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Relationships between multidrug resistance (MDR) and stem cell markers in human chronic myeloid leukemia cell lines

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ABSTRACT

The K562 cell line (chronic myeloid leukemia), sensitive to chemotherapy (non-MDR), and the Lucena cell line, resistant to chemotherapy (MDR) were investigated. The results suggest that both cell lines possess CD34+CD38− profiles of hematopoietic stem cell markers. The promoter regions of ABCB1, ABCG2 and ABCC1 genes contain binding sites for the Oct-4 transcripton factor, which is also considered a marker of tumor stem cells. Lucena cells showed an over-expression of the ABCB1 gene and a high expression of the Oct-4, ABCG2 and ABCC1 genes as compared to K562 cells.

1. Introduction

The available information regarding the multidrug resistance (MDR) phenotype of tumoral cells relates the function and regulation of membrane transport proteins of the ATP binding cassette (ABC) superfamily. The activation of these proteins may be associated with changes in the activity of protection systems and signal transduction pathways involved in regulation, proliferation, differentiation and apoptosis in different cellular types [1]. MDR is a cellular protection system characterized by the capacity to export many compounds, including drugs with varying chemical structures and different mechanisms of intracellular activity [1,2]. The ABC superfamily, of which MDR is a part, comprises nearly 300 transport proteins [3–6]. Humans express seven ABC subfamilies [5,6]. The ABC transporters investigated in the present study include: ABCB1 (MDR1 – Pgp), ABCC1 (MRP1), and ABCG2 (BCRP ou MXR).

A fascinating fact regarding ABC transporters is the documented hyper-expression of some proteins of this family by stem cells [7]. Some data show that tumor initiation and progression are associated with the proliferation of tumor stem cells whose phenotype resembles normal stem cells of the affected tissues [8,9].

The concept of "tumor stem cells" was established by *in vitro* experiments with acute myeloid leukemia (AML). In these studies, a small proportion of undiferentiated cells were able to reconstitute the tumor when injected into mouse. Along with potent tumor initiation, these cells also showed both self-renewal and differentiation [10,11].

Normal or leukemic stem cells comprise a side population (SP) of haematopoietic cells, the first property of this population was characterized by their ability to export Hoescht 33342 and Rhodamine 123 fluorescent dyes from cells, which are transported by proteins of the ABC superfamily [7]. There are data showing that Rh 123 is removed from cells by Pgp and Hoechst 33342 is exported by ABCG2 (BCRP) [12]. Also, investigations with ABCG2 knockout mice have shown that bone marrow cells acquire an increased sensitivity to toxic agents [13].

Leukemic stem cells with the phenotype CD34+CD38− possess resistance to chemotherapy, contrary to CD38+ cells [14]. Hematopoietic stem cells display the CD34+CD38− marker phenotype [15]. Additionally, cells which hyper-express Pgp show the phenotype CD34+ in human hematopoietic stem cells [16]. Also, the ABC carrier, ABCG2, is expressed in hematopoietic stem cells [17] and is considered as a stem cell marker. Recent data have shown that hematopoietic stem cells express a significant number of different ABC transporters [18]. The role of these transporters remains unclear, however, it is most often assumed that these transport pro-

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teins play an important role in protecting both normal and tumor stem cells against toxic substances [7,12].

It as also been suggested that ABC transporters may be involved in the regulation of key processes of stem cells, perhaps may be involved in their capacity for self-renewal and differentiation. This effect on differentiation is associated with the efflux of cell differentiated factors [19].

In turn, the regulation of ABC proteins is closely linked to signal transduction pathways, elements which are remodeled after malignant transformation. Studies show a variety of signal transduction pathways, different transcription factors, and many molecular events are involved in the regulation of ABC transporters [1]. Oct-4 is a transcription factor first described in 1990 [20–22]. Expression of this factor plays a crucial role in maintaining the self-renewing, cancer stem-cell-like, and chemoradioresistant properties in lung cancer-derived CD133+ cells [23].

Oct-4, a member of the family of POU-domain transcription factors, is normally found in the totipotent and pluripotent stem cells of embryos [24]. This protein activates transcription via octamer motifs, and Oct-4 binding sites have been found in various genes [25,26].

Also, Tai et al. [27] showed the activation of the Oct-4 gene and the presence of its protein in several types of adult pluripotent stem cells including: kidney, breast, epithelial, pancreatic, mesenchymal, gastric and liver; as well as in tumor cell lines derived from pancreas and liver of rats and humans. This activation and expression was not observed in the normally differentiated cells derived from these stem cells. The authors suggested that adult cells that express the Oct-4 gene are potential pluripotent stem cells, and may be the targets of carcinogenesis. Thus, instead of speculating that embryonic genes are re-expressed during carcinogenesis it is possible that the carcinogenic process prevents the down-regulation of genes such as Oct-4, which would normally begin the process of cell differentiation.

Therefore, it is clear that research on the relationship between stem cells and ABC transporters is important. Besides, many issues emerge during the consideration of this type of investigation, including the regulation of various groups of ABC transporters and the significance of the expression of these transporters in the evolution of a population of malignant cells. These proteins are highly conserved in all living cells, indicating their role in vital cell activities.

The purpose of this study was to investigate the CD34 and CD38 cell surface markers profile of hematopoietic stem cell in two cell lines, human chronic myeloid leukemia K562, chemotherapy sensitive (non-MDR), and another, selected from its parental K562, called K562-Lucena (Lucena) chemoterapy resistant (MDR). In addition to investigate representatives of the ABC transporter superfamily (ABCB1, ABCG2 and ABCC1), their gene expression and its relationship with the Oct-4 transcripition factor gene expression. Suggesting that Oct-4 may be a putative tumoral stem cell marker, and moreover, may be involved with the multidrug resistance phenotype in Lucena cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines were obtained from the Tumoral Immunology Laboratory at the Medical Biochemistry Institute – Federal University of Rio de Janeiro (Brazil). The cells were grown at 37 °C in disposable plastic flasks containing RPMI1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g/L) (Vetec), L-glutamine (0.3 g/L) (Vetec), Hepes (25 mM) (Acros), β-mercaptoethanol (5×10^{-5} M) (Sigma), fetal bovine serum (FBS-10%; Gibco), 1% antibiotic (penicillin –100 U/mL) and streptomycin (100 mg/mL) (Gibco) and antimicotic (amphotericin-B 0.25 mg/mL – Sigma). Lucena cells were grown under the same conditions as K562 cells, with the addition of 60 nM of vincristine (VCR – Sigma) in the culture medium. Cell viability was determined by trypan blue exclusion.

2.2. Flow cytometry analysis of CD34 and CD38 cell surface marker

A single amount of 10^6 cells in suspension were used for the cell surface marker identification from K562, non-MDR, and Lucena, MDR cells lines. The cells were labeled with Pharmingen/BD Bioscience R-PE conjugated monoclonal anti-human CD34 antibody and Pharmingen/BD Bioscience R-PE conjugated monoclonal anti-human CD38 antibody as indicated by manufacturer. Cells were analyzed with a FACS Calibur cytometer equipped with a 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected. The negative control was cells not labeled with antibody.

2.3. Oct-4 binding sites on ABC transporters gene promoters

Considering the Oct-4 core sequence in the analyzed genes, we identified sequences containing ABCB1, ABCG2 and ABCC1 proximal promoters from GenBank. For the three pumps we used the Oct-4 core sequence (ATGCAAAT) identified by Tantin et al. [28]. Within these sequences, the transcription starting point for each gene was identified and the upstream 10,000 bp were selected. The potential transcription factor-binding sites for Oct-4 were localized using the MatInspector program [29].

2.4. Total RNA extraction and cDNA synthesis

Total RNAs were extracted from five samples of each line cell with an amount of 3×10^6 per sample cells according to the protocol of the manufacturer of TRIzol Reagent (Invitrogen, Brazil). The RNAs were quantified by fluorometry in a Qubit™ fluorometer, using Quanti-iT™ RNA Assay Kit (Invitrogen, Brazil). The RNA integrity was confirmed by electrophoresis in 1.5% agarose gel with ethidium bromide 0.5 μg/mL. The RNAs were reverse transcribed to cDNA (complementary DNA) using 2 μg of total RNA, and following the protocol of the manufacturer of the reverse transcriptase enzyme (RT Superscript III, Invitrogen, Brazil).

2.5. Gene expression analyses

The gene expression analyses were performed using Real-time PCR System 7300 equipment (Applied Biosystems, Brazil). Each PCR reaction had 12.5 μL final volume, containing 6.25 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), 0.3125 μL of each primer, 4.625 μL of Ultra Pure Water (Invitrogen, Brazil), and 1.0 μL diluted cDNA of the line cells K562 or Lucena (1:10). The primers used in this stage were designed with basis in the consensus coding sequences database (CCDS) available at GenBank (http://www.ncbi.nlm.nih.gov): Oct-4 (CCDS34391.1), ABCB1 (CCDS5608.1), ABCC1 (CCDS42122.1), ABCG2 (CCDS3628.1). The primer sequences are the following: forward Oct-4 (5′-GGGTTTTTGGGATTAAGTTCTTCA-3′), reverse Oct-4 (5′-GCCCCCACCCTTTGTGTT-3′), forward ABCB1 (MDR1) (5′-TCCTCAGTCAAGTTCAGAGTCTTCA-3′), reverse ABCB1 (MDR1) (5′-TCTCCACTTGATGATGTCTCTCACT-3′), forward ABCG2 (BCRP) (5′-CCAGGTGTGCGTCAGAATCA-3′), reverse ABCG2 (BCRP) (5′-GGAGCTACTTAGGCCAGATTTTTG-3′), forward ABCC1 (MRP1) (5′-GGATCTCTCCAGCCGAAGTCT-3′) and reverse ABCC1 (MRP1) (5′-GTGATGGGAGCCAGAAGCA-3′). The β-actin gene (CCDS5341.1) was used as endogenous control for data normalization (forward β-actin 5′-CCACCCCACTTCTCTCTAAGGA-3′, reverse β-actin: 5′-ACCTCCCCTGTGTGGACTTG-3′).

The data normality and variance homogeneity were previously tested. The results are expressed as mean ± S.E.M. Data were analyzed correlating expression ratio by the software REST [30] and considering statistically significant $p < 0.05$.

3. Results

3.1. Cell surface markers CD34 and CD38 on K562 (non-MDR) and Lucena (MDR) cell lines

As shown in Figs. 1A (K562 cells) and 2A (Lucena cells), both cell lines display a positive profile for the cell surface marker CD34 (CD34+). However, regarding the CD38 cell surface marker, Figs. 1B (K562 cells) and 2B (Lucena cells) show a negative profile (CD38−). Both K562 and Lucena cell lines exhibited similar CD34+CD38− markers profiles when compared with the left curve, which represents the negative control, not labeled with antibody.

3.2. Identification of Oct-4 binding sites in promoters of ABCB1, ABCG2 and ABCC1 genes

To determine the relationship between stem cells and the MDR phenotype, we analyzed 10,000 bp from the 5′ regulatory regions of the ABC transporter genes (ABCB1, ABCG2 and ABCC1), in search of

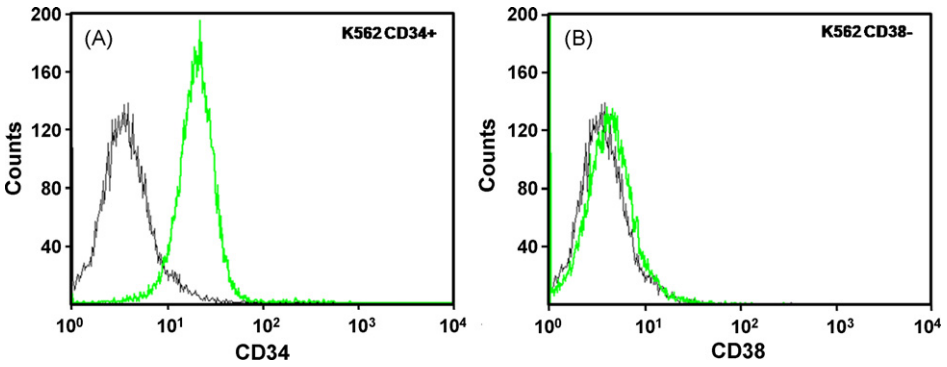

Fig. 1. FACS analysis of CD34 and CD38 cell surface markers on K562 cells. (A) CD34+ cells. (B) CD38− cells. The negative control (left curve) was cells not labeled with antibody.

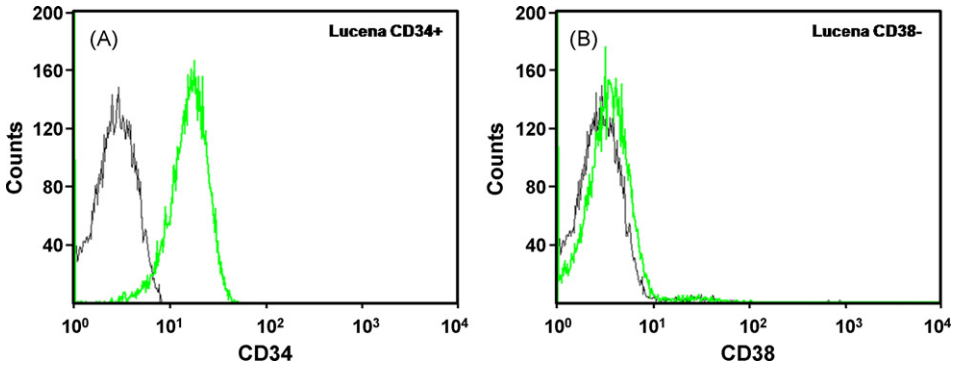

Fig. 2. FACS analysis of CD34 and CD38 cell surface marker on Lucena cells. (A) CD34+ cells. (B) CD38− cells. The negative control (left curve) was cells not labeled with antibody.

Oct-4 binding sites. In all three gene promoters analyzed we found Oct-4 binding sites, been two core sequences in ABCB1 and ABCG2 and only one in ABCC1 (Table 1).

3.3. Gene expression: Oct-4 and ABC transporters on K562 (non-MDR) and Lucena (MDR) cell lines

The results for Oct-4 gene expression are shown in Fig. 3. The Lucena cells (MDR) proved to be a putative tumoral stem-like cell profile since these cells showed a significant 3-fold induction of this stem cell marker compared as K562 cell line.

In addition, we investigated these same cell lines with regard gene expression of some members of ABC transporters superfamily. Fig. 4A shows the result that follows the ABCB1 (MDR1) gene over-expression with an amount of 1400-fold induced in the Lucena cells when compared with its parental K562. Also the ABCB1 gene expression was just significantly higher in Lucena cells. The data shown in Fig. 4B displays a similar profile for the ABCG2 transporter in the cell lines due to increased in the induction of this gene. The Lucena cell line showed an induction of approximately 4-fold in the gene expression of this transporter compared to K562 cells. Moreover, Fig. 4C shows that the ABCC1 transporter displays a significant difference in gene expression in these two lines but this difference was not very large regarding this transporter. The Lucena cells exhibited an ABCC1 gene expression slightly 1.5-fold induced when compared to K562 cell line. All results were statistically different.

The summary of all results are presented in Table 2.

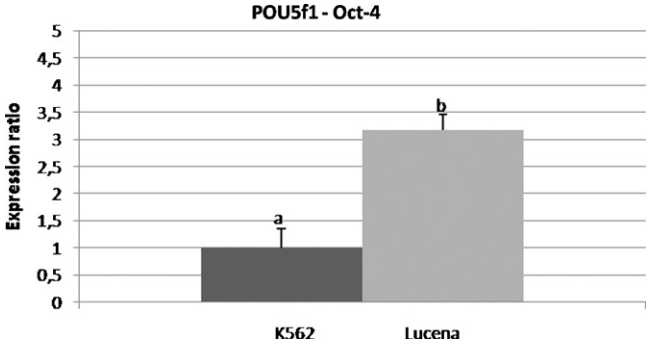

Fig. 3. Relative gene expression of Oct-4 in K562 (non-MDR) and Lucena (MDR) cell lines. Different letters show significant difference between means of each group ($p < 0.05$).

Table 1
Analysis of the Oct-4 core sequences in the promoters of the ABC transporters superfamily (ABCB1, ABCG2 and ABCC1).

Promoter	Position	Strand
ABCB1(MDR1)	−3949 to −3956	(−)
	−4038 to −4045	(−)
ABCG2(BCRP)	−5698 to −5705	(+)
	−6895 to −6902	(−)
ABCC1(MRP1)	−6712 to −6719	(−)

Table 2
Molecular profile to CD34 and CD38 (hematopoietic stem cell markers), Oct-4 (tumoral stem cell marker) and ABCB1, ABCG2 and ABCC1 transporters in K562 (non-MDR) and Lucena (MDR) cell lines (LMC).

LMC	CD34	CD38	Oct-4	ABCB1	ABCC1	ABCG2
K562	+	–	1	1	1	1
Lucena	+	–	3×	1400×	1.5×	4×

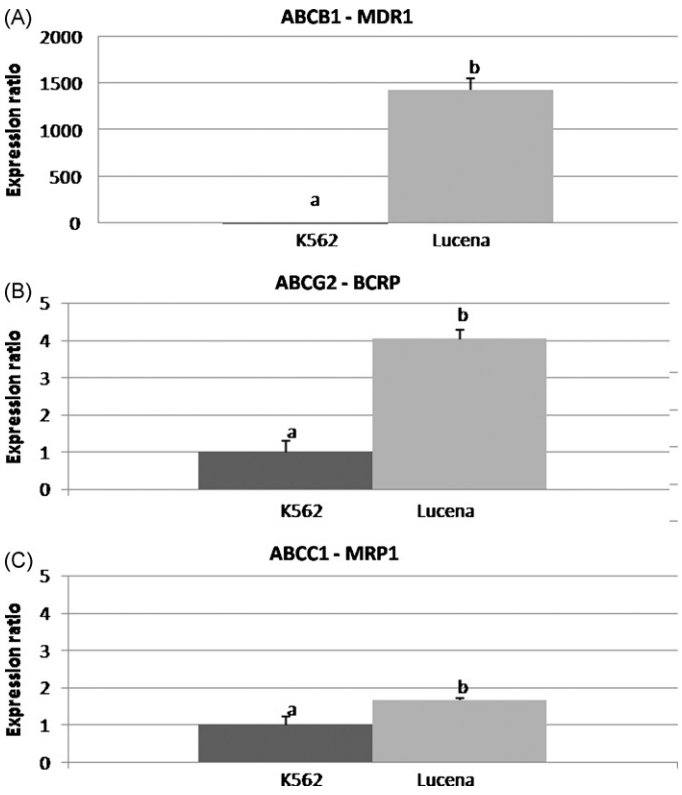

Fig. 4. Relative gene expression of ABC transporters in K562 (non-MDR) and Lucena (MDR) cell lines. (A) ABCB1 (MDR1). (B) ABCG2 (BCRP). (C) ABCC1 (MRP1). Different small letters show significant differences between means of each group ($p < 0.05$).

4. Discussion

Given the general acceptance of CD34 as a cell surface marker for hematopoietic stem cells, our purpose was to test the hypothesis that leukemic cells might also express the CD34 marker. This question was generated by the observation that some cancer cells expressed CD34 as identified by Kubonishi et al. [31], who established leukemic stem cell (Ph1+, CD34+) in chronic myeloid leukemia (CML). The surface phenotype of human hematopoietic stem cells has been defined as being CD34+CD38− [15,32,33], which is in agreement with our results, as both K562 and Lucena cell lines showed a CD34+CD38− cell surface marker profile.

The similar CD34+CD38− profile displayed by K562 and Lucena cells is not consistent with the idea that CD38− is unique for the MDR phenotype, since K562 (non-MDR) as well as Lucena (MDR) cell lines demonstrated the same result (Figs. 1B and 2B). Until this work, data comparing the expression of markers of multidrug resistance in these cell lines were limited to Pgp, and its over-expression in Lucena cells and its absence in K562 cells [34].

Considering the MDR phenotype, it is difficult to explain the CD34+CD38− phenotype of the K562 line. However, it is important to consider that this study also assessed the expression of two additional genes related to resistance and that, although they exhibit a lower expression than the ABCB1 gene expression exhibited by Lucena cells, the ABCC1 and ABCG2 genes are present in K562 and Lucena cells.

Another consideration is that these results suggest the maintenance of MDR phenotype of Lucena cells may be linked to certain agents such as ultraviolet radiation, even when Pgp is altered [35]. That particular study did not investigate the existence of other MDR proteins in the Lucena line. From our present work, it is possible to suggest that the protein encoded by the ABCG2 gene may also be involved in the resistance phenotype in Lucena cells. As well, it is possible to suggest that a direct correlation between the CD38− marker and the MDR phenotype may not exist in these cells.

This work demonstrates that a difference exists in the gene expression profile between K562 and Lucena cell lines with regard to the Oct-4 transcription factor. Our results show a higher induction of the Oct-4 gene expression in Lucena cells as compared to K562 cells. It has been reported that the Oct-4 gene and Oct-4 protein are expressed in several adult pluripotent stem cells, as well as in several human and rat tumor cells [27]. Therefore, cells expressing the Oct-4 gene, such as the Lucena cell line, are potential pluripotent stem cells that may possess a greater invasive capacity. Additionally, the fact that the Lucena cells displayed a higher Oct-4 gene expression compared to K562 cells, is in accordance with that which has recently been demonstrated in lung cancer LC-CD133+ cells, which display a higher Oct-4 expression with the ability to self-renew, plus possesses proliferative potential, and chemoradiotherapy resistance [23]. The authors suggested that these abilities are regulated by the activation of Oct-4 gene expression.

The MDR phenotype is quite complex and not fully understood. The regulation of stem cell biology by ABC transporters has emerged as an important new field of investigation. In light of these findings, it is critical to characterize this family of proteins in leukemic cell lines as putatives tumoral stem-like cells resistant. Our results suggest that ABCB1 may be responsible for several functional differences between the K562 and Lucena cell lines, due to the possible Oct-4 participation in provoking changes in Pgp expression in the Lucena cell line.

ABCG2 was subsequently identified and characterized as a novel stem cell transporter in human cells [36]. ABCG2 appears to be highly expressed in phenotypically defined populations of primitive hematopoietic stem cells [37]. The same was observed in this work where a high level of ABCG2 gene expression in Lucena cells was observed as compared to K562 cells.

Recently, it has been shown that hematopoietic stem cells express a significant number of different transporters from the ABC family [17], which encourages us to suggest that K562 and Lucena cells are pluripotent leukemic stem cell lines, and these cell lines assume features concerning the tumoral stem-like cells resistant from blastic phase of CML.

The two ABC transporter-encoding genes that have been studied most extensively in stem cells are ABCB1 and ABCG2 [36–38]. Along with ABCC1, they represent the three principal multidrug resistance genes that have been identified in tumor cells [6,39]. Thus, this work emphasizes that Lucena revealed a putative profile of tumoral stem-like cells by exhibiting a high ABCB1 and ABCG2 gene expression in conjunction with a lower induction of ABCC1 gene expression. Also, these MDR cells showed a high Oct-4 gene induction, suggesting that Oct-4 expression may be a possible key regulator for ABCB1, ABCG2 and ABCC1 transporters.

It is also possible that the resistance phenotype developed by Lucena cells is determined by ABC transporter expression which was probably activated by the induction of the Oct-4 transcription factor, such as demonstrated in Table 1. The ABCB1, ABCG2 and ABCC1 transporters exhibit binding sites (octamer-ATGCAAAT) for the Oct-4 transcription factor. The presence of these binding sites in the gene promoter of these transport proteins suggests that the transporter regulation pathways may be initiated at the Oct-4 recognized binding sites. However, the presence of Oct-4 alone is not always sufficient for induction of transporter genes. Transporter expression levels are often dependent upon Oct-4 interactions with other transcription factors. In fact, some regions termed multiple transcription factor-binding loci (MTLs) were bound by several factors [40]. Specifically, clusters of Oct-4, NANOG and SOX2 are known to cooperate in the regulation of the mouse embryonic stem cell transcriptome [41]. The super-expression of ABCB1 could suggest that ABCB1 has a greater number of specific Oct-4 core

sequence binding sites. However, the present study indicates that both genes possess only two Oct-4 core sequence binding sites (Table 1), suggesting that the ABCB1 super-expression might be explained by a possible relationship between Oct-4 and other transcription factors, such as the complex Sox-Oct [42]. Research into interactions surrounding the Oct-4 transcription factor and its binding sites are necessary to better understand its role in the MDR phenotype.

The existence of tumoral stem-like cells may explain why anticancer therapies may not completely eradicate tumors, which, in turn, assures eventual recurrences [43]. The identification and possible manipulation of the tumoral signaling pathways represent a possible means to confront the development of leukemia. Moreover, the phenotypic identification of tumoral cells by marker expression may be an important diagnostic methodology. This work provides a molecular basis for further studies that seek the reversal of the chemotherapeutic resistance phenotypes.

5. Conclusions

The profiles displayed by the two blastic phase CML cell lines are summarized and presented in Table 2. Both lines exhibit the CD34+CD38− cell surface marker profile and may be considered to be hematopoietic stem cells. The CD38 phenotype is not related with the MDR phenotype in this leukemia. The prominent feature appears to be the concomitance of Oct-4 gene expression and ABC transporter expression.

The Lucena cells may be considered as a putative tumor stem-like cell, and perhaps also K562 may be considered as such. The expression of the Oct-4 transcription factor in the K562 cells suggests that this cell line is somewhat compromised with respect to some hematopoietic signaling pathways, while the Lucena cell line may have greater plasticity and the ability to differentiate into other cell types that are unrelated to hematopoietic lines. The higher expression of Oct-4 in Lucena cells and the presence of binding sites of this transcription factor in the promoter region of the transport proteins provides evidence of its relationship with the MDR phenotype in these leukemic stem cells (K562 and Lucena – LMC).

Conflicts of interest statement

The authors have no conflict of interest.

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