Another group (n=30) was selected because the CML patients were in early CML-CP and had not undergone any type of chemotherapy. After their leukocytes had been collected for this study, 7 out of 30 patients were submitted to bone marrow transplantation and 23 out of 30 patients were treated with interferon-α as first treatment option. After interferon-α failure or intolerance, 20 out of these 30 patients were treated with imatinib. This study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee.

Controls. K562 cell line (derived from a CML-BP) was maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), 10% fetal bovine serum (Gibco). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. This cell line was used as positive control for survivin expression. Peripheral blood cells (PBC) from healthy donors were used as negative control for survivin and as positive control for Smac/DIABLO expressions. The median of the densitometry values was used as a cut-off point for the increase or decrease of proteins determination in the patient samples. For the mRNA expressions, a pool of mononuclear cells was obtained from 15 healthy donors.

Determination of survivin and Smac/DIABLO expression by Western blot. Cells were lysed by incubation at 4°C for 20 min in lysis buffer consisting of 30 mM Tris-HCl (pH 7.5), 1% SDS. The protein content of lysates was determined using an adapted Lowry assay. Equal amounts of protein (50 μg) were resuspended in SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, bromophenol blue) and boiled in a bath for 10 min. After that, samples were submitted to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred into a nitrocellulose membrane (Hybond-GE Healthcare, Piscataway, NJ, USA) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm for 2 h at 4°C. The membrane was blocked with TBS (pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% non-fat dry milk for 2 h. Subsequently, the membrane was washed three times (10 min for each washing) with TBS-T and then incubated with each antibody anti-survivin (1:1000) (R&D Systems, Minneapolis, MN, USA), anti-Smac/DIABLO (1:1000) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. After washing (3x10 min), the membrane was incubated with anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:1000), and the antibody complexes were visualized by the ECL detection system through the manufacturer's instructions (GE Healthcare). The membrane was reprobed with a monoclonal antibody against β-actin (1:2000) (Sigma-Aldrich) as a loading control. The relative expressions of survivin and Smac/DIABLO proteins were determined with a chemiluminescence imaging system and quantified by image analysis (Labworks software). The relative value of protein expressions obtained after image analysis was used to compare the expression levels among samples from patients. Protein expressions were considered as negative when the bands were not observed.

Determination of survivin and Smac/DIABLO mRNA levels by real-time qRT-PCR. Total RNA from cells was isolated using the guanidinium thiocyanate method (TRIzol®, Invitrogen, Carlsbad, CA, USA) from three late and eight early CML patients. RNA was treated with DNase (Ambion, Austin, TX, USA) to eliminate contaminating DNA. cDNA was synthesized with the Ready-To-Go T-Primed First-Strand Kit (GE Healthcare). Survivin was amplified by real-time qRT-PCR using an assay on demand (Applied Biosystems, Foster City, CA, USA). Real-time monitoring of PCR amplification of cDNAs was carried out with a Taqman Universal master mix (Applied Biosystems) using 200 nM of probe and 100 nM of each both survivin and β-actin primers. Relative quantification (RQ) of target gene expression was performed by a comparative Ct method. We used arithmetic formulas as described in the Taqman user manual. β-actin (Applied Biosystems) was used as a reference gene for all samples to normalize the Ct values for the target gene. RNA from granulocytes from healthy individuals pool (n=15) was used as a calibrator and their survivin
expression levels were assigned the value of 1 as an arbitrary unit. All experiments were carried out in the StepOne™ Real-Time PCR System (Applied Biosystems).

**Determination of Pgp expression by flow cytometry.** Detection of Pgp expression was carried out as previously described (24). Briefly, one million cells were washed twice in PBS/BSA, incubated with or without 10 µl of 4e3 monoclonal antibody for 30 min, washed twice in PBS/BSA and were incubated with 10 µl rabbit anti-mouse FITC-conjugated secondary antibody (diluted 1:20) for 30 min. After washing twice, cells were suspended in PBS/BSA and analyzed by flow cytometry. The technique was performed according to manufacturer’s instructions. The results were expressed as the ratio of mean fluorescence intensities (MFI) of cells incubated with 4e3 on cells with secondary antibody. The cut-off of positivity was MFI ≥1.1 (24).

**Determination of functional MDR status by flow cytometry.** Efflux pump activity was investigated as described before by our group (24). Briefly, cells (5x10^5) were first incubated with Rhodamine-123, 200 ng/ml (Rho-123) with or without cyclosporine A (CSA) 200 ng/ml, for 45 min at 37˚C. CSA is able to block Pgp efflux pump and the intracellular fluorescence increases. Cells were washed in ice cold PBS and incubated with CSA in dye-free medium for another 45 min at 37˚C. Cells without dye or CSA were used to evaluate cell autofluorescence. The results were expressed as the ratio of MFI of cells with Rho-123 plus CSA subtracting MFI of cells with Rho-123 only. The cut-off of positivity was MFI ≥1.1 (24).

**Statistical analysis.** Spearman test was used to correlate the survivin, Smac/DIABLO and Pgp expressions in CML samples. Mann-Whitney test was used to compare the protein expressions in all groups according to patients’ clinical features. ANOVA test was used to evaluate the differences between expression and activity of Pgp. p<0.05 was considered statistically significant. Statistical analysis was performed using the Graph Prism 5 software (GraphPad Software, La Jolla, CA, USA).

### Results

**Survivin and Smac/DIABLO expressions in cells from healthy individuals (controls) and in samples from CML-CP patients by Western blot.** Survivin expression was not detected by Western blot in samples from five healthy individuals, whereas the median of Smac/DIABLO expression in the same samples was equal to 2.27 (range: 1.8-2.5).

The demographic, clinical and biological characteristics of patients in chronic CML phase.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Early chronic (n=30)</th>
<th>Late chronic (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>35.5 (21-50)</td>
<td>41.5 (18-81)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male</td>
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<td>12</td>
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<td>8</td>
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<tr>
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<td>Low</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>Intermediate</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>High</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Median survivin* (range)</td>
<td>1.23 (0-3.43)</td>
<td>1.57 (0-3.60)</td>
</tr>
<tr>
<td>Median Smac/DIABLO* (range)</td>
<td>2.1 (0-3.53)</td>
<td>2.07 (0-3.55)</td>
</tr>
<tr>
<td>Median Pgp expression† (range)</td>
<td>1.7 (0.89-6.01)</td>
<td>1.55 (0.86-4.4)</td>
</tr>
<tr>
<td>Median Pgp activity‡ (range)</td>
<td>1.2 (0.84-1.64)</td>
<td>1.119 (0.53-3.88)</td>
</tr>
</tbody>
</table>

*Relative protein expression; †protein expression (MIF); ‡Rhodamine-123 efflux MIF, ratio of mean fluorescence intensity by flow cytometry; CML, chronic myeloid leukemia.
when compared with those in the high/intermediate Sokal score (n=20; median = 2.1) (p=0.012) as shown in Fig. 3A. Analyzing only the group of patients in early CML-CP (n=30) we verified that patients grouped as low Sokal score (n=21; median = 1.12) also exhibited lower levels of survivin expression when compared with high/intermediate Sokal score group (n=9; median = 1.45). However, due to the small number of samples in these conditions, no statistical test was performed.

In this group of patients, we evaluated the response to imatinib in 20 out of 30 patients in which only 4 out of 20 did not display survivin expression, 13 out of 20 patients reached major cytogenetic response, despite their survivin levels. The survivin levels among patients who did not reach cytogenetic response were not different from those who achieved remission. The median of the period of time, since imatinib was introduced until cytogenetic remission was 27 months (range: 2.5-30).

In Fig. 3B, the expression of Smac/DIABLO was lower (median = 1.40) in samples from patients with low Sokal score (n=30) than in high/intermediate Sokal score group (n=20), in which median was 2.54 (p=0.009). Nevertheless, no significant statistic correlation was observed between survivin and Smac/DIABLO expressions (p=0.477). Smac/DIABLO expression did not show relevance in cytogenetic response.

Survivin and Smac/DIABLO mRNA relative expression by real-time qRT-PCR and association with protein expressions. Given the complex interplay among the many factors that
can influence IAPs expression, we further explored survivin and Smac/DIABLO mRNA levels by real-time qRT-PCR to observe if there was an association between protein and mRNA expression in CML samples. After Western blot assay, we analyzed 11 out of these 50 samples by real-time qRT-PCR assay. Survivin mRNA expression was significantly higher (p=0.006) (RQ range: 0.16-61.58) in CML patients (Fig. 4A), while Smac/DIABLO expression was lower (p=0.04) (RQ range 0.13-7.5) (Fig. 4B), in relation to healthy individual samples (n=15). There was a good agreement between the two assays, although we did not observe an association between mRNA and protein in two samples, since only mRNA, but not protein expression, was detected in these samples. As seen in Fig. 4A, three patients exhibited marked survivin mRNA levels. All of them also presented survivin expression levels above the median of the samples (1.23) as analyzed by Western blot.

Pgp expression and activity in early and late phases of CML-CP. Using flow cytometry, we evaluated the Pgp expression as well its activity, through MIF, in CML samples. In 26 early CML-CP patients it was possible to obtain samples to evaluate Pgp expression. The MIF median of these expressions was 1.7 (range: 0.89-6.01). Pgp expression was evaluated in 17 patients at late CML-CP where the median was 1.55 (range = 0.86-4.4). Pgp activity, analyzed by Rho-123 efflux assay, ranged from 0.84 to 1.64 of the MIF (median: 1.2) in 26 early CML-CP patients. In 17 late CML-CP, the MIF of the Rho-123 efflux ranged from 0.53 to 3.88 (median 1.19).

By using Mann-Whitney test, there was no significant difference among Pgp expressions (p=0.142) or Pgp activities (p=0.806) in early and late phases of CML samples. The dashed line represents the cut-off point (< or >1.1) expressed as the ratio of mean fluorescence intensities (MFI).

Figure 4. Relative quantification of mRNA levels by real-time qRT-PCR survivin and Smac/DIABLO in chronic myeloid leukemia (CML) patients. mRNA from CML patients and from healthy individuals were analyzed by Ct comparative method. (A) Relative quantification of mRNA survivin in the CML patients (n=11; open squares) was higher as compared with mRNA survivin in the healthy individuals (n=15; open circles) (p=0.006). (B) mRNA Smac/DIABLO expressions were lower in CML patients in relation to mRNA Smac/DIABLO expressions from healthy individuals (p=0.04).

Figure 5. P-glycoprotein (Pgp) expression and Pgp activity according to early and late chronic phase of the chronic myeloid leukemia (CML) patients. Using Mann-Whitney test, no significant difference was observed among Pgp expressions (p=0.142) or Pgp activities (p=0.806) in early and late phases of CML samples. The dashed line represents the cut-off point (< or >1.1) expressed as the ratio of mean fluorescence intensities (MFI).
expressions and Pgp activities in early (p=0.483) as well as in late (p=0.341) CML.

**Correlation between survivin expression and Pgp status.** We carried out a correlation analysis between survivin and Pgp expressions in samples from early and late CML-CP patients. We observe a significant statistically-positive correlation between positive samples from patients in late CML-CP group (p=0.018) (Fig. 6A), but not in early CML-CP group (p=0.5) (data not shown). On the other hand, despite the important positive correlation observed between survivin and Pgp expressions, a trend of negative correlation was found between survivin expressions and Pgp activities (p=0.06) (Fig. 6B).

**Discussion**

Despite the findings on survivin overexpression in several types of malignant disease (25-28), only three reports have demonstrated the expression of survivin in CML patients (12-14). These studies described survivin overexpression in CML-CP as well as in previously treated CML-BP patients.

In our study, we analyzed the levels of survivin as well as Smac/DIABLO expressions in two distinct groups of CML-CP patients: early (untreated) and late (previously treated). Therefore, it was possible to observe different status of these proteins before and after the exposure of the patients to chemotherapeutic drugs.

The levels of survivin were higher in late CML-CP compared to newly diagnosed (early CML-CP) patients (p=0.044). Therefore, our data suggest that survivin expression may emerge during the natural course of the disease as a result of the occurrence of anti-apoptotic mechanisms in the later stage of CML-CP, or may reflect previous clinical treatment effect. In any of these situations, survivin could be conferring to the CML cells a selective survival advantage. From this point of view, it is possible to infer that the high levels of survivin reported by other authors in CML-BP may be related to the latest stages of CML or exposition to chemotherapeutic drugs, once those patients had been previously treated (12-14).

It is important to emphasize that in our study, the treatment employed in patients at the late phase of the CML-CP was greatly diversified but all patients received hydroxyurea before interferon-α and some of them also received imatinib.

Previous work demonstrated that imatinib was capable of inhibiting survivin overexpression besides several tumors antigens, such as Bcl-2 and Bcl-xL (29). Moreover, data in literature suggest that survivin is regulated by Bcr-Abl/MAPK cascade in CML (30,31). Our results show that a small number of patients at the late CML-CP achieved cytogenetic remission, or had their disease progress to the advanced phase after imatinib treatment. We were interested in whether the imatinib failure could be attributed to a malignant clone proliferation that was controlled by survivin overexpression after chemotherapy.

Another aspect of our work was to verify the relationship between Sokal score and survivin expression and also between Sokal score and Smac/DIABLO expression. The highest levels of survivin in high/intermediate Sokal score group compared to the low Sokal group of patients (p=0.012), suggest that survivin can be involved in more aggressive evolution of CML. This is in line with Badran et al (14) who showed that up-regulation of survivin gene expression may be involved in typical CML evolution from CP to BP. Evolution of CML from CP to BP is often associated with additional chromosomal and molecular changes resulting in cells with enhanced proliferation, survival and differentiation arrest. Most of these abnormalities have a direct or indirect effect on p53. The loss of p53 or other protein functions in CML evolution decreases the expression of natural inhibitors of survivin (30-32). Indeed, the higher Smac/DIABLO expression in the same high/intermediate Sokal group compared to low Sokal group (p=0.009), suggests a compensatory mechanism in favor of apoptosis activation. CML patients exhibiting poor prognosis are those
who frequently exhibit additional cytogenetic alterations and mutations (33). These alterations can produce a higher apoptotic response, such as a Smac/DIABLO increase, which may be regulated by cAMP (34) and E2F1 (35). The genetic and molecular changes associated with malignant transformation lead to the activation of the apoptotic cascade. Cancer cells are able to avoid death by up-regulating IAPs, which inhibit the activity of the caspases and block apoptotic cascades. Pgp/DIABLO overexpression may be related to cancer progression as a response to the enhanced levels of IAPs (36,37). According to Yang et al, some types of carcinoma overexpress both pro-apoptotic and anti-apoptotic proteins. In this situation, high levels of pro-apoptotic protein could trigger cell death (25). However, our study is the first to report results of Smac/DIABLO expression in CML. Therefore, it is not possible to compare our data with other studies. It has been recently described that in renal carcinoma, low levels of Smac/DIABLO are associated with poor prognosis (38). On the other hand, in non-Hodgkin lymphoma, the levels of Smac/DIABLO were heterogeneous suggesting that cell death mechanisms may be involved in the pathogenesis of these diseases in different ways (39). A recent work demonstrated that a greater expression of IAPs and Smac/DIABLO revealed a mechanism towards dynamic homeostatic equilibrium between the quantities of these proteins. The role of the Smac/DIABLO in disease progression remains a controversial subject since it can be a negative or a positive indicator of prognosis according to the type of disease (40).

Here, we showed the survivin and Smac/DIABLO mRNA levels by real-time qRT-PCR in CML patients relative to healthy individuals. mRNA and protein expressions were in good agreement, except in two CML samples, where the proteins were not detected. A possible explanation for this finding is that the mechanism of survivin and Smac/DIABLO synthesis is differently regulated in patients in comparison to healthy individuals.

We clearly observed that remarkable survivin levels, as analyzed by mRNA, were indistinctly found in previously treated as well in the untreated CML patients. The fact that the high levels of survivin can be found in untreated patient suggests that this anti-apoptotic protein may emerge spontaneously and is not only triggered by drugs. Therefore, this finding could be associated to the natural history of CML.

We also demonstrated that there was an important dissociation between the levels of Pgp expression and activity in samples obtained either from early or late CML-CP patients. The dissociation between Pgp expression and activity was previously observed in other studies (22,23), including ours (24). It is noticeable that survivin expression was positively correlated with Pgp expression in late but not in early CML-CP patients. Therefore, this association might be correlated with the previous treatment recieved by patients. Besides, we observed no correlation between survivin expression and Pgp activity. This suggests that Pgp in these patients may be associated to drug-resistant mechanisms independent of its drug-efflux pump role, as previously shown by others (23). In our study, correlation between Pgp and survivin expressions were observed in late but not in early CML-CP. This phase did not exhibit discordance between Pgp expression and activity. Thus, this finding indicates that an agreement between Pgp expression and activity levels can be necessary for survivin in inhibiting apoptosis of the CML cells. The possible participation of Pgp in cell death inhibition has been described in many studies. Since this molecule can also influence caspase-3 activation, it also inhibits the caspase-dependent apoptosis (41). Another possibility is that survivin could modulate the Pgp turnover or its function as efflux pump, thus interfering with cell death and drug resistance (23).

In summary, this study showed the highest levels of survivin expression in late CML-CP patients, and also in patients with the highest Sokal score. This finding may help in understanding the influence of survivin overexpression in the evolution of CML. In addition, the significant correlation found between Pgp and survivin expressions in the late phase of CML suggests a possible role for this association in the evolution of CML. The lack of the same association between Pgp activity and survivin expression reinforce previous data which suggest that Pgp is not only a drug efflux pump molecule, it can also participate in the anti-apoptotic mechanism of drug resistance in cancer cells (41). The association between Pgp and survivin may play a biological role in late CML phase and might offer an important target for the development of new therapies.

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