Original article

The Wnt signaling pathway regulates Nalm-16 b-cell precursor acute lymphoblastic leukemic cell line survival and etoposide resistance

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ABSTRACT

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy in children. The Wnt signaling pathway has been found to be extensively involved in cancer onset and progression but its role in BCP-ALL remains controversial. We evaluate the role of the Wnt pathway in maintenance of BCP-ALL cells and resistance to chemotherapy. Gene expression profile revealed that BCP-ALL cells are potentially sensitive to modulation of Wnt pathway. Nalm-16 and Nalm-6 cell lines displayed low levels of canonical activation, as reflected by the virtually complete absence of total β-catenin in Nalm-6 and the β-catenin cell membrane distribution in Nalm-16 cell line. Canonical activation with Wnt3a induced nuclear β-catenin translocation and led to BCP-ALL cell death. Lithium chloride (LiCl) also induced a cytotoxic effect on leukemic cells. In contrast, both Wnt5a and Dkk-1 increased Nalm-16 cell survival. Also, Wnt3a enhanced the in vitro sensitivity of Nalm-16 to etoposide (VP-16) while treatment with canonical antagonists protected leukemic cells from chemotherapy-induced cell death. Overall, our results suggest that canonical activation of the Wnt pathway may exerts a tumor suppressive effect, thus its inhibition may support BCP-ALL cell survival.

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1. Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a malignancy characterized by progressive accumulation of immature clonal B cell precursors in the bone marrow (BM). Nowadays, roughly 80% of all newly diagnosed pediatric patients become long-term survivors after adequate multiagent chemotherapy protocols administered according to the individual patient risk category [1]. However, a subset of children relapses despite this strategy, suggesting that other factors than those currently used to define patients risk could have an impact on the behavior of the disease and response to therapy.

At present, it is well known that BM microenvironment plays an essential regulatory role on the proliferation and survival of BCP-ALL neoplastic cells; at the same time it represents the primary site for disease relapse [2,3]. Understanding the specific molecular signaling pathways activated and/or intensified by stromal cells is crucial to be able to disrupt BCP-ALL cell maintenance and abrogate resistance to chemotherapy.

The Wnt signaling is a highly conserved pathway that mediates cell-to-cell communications during embryogenesis. In addition, it is also involved in other relevant cell functions such as cell proliferation, differentiation, migration, cell fate decisions, apoptosis, and stem cell self-renewal [4,5]. Wnt molecules are secreted lipid-modified cysteine-rich glycoproteins that bind to and signal through Frizzled (Fz) receptors [6]. Different types of Wnt/Fz complexes may signal through the so-called canonical (β-catenin mediated signaling) or non-canonical Wnt pathways (β-catenin-independent signaling). In the canonical pathway, this interaction leads to activation of Dishevelled (Dsh) proteins that block the GSK3-β kinase activity, required for β-catenin accumulation in the cytoplasm. As a result, cytoplasmic β-catenin is translocated into the nucleus where it converts the TCF/LEF complex from a transcriptional repression form to a transcriptional activating
configuration, modulating the expression of several genes involved in different biological phenomena such as those described above [7].

In non-canonical signaling, Wnt agonists such as Wnt5a may stimulate intracellular Ca²⁺ release, activation of protein kinase C (PKC) and Ca²⁺/calmodulin-dependent kinase II (CaMKII), or they may inhibit the canonical pathway through degradation of β-catenin [8,9].

Deregulation of the Wnt signaling pathway has been extensively found to be involved in cancer onset and progression [10–12]. In recent years, several reports have shown that members of the Wnt pathway could be involved in regulating cell proliferation and survival in different hematological malignances [13–15]. However, more recent data indicates that antagonists of the Wnt pathway may increase tumor cell growth [16,17] and sensitivity to chemotherapy [18,19] depending on the model studied. In fact, at present no consensus exists on the precise role of the Wnt pathway in BCP-ALL [20–22].

In this study, we evaluate the role of the Wnt pathway in maintenance of Nalm-16 BCP-ALL neoplastic cells, and its effects on determining the sensitivity to chemotherapy. Gene expression profiling studies revealed that BCP-ALL cells do express several members of the Wnt pathway and BCP-ALL cells are potentially sensitive to modulators effects of the members of the Wnt pathway. Our results show that Nalm-6 cells are virtually devoid of β-catenin, while Nalm-16 cells express higher levels of β-catenin but the protein is mainly located at the cell membrane, suggesting that canonical signaling is not required for BCP-ALL maintenance. Activation of the canonical Wnt pathway through the Wnt3a protein induced BCP-ALL cell death and an increased sensitivity to etoposide (VP-16). This cytotoxic effect was also induced by lithium chloride (LiCl), suggesting that GSK-3β is involved in it. In turn, incubation with Wnt5a or Dkk-1, increased survival rates of Nalm-16 cells and treatment with canonical antagonists protected leukemic cells from chemotherapy-induced cell death. Based on these results, it could be speculated that activation of canonical Wnt signaling could be a novel therapeutic target to induce cytoreduction and enhance the sensitivity to chemotherapy of BCP-ALL cells.

2. Materials and methods

2.1. Leukemic cell lines and patient samples

BCP-ALL cell lines were kindly provided by Dr. Maria Isabel Doria Rossii, Federal University of Rio de Janeiro, Brazil. Nalm-16 and Nalm-6 had been originally established from peripheral blood of BCP-ALL relapsed patients. According to the EGIL criteria, Nalm-16 and Nalm-6 were characterized as BII/common BCP-ALL (CALLA⁺) and BIII/pre-B ALL, respectively [23]. Cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FCS; Cultilab, Campinas, SP, Brazil), 100 U/mL G sodium penicillin, 100 μg/mL streptomycin, 2 mM l-glutamine, 1 mg/mL pepstatin, 1 mM PMSF, 1 mM NaF, 1 mM Na₂VO₃. The protein concentration in the cell lysate was determined by the method of Lowry et al. [24]. Samples were mixed with sample buffer – 0.02 mM dithiothreitol (DTT); 1.38 mM sodium dodecyl sulfate (SDS); Tris·HCl 125 mM, pH 6.8 and 20% glycerol – and resolved using an SDS-polyacrylamide 12% gel electrophoresis (SDS-PAGE); then they were electroblotted and transferred to a nitrocellulose membrane (HybondTM-P, Amersham Biosciences, São Paulo, Brazil). Membranes were treated with a blocking solution containing 5% non-fat dry milk in 0.001% Tris-buffered saline-Tween 20 (TBS-T) for 1 h and then incubated overnight with the primary polyclonal antibody for β-catenin (Sigma, 1:2000 dilution). Monoclonal anti α-actin (clone C4, Chemicon, Hofheim, Germany, 1:2000 dilution) was also employed to compare loading of the samples. Secondary anti-mouse HRP-conjugated antibodies (1:2000, Promega, Madison, WI) were then added and cells incubated for 1 h at room temperature. The blot reaction was visualized using the chemiluminescence detection kit luminol (Super Signal West Pico, Pierce, Rockford, IL, USA). For the analysis of the intensity of the stained band, Image J software (NIH) was used, results being expressed as arbitrary units (AU).

2.3. Generation of Wnt3a-conditioned medium

Mouse Wnt3a transfected cells (L-Wnt3a) and control non-transfected L-cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s medium (DMEM; Sigma) supplemented with 10% FCS, 4 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. L-Wnt3a culture medium was supplemented with 0.4 mg/ml G-418 (Gibco-BRL) to maintain transgene expression during cell culture expansion. Conditioned medium from L-Wnt3a or control L-cells were collected according to the manufacturer’s instructions. Briefly, cells were passed at 1:10 dilution in 10 mL medium without G-418 and left to grow for four days. Medium was collected from each cell line and replaced with 10 mL of fresh medium for another three days. The second batch of medium was then collected and the cells discarded. Both batches were mixed, sterile-filtered (0.2 μm) and stored at −20 °C until required. Activity of the conditioned medium was tested by the TCF/LEF luciferase reporter assay as described below.
2.4. Cell Transfection and Luciferase Activity Assay

HEK 293T cells cultured in 96-well plates to 80% confluence were transfected with pGal (for β-galactosidase expression), Fop-Flash (negative control luciferase reporter mutated in the TCF/LEF binding site) and Top-Flash (luciferase reporter containing TCF/LEF binding site) plasmids using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad CA). A DNA/Lipofectamine mixture was added to cells and maintained for 6 h at 37°C. DMEM with 10% FCS was added to cells that were let to recover overnight. Then, cells were incubated for 18 h with 10% diluted conditioned medium from L-Wnt3a or L-control cells. After this incubation period, cells were lysed with lysis buffer (Promega, Madison, WI). Luciferase activity was detected by adding the enzyme substrate according to the manufacturer’s protocol and samples were read in a Tecan GENios Luminometer (Tecan Group Ltd., Mannedorf, Switzerland). β-galactosidase activity was measured by adding ONPG reagent to each well, and read using a spectrophotometer. In order to normalize the data, the luciferase activity index was calculated by dividing the luciferase values by the β-galactosidase ones.

2.5. Immunofluorescence microscopy and digital image acquisition

For immunofluorescence microscopy, untreated BCP-ALL cells and leukemic cells treated with conditioned medium from L-Wnt3a or control L-cells were incubated for 6 hours at 37°C in a humidified atmosphere with 5% CO2. Then, cells were placed on glass coverslips coated with poly-L-lysine (Sigma) and incubated at 37°C for 50 minutes. Subsequently, cells were rinsed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (RT). Cells were then permeabilized and washed with 0.5% Triton-X 100 (Sigma) in PBS and were incubated with a rabbit anti-β-catenin polyclonal antibody (Sigma), at a 1:100 vol/vol dilution, for 1 h at 37°C. After this incubation period, cells were washed and incubated with an Alexa Fluor 546-conjugated goat anti-rabbit IgG antibody (Molecular Probes) for 1 h at 37°C and washed in PBS. Then the cell nuclei was stained by adding a solution containing DAPI (0.1 µg/mL in 0.9% NaCl) for 5 min (RT). Finally, cells were washed and mounted in glycerol containing 5% propyl gallate, 0.25% DABCO (1,4-diazabicyclo(2,2,2)octane) and 0.0025% para-phenylenediamine (w/w; all from Sigma). Cells were examined with an Axiosvert epifluorescence inverted optical microscope (Carl Zeiss, Oberkochen, Germany) and images acquired with a C2400i integrated CCD camera (Hamamatsu Photonics, Shizuoka, Japan) using an Argus 20 image processor (Hamamatsu Photonics). Digital images were transferred to a Dell Optiplex GX270 computer (Dell Corporate, Round Rock, TX) and plates were mounted using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). Control experiments with no primary antibodies showed only a faint background staining (data not shown).

2.6. Modulation of the Wnt pathway

Nalm-16 cells were plated at 3 x 10^5 cells/mL in 24-well plates in the presence or absence of 10^{-6} M etoposide (VP-16, Onconespo; Quiral Quimica SA, Juiz de Fora, MG, Brazil) and 10% L-control or L-Wnt3a conditioned medium, as indicated. To test the effect of purified proteins of the Wnt pathway on cell proliferation and survival, either the canonical activator Wnt3a protein or the Wnt5a, Dkk-1, SFRP-1 canonical inhibitors, (R&D Systems Minneapolis, MN) were added (100 ng/mL) to the cell culture medium. To investigate the potential role of GSK3-β in survival of BCP-ALL Nalm-16 cells were also incubated with LiCl (Sigma) at 3 mM.

2.7. Analysis of cell cycle and cell death

After 24, 48 and 72 h of culture, cells were collected and their viability was measured by Trypan dye exclusion assay. Double stainings with Annexin V-FITC (1:1 dilution, Molecular Probes, Eugene, OR) for apoptotic cells and Propidium Iodide (PI; Sigma) for dead cells, were performed according to the manufacturer’s instructions and measured in a FACSCalibur flow cytometer (BD, San José, CA) for a total of 30,000 events. DNA cell contents were monitored by flow cytometry after PI staining according to Vindelov et al. [25]. At 24, 48 and 72 h. Cells with subdiploid DNA content peaks (sub-G0) were excluded from cell cycle analysis. FACS data were analyzed using the Infinicyt software (Cytognos, Salamanca, Spain).

2.8. RNA Isolation and Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from leukemic cells and normal BM stromal cells using TRIzol® Reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was first synthesized from 2 µg total RNA using 1 µL oligo dT primers (500 µg/mL) and 1 µL Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT; 200 U/µL) (both from Invitrogen). The resulting cDNA was used with gene-specific oligonucleotide primers for PCR amplification. The primer sequences with annealing temperature and product size are listed in Table 1. Forward and reverse oligonucleotide primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi). RT-PCR was performed in a TGradient thermal gradient cycler (Biometra, Göttingen, Germany); PCR products were submitted to electrophoresis in 2% agarose gels and stained with ethidium bromide. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control. Negative control was the PCR reaction mix lacking cDNA. As a positive control for Wnt3a expression, total RNA from whole B6CBA mouse embryos on days 8–10 after fertilization, was used; for LEF-1, Fzd-3, Dkk-1, SFRP-1 and SFRP-2, normal BM stroma was used as positive control, and; for Wnt5a, Jurkat cells were used as positive control.

### Table 1

<table>
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<th>Primers</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
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<th>Product Size (pb)</th>
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<td>358</td>
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<td>CCTGCTCCACACCTTCTG</td>
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<td>571</td>
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</tbody>
</table>
2.9. Statistical methods

Statistical significance was determined using the unpaired student T and the non-parametric Mann-Whitney U tests (Prism 2.01 software; GraphPad, San Diego, CA) \((p < 0.05\) was considered to be associated with statistically significant).

3. Results

3.1. Nalm-6 is virtually devoid of β-catenin while Nalm-16 expresses higher levels of cell membrane but not nuclear β-catenin; Wnt3a treatment induces nuclear β-catenin translocation

Since nuclear β-catenin is the hallmark of the activated canonical Wnt signaling pathway, our first goal was to evaluate the expression and distribution of β-catenin on BCP-ALL cell lines. Our results showed that Nalm-6 was virtually devoid of β-catenin expression as found by Western Blot assay (Fig. 1A). In contrast, Nalm-16 cells expressed β-catenin at levels similar to the positive control (Jurkat cells) as depicted in Fig. 1B. However, in Nalm-16 cell line, the distribution of β-catenin as assessed by immunofluorescence was predominantly located on the cell membrane (Fig. 1C, control and control-CM), being confined to cell adhesion complexes at cell membranes. However, following Wnt3a stimulation, β-catenin was clearly translocated into the nucleus of Nalm-16 BCP-ALL cells (Fig. 1C, Wnt3a-CM).

3.2. Activation of the canonical Wnt pathway induces death of Nalm-16 cells while Wnt5a and Dkk-1 increase their survival rates

In order to investigate the role of canonical Wnt activation on BCP-ALL cell survival, we treated Nalm-16 cells with either a classical canonical Wnt activator (Wnt3a) or canonical Wnt inhibitors (Wnt5a, Dkk-1, SFRP-1). Wnt3a clearly induced death of Nalm-16 cells, decreasing the viability by around 50% at 72 h (Fig. 2 A). Conversely, incubation with either Wnt5a or Dkk-1, increased survival rates of Nalm-16 cells over control values. Although SFRP-1 at the concentration tested showed a slight tendency to increase cell survival, no statistically significant differences were observed (Fig. 2 A and B). In addition, we also tested whether LiCl, which inhibits GSK-3β and mimics Wnt signaling by stabilizing β-catenin, could also play a similar role to the activation of canonical Wnt pathway on cell survival. LiCl clearly decreased cell survival, suggesting that GSK-3β activity may regulates survival of BCP-ALL cells, although we cannot rule out others GSK-3β-unrelated effects (Fig. 3). These results suggest that inhibition of canonical Wnt signaling may play an important role on BCP-ALL maintenance.

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Fig. 1. Overall expression of β-catenin in BCP-ALL cell lines. (A) Western blot analysis for total β-catenin showed that Nalm-6 (N6) cells were virtually devoid of β-catenin protein in contrast to Nalm-16 (N16). For these experiments, Jurkat cells were used as positive control. Total β-catenin levels (mean ± one standard deviation) from two independent experiments (expressed as arbitrary units [AU]) are shown in B. (C) Immunofluorescence microscopical profiles of NALM-16 cells for total β-catenin, DAPI and both β-catenin and DAPI. (Bar = 10 μm). As shown by arrows, the majority of β-catenin was located at cell membrane in untreated cells and control-CM treated cells. However, under Wnt3a stimulation, β-catenin was translocated into the nucleus of Nalm-16 BCP-ALL cells as indicated by arrows in Wnt3a-CM ("p < 0.0001 - N6 versus N16").
3.3. Activation of the canonical Wnt pathway enhances sensitivity of Nalm-16 cells to DNA topoisomerase-II inhibitor while canonical antagonists induce drug resistance

To determine the effects of Wnt activation on BCP-ALL cell survival after chemotherapy, Nalm-16 cells were treated with purified Wnt3a-canonical agonist or canonical antagonists as indicated. Viability was monitored by an Annexin V-FITC / Propidium Iodide (PI) double staining followed by FACS analysis. Canonical activation reduced BCP-ALL cell survival while Wnt5a and Dkk-1 increased survival rates. SFRP-1 did not show a significant impact on cell survival. Viability fold change was obtained from absolute Annexin-V–/PI– cell count (panel A, arbitrary units) from three independent experiments carried out in triplicate (mean values and standard errors are shown). FACS profiles show results from 72 hours and is representative of three independent experiments (panel B). (*) p < 0.01 [Mann-Whitney U test].

Fig. 2. Wnt3a induces death of Nalm-16 cells while Wnt5a and Dkk-1 increase their survival. Nalm-16 cells were treated with purified Wnt3a-canonical agonist or canonical antagonists as indicated. Viability was monitored by an Annexin V-FITC / Propidium Iodide (PI) double staining followed by FACS analysis. Canonical activation reduced BCP-ALL cell survival while Wnt5a and Dkk-1 increased survival rates. SFRP-1 did not show a significant impact on cell survival. Viability fold change was obtained from absolute Annexin-V–/PI– cell count (panel A, arbitrary units) from three independent experiments carried out in triplicate (mean values and standard errors are shown). FACS profiles show results from 72 hours and is representative of three independent experiments (panel B). (*) p < 0.01 [Mann-Whitney U test].
3.4. Modulation of the Wnt pathway does not modify the cell proliferation status

Since maintenance of the cellular pool is a balance between cell proliferation and survival, we also investigated whether modulation of the Wnt pathway could also play a role in modulating cell cycle distribution of BCP-ALL cells. Accordingly, Nalm-16 cells were treated with purified proteins of the Wnt pathway (activators and inhibitors) and DNA cell contents were determined by PI staining at 24, 48 and 72 h. Surprisingly, neither the activation nor the inhibition of Wnt pathway had an impact on the cell cycle distribution of viable Nalm-16 cells in culture (Fig. 6).

3.5. BCP-ALL gene expression profile corroborates the functional findings related to the Wnt pathway.

Molecular analysis of mRNA expression by BCP-ALL cell lines and CD19+ BCP-ALL patients’ cells using RT-PCR is shown in Fig. 7. As shown there, both BCP-ALL cell lines and patient samples expressed LEF-1 and Fzd-3 transcripts, indicating that leukemic cells are potentially sensitive to Wnt signals. In line with our findings indicating that Wnt3a could act as a tumor suppressor gene for BCP-ALL cells, Wnt3a mRNA expression was completely absent in both cell lines as well as in all patient samples tested. In addition, neither BCP-ALL cell lines nor patient samples expressed Wnt5a transcripts while Dkk-1 mRNA was expressed in patient samples but not in the cell lines tested, suggesting that primary leukemia cells are still dependent on external survival signals. SFRP-1 and SFRP-2 mRNA were absent in all cell lines tested while SFRP-2 transcripts were expressed at low levels by two patients.
and absent in another two samples, also suggesting that, at least in some cases, primary leukemia cells are dependent on survival signals. Also, Dkk-1, SFRP-1 and SFRP-2 mRNA were expressed by normal BM stromal cells.

4. Discussion

The idea of designing targeted-therapy was first conceived in the early 1900s by Paul Ehrlich, who named “magic bullets” the compounds that could specifically attack diseased cells. In order to develop such specific treatment against tumor cells, it is mandatory to understand the deregulated signaling pathways underlying neoplastic conditions. Here, we describe the involvement of the Wnt signaling pathway in the maintenance of BCP-ALL cells before and after chemotherapy.

An increasingly body of evidence has emerged in the literature concerning the role of Wnt/β-catenin pathway in B cell malignancies. Inhibition of GSK-3β activates β-catenin-mediated
transcription and enhances survival of chronic lymphocytic leukemia (CLL) cells [14]. Malignant multiple myeloma cells overexpress β-catenin and stimulation with either Wnt3a or LiCl induces further accumulation of nuclear β-catenin and increases cell proliferation [15]. Conversely, Yaccoby et al. [16] have shown that Dkk-1 enhanced tumor-induced bone resorption and multiple myeloma growth in vivo, providing conflicting results about the precise role of the Wnt/β-catenin pathway.

After canonical Wnt activation, β-catenin is translocated into the nucleus where together with LEF-1 induces transcriptional activation [26]. Although the BCP-ALL cell lines studied here constitutively expressed LEF-1 mRNA, low levels of total β-catenin and predominant membrane distribution were found in Nalm-6 and Nalm-16 cells, respectively, strongly suggesting downregulation of the canonical Wnt pathway in these cells. Serinsöz et al. [22] have shown that β-catenin is inversely expressed in acute myeloid leukemia (AML) and ALL, no β-catenin expression being identified in ALL cells obtained from formalin-fixed, paraffin-embedded BM trephines. However, expression of both the LEF-1 and Fzd-3 receptor mRNAs shown here suggest that leukemic cells are potentially sensitive to Wnt external stimulation. Indeed, we demonstrate here that following Wnt3a stimulation, β-catenin was clearly translocated into the nucleus of BCP-ALL cells. Thus, although the canonical pathway is not constitutively activated, the Wnt/β-catenin pathway could be used to modulate leukemic cells behavior.

Wnt3a has been by far the most widely investigated canonical agonist of the Wnt pathway, even though its role in BCP-ALL remains controversial. Accordingly, while some authors have described Wnt3a as an important mediator of cell survival and proliferation, others have found that Wnt3a inhibits proliferation of BCP-ALL cells but does not modify their survival rates [20,21]. Indeed, these two works were in part done with the same BCP-ALL cell lines (Nalm-6 and Reh) and yielded opposite results concerning survival and proliferation [20,21]. In the present study, Wnt3a strongly decreased survival of leukemic cells without modifying the cell cycle distribution of viable cells. These discrepancies may reflect different methodological approaches such as cell culture conditions or stress the idiosyncrasy of different BCP-ALL cell lines tested which may reflect heterogeneity of leukemic cell population in a tumor bulk. Besides this, we cannot rule out that different cell lines origins may be generating these conflicting results.

Although this, our data is in accordance to a work by Zhao et al. [27] that have shown that lack of β-catenin in BCR-ABL-transduced hematopoietic cells allows progression of B-ALL in a murine model. In line with this, we found that treatment with LiCl, an agent that
mimics canonical Wnt signaling by blocking GSK-3β, also increased leukemic cell death. Although LiCl is generally thought to have antiapoptotic effects, particularly in neural cells, it has also been shown to induce apoptotic death in several cell lines, such as promyelocytic leukemia HL-60 cells, erythroleukemia K562 cells, and monkey kidney COS7 cells. The factors responsible for such apoptotic effects are largely unknown, but studies of Van Gijn et al. [28] have shown that accumulation of β-catenin can induce apoptosis, further supporting a role for β-catenin in LiCl-mediated cell death. Moreover, β-catenin overexpression by itself can induce apoptosis independently of LEF-1 [29]. Therefore, canonical activation of the Wnt pathway may have a negative impact on leukemic cell survival. We also demonstrated here that incubation with the canonical Wnt inhibitors Wnt5a and Dkk-1 was associated with an increased cell survival, reinforcing the benefits of canonical inhibition for maintenance of BCP-ALL cells.

The increased sensitivity to VP-16 induced by Wnt3a together with the absence of Wnt3a mRNA in all patient samples and cell lines studied emphasizes its negative role on leukemic cell survival. BM stromal cells, known to play a pivotal role on leukemic cell maintenance by providing survival signals, are also completely devoid of Wnt3a [30]. Altogether, our results clearly suggest that, at least in our model, Wnt3a behaves as a bona fide tumor suppressor gene, inducing cell death in malignant cells, both under normal conditions as well as after genotoxic challenge with etoposide.

The mechanism underlying Wnt3a-induced sensitivity to etoposide remains largely unknown. We showed here that none of the Wnt modulators was able to modify cell proliferation, and this mechanism is probably not involved in increased sensitivity. In turn, sensitivity to etoposide could be at least partially explained by the increased expression of topoisomerase IIα following Wnt3a treatment, as demonstrated by Khan et al. [21]. Since etoposide is a specific DNA topoisomerase-II inhibitor, the increased amount of molecular drug target induced by Wnt3a may increase the sensitivity to this drug.

In contrast to recent findings suggesting that Wnt5a acts as a tumor suppressor gene and its inactivation may confer a poor prognosis [31], we found that Wnt5a protects leukemic cells from chemotherapy-induced cell death. Such discrepancy may indicate that in multitarget chemotherapy schemes, the DNA topoisomerase-II inhibitor resistance induced by Wnt5a may be circumvented by other chemotherapy drugs.

In line with our findings, Wnt5a has been shown to inhibit the canonical Wnt pathway by promoting β-catenin degradation through a GSK3-β independent mechanism [9]. Here we showed that two other canonical Wnt antagonists (e.g. Dkk-1 and SFRP-1), also induce BCP-ALL drug resistance. These results support previous observation [19] showing that the same canonical Wnt antagonists are capable of inducing drug resistance in AML as efficiently as cell adhesion; in this work De Tonti et al. [19] also demonstrated that leukemic cell adhesion to osteoblasts induced secretion of SFRP-1, reinforcing resistance to chemotherapy by a double resistance mechanism (adhesion and anti-Wnt production).

Production of Wnt antagonists by leukemic cells or BM stroma could contribute to the protective effects of BM niches against chemotherapy in BCP-ALL. Here, we show the existence of constitutive Dkk-1 and SFRP-2 mRNA expression by CD19+ cells from BCP-ALL patients, but not by established BCP-ALL cell lines, which may reflect dependency of primary leukemia on external survival signals. The absence of SFRP-2 mRNA in two patient samples may be due to undetectable expression levels or indicate variability among patients. Importantly, Dkk-1, SFRP-1 and SFRP-2 mRNAs were expressed by normal stromal cells, which could contribute to BCP-ALL cell survival through crosstalk between leukemic cells and the supportive BM microenvironment. Although we could not find Wnt5a mRNA expression neither in BCP-ALL cell lines nor in patient samples, we found Wnt5a expression in BM stromal cells (data not shown) in accordance to a work by Etheridge et al. [30], which could provide Wnt5a signal to leukemic cells.

In summary, our results suggest that canonical Wnt3a behaves as tumor suppressor gene and inhibition of this pathway may support BCP-ALL cell survival. In addition, canonical activation of the Wnt pathway may enhance sensitivity to DNA topoisomerase II inhibitor and its inhibition promotes drug resistance in Nalm-16 cell line. These results suggest that canonical agonists may be developed into novel effective agents capable of inducing leukemic cell death, and enhancing response to chemotherapy in BCP-ALL, although further studies with patient samples are necessary.

5. Contributors

L.S. Thiago and R. Borovec contributed to the concept and design, interpreted and analyzed data, provided drafting of the article, provided critical revisions and important intellectual content, obtained a funding source, gave final approval, supplied statistical expertise, collected and assembled data. E.S. Costa collected and analyzed data, provided drafting of the article and critical revisions. D.V. Lopes, F.A. Mendes, D.M. Portilho, J.C. Abreu, C.S. Meremstein collected and analyzed data. I.B. Otazu obtained a funding source, gave final approval, supplied critical revisions and important intellectual content.

6. Conflicts of interest

The authors declare no competing financial interests.

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References


