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BRG1 regulation by miR-155 in human leukemia and lymphoma cell lines

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Abstract

Introduction/purpose BRG1 is a key regulator of leukemia stem cells. Indeed, it has been observed that this type of cells is unable to divide, survive and develop new tumors when BRG1 is down-regulated.

Materials and methods We assessed BRG1 and miR-155 expression in 23 leukemia cell lines, and two no pathological lymphocyte samples using qPCR. MiR-155 transfection and western blot were used to analyze the relationship between miR-155 and its validated target, BRG1, by measuring protein expression levels. The effect of miR-155 on cell proliferation and prednisolone sensitivity were studied with resazurin assay.

Results BRG1 expression levels could correlate negatively with miR-155 expression levels, at least in Burkitt's lymphoma and diffuse large B cell lymphoma (DLBCL) cell lines. To clarify the role of miR-155 in the regulation of BRG1 expression, we administrated miR-155 mimics in different leukemia/lymphoma cell lines. Our results suggest that miR-155 regulate negatively and significantly the BRG1 expression at least in the MOLT4 cell line.

Conclusion Our study revealed a previously unknown miR-155 heterogeneity that could result in differences in the treatment with miRNAs in our attempt to inhibit BRG1. However, the expression levels of BRG1 and miR-155, before prednisolone treatment were not statistically significantly associated prednisolone sensitive leukemia cells.

Keywords BRG1 · miR-155 · Leukemia · Prednisolone

Introduction

The chromatin-remodeling complex SWI/SNF is involved in gene expression control, regulating the accessibility of DNA to the transcription machinery and transcription factors. In mammals, this complex is comprised one catalytic ATPase subunit that can be *BRM* (also known as *SMARCA2*) or *BRG1* (*SMARCA4*), and a set of highly conserved 'core' subunits, and variant subunits that are thought to contribute to the targeting, assembly and the regulation of lineage-specific functions of the complexes [1]. Several evidences have shown that dysfunctional SWI/SNF activity may lead to epigenetic dysregulation that can contribute to cancer development.

Moreover, most of the bibliography attribute to BRG1 tumor suppressor roles in solid tumors. Some reports have found elevated levels of *BRG1* in gastric [2], prostate [3] and skin [4] cancer, and correlated with tumor progression [2, 3, 5]. However, in contrast to most solid tumors, several evidences indicate that *BRG1* is required for leukemia cells propagation, to maintain tumoral phenotype in a mouse model of acute myeloid leukemia [6, 7], and also it appears to be associated with glucocorticoid resistance [8], suggesting that inhibition of *BRG1* expression could make the leukemia cells unable to divide, survive, develop new tumors or determine the response to treatment.

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MicroRNAs (miRNAs) are emerging as important regulators of different biological processes based on their ability to bind to the complementary sequences in 3′ UTRs of their target mRNAs, around 30% of all genes [9], and negatively regulate their translation. Evidences indicate that miRNAs play an important role in the development of human cancers including leukemias [10]. One example that highlights the importance of these non-coding RNAs is miR-155 which has multiple biological roles in the immune system [11]. In fact, the miR-155 knockout mice demonstrated that microRNA-155 is necessary for normal immune function [12, 13]. In addition, miR-155 acts as an oncomiR [6, 7], and is highly expressed in B cell lymphomas [14, 15]. Therefore, identification of miR-155 targets is critical for understanding its biological function, enabling us to use it as a novel therapeutic drug in these tumors.

In this study, *BRG1* and miR-155 expression levels were determined by qPCR in a panel of 23 human leukemia cell lines. Our data showed that miR-155 expression was inversely correlated with *BRG1* expression levels, at least in Burkitt's lymphoma and diffuse large B cell lymphoma. Second, the administration of synthetic miR-155 mimics results in reduced level of BRG1 protein in MOLT4, suggesting that miR-155 could be an important regulator of *BRG1* expression. Finally, to assess whether expression of *BRG1* and miR-155 differed between leukemia cells that are resistant to glucocorticoids and those that are sensitive to glucocorticoids, we measured prednisolone sensitivity in four cell lines (MOLT4, REH, SD-1, and U937).

Materials and methods

Samples

We analyzed 23 cell lines from different subtypes of leukemia and lymphoma and two samples of normal lymphocytes (Table 1) from peripheral blood samples.

Cell culture

The leukemia cell lines were grown under standard conditions (37 °C, 5% carbon dioxide) in RPMI1640's medium supplemented with glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin. Protein and RNA from the cultured cells were extracted with RIPA buffer and Trizol reagent (Sigma), respectively.

In silico analysis of *BRG1* alterations and methylation status

The Cancer Cell Line Encyclopedia (CCLE) and canSAR databases were used to carry out an in silico analysis of *BRG1* mutation in 23 leukemia cell lines. The MethHC, Methy Cancer, c-BioPortal, and DiseaseMeth databases were used to perform in silico analysis of the *BRG1* promoter.

Systematic review of the literature

A systematic review was carried out using an online database (MedLine). The articles were selected according to the following inclusion criteria: (1) leukemia or lymphoma cell lines, (2) with methylation assays, (3) analysis of BRG1 or SMARCA4, and (4) published in any language.

Real-time quantitative reverse transcription PCR

Real-time quantitative reverse transcription PCR (qPCR) was optimized using the Applied Biosystems 7900HT Real-Time PCR System containing cDNA prepared from 1 µg total RNA. PCR reactions contained 1 µL cDNA, 5 µL qPCR SyBrGreenMaster Mix, 2 uL water and 1 uL primers for each gen. PCR conditions were 30 s at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles. The cycle threshold (Ct, PCR cycle at which probe signal reaches the threshold) was determined for each gene. Before performing this study, *GAPDH* and *TBP* were selected as candidate housekeeping genes, while *SNORD44* and 5S were used as housekeeping for the analysis of miRNA expression. Among all the housekeeping genes tested, *GAPDH* and *SNORD44* emerged as the most stable genes in these series of tumors.

Expression was quantified following the analysis of cDNAs in triplicate. For each experimental sample, the amount of each gene and its endogenous reference (*GAPDH* or *SNORD44*) was determined using standard curves. These standard curves were composed of five points obtained from fivefold serial dilutions (20, 10, 5, 2.5, and 1.250 ng/µL) of cDNA from a cell line pool. We only considered experiments in which the linear relationship between Ct (threshold cycle) and the log of the amount of each gene were higher than 0.99 (correlation coefficient). The average Ct of the triplicates was calculated, excluding outliers (replicates with Ct differing by more than one cycle from the median). If the sample failed to meet these criteria a third time, it was classified

Table 1 Characteristics of 23 cell lines included in this study

Cell line	*BRG1* gene alterations	BRG1 protein expression	Tumor subtype (ATCC)
Jurkat	WT[b]	Yes	Acute T cell leukemia
MOLT4	Deletion frameshift[b]	Yes	Acute T cell lymphoblastic leukemia
DB	NA[b]/WT[a]	Yes	Diffuse large B cell lymphoma (DLBCL)
Karpas 1106	NA[ba]	Yes	
MHH-PREB-1	WT[b]	Yes	
Oci Ly7	Substitution missense[b]	Yes	
U2932	WT[b]	Yes	
Karpas 422	WT[b]	Yes	Germinal center B cell like diffuse large B cell lymphoma (GCG-DLBCL)
Oci Ly1	WT[b]	Yes	
Riva	NA[ab]	Yes	
RL	NAb/WT[a]	Yes	
SU-DHL4	WT[b]	Yes	
Daudi	NA[b]/WT[a]	Yes	Burkitt's lymphoma
Namalwa	Deletion in frame[b]	Yes	
Raji	WT[b]	Yes	
Ramos	NA[ba]	Yes	
Wien 133	NA[ba]	Yes	
REH	NA[b]/WT[a]	Yes	Acute B cell lymphoblastic leukemia
RS4;11	Substitution missense[b]	Yes	
SD-1	NA[ba]	Yes	
JY	NA[ba]	Yes	B cell immortalized
NB4	NA[b]/WT[a]	Yes	Acute promyelocytic leukemia
U937	WT[ab]	No	Monocytic-like histiocytic lymphoma

NA data no available in database

[a] Cancer Cell Line Encyclopedia (CCLE)

https://www.broadinstitute.org/ccle/home

[b] canSAR, https://cansar.icr.ac.uk/cansar/#main_tab_holder:tab_search_main_protein:tab_search_1

as an assay failure. The expression values of each gene were then divided by the amount of *GAPDH* or *SNORD44* to obtain a normalized value. *GAPDH* or *SNORD44* genes were used as an internal control for RNA quality reverse transcription and to correct the variations due to RNA degradation.

Transfection studies

To know if BRG1 expression is directly regulated by miR-155 expression on leukemia cells, we selected three cell lines (MOLT4, REH, SD-1, and U937) because they showed distinct expression levels of these genes. We transfected the cells with a miR-155 (miRcuryLNA microRNA, BioNova), or miR scrambled negative control (mercury LNAmicrRNA. Mimic control negative, BioNova), using the Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's guidelines. All transfections were performed in 6-well plates using 5×10^5 cells/well in 2 mL of medium.

Determination of viability and metabolic activity with resazurin method

Responsiveness of leukemia cells to miR-155 was determined by the 2-day in vitro resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay. Resazurin dye has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. The resazurin-based method follows the reduction of the redox dye resazurin by metabolically active cells that convert it into a highly fluorescent product (resorufin). Nonviable cells have no metabolic capacity, and will not reduce the dye, so therefore it can be said that the fluorescence intensity observed is a measure of the viable cells. 5×10^4 cells were plated in 96-well plates and transfected with control miRNA or miR-155. After 48 h, 20 μL of resazurin solution were added to each well, and the cells were incubated for 4 h at 37 °C. After that, we added 50 μL of SDS 3%, and fluorescence was measured at 595 nm using a microplate reader according to the manufacturer's

recommendations. Relative cell number was calculated by normalizing the absorbance to the value obtained with untreated cells.

BRG1 and sensitivity to prednisolone

Prednisolone sensitivity was determined for MOLT4, REH, SD-1, and U937cell lines. Drug sensitivity was determined by resazurin assay after 48 and 72 h of continuous drug incubation. Tumor cells were seeded at a concentration of 5×10^4 cells/well in 200 μL culture medium in a 96-well microculture plate with the indicated drugs, and incubated at 37 °C in 5% CO_2. To estimate the appropriate concentrations for prednisolone, cells were treated with a wide range of concentrations. Concentrations used were from 50 mM to 50 μM.

Western blot

Total protein was extracted from cells using cell lysis RIPA buffer (50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, 25 mM $MgCl_2$, and supplemented with a phosphatase inhibitor cocktail). Protein concentration was determined by Bradford assay. 50 μg of protein lysate was resuspended in sample buffer, electrophoresed, transferred to PVDF membranes and incubated overnight with primary antibodies anti-BRG1 (Santacruz) and anti-ACTIN (SantaCruz). The membranes were then treated with conjugated anti-rabbit and antimouse secondary antibodies, respectively (Dako). The target protein bands were determined using the reagents provided in the ECL+ plus kit (GE healthcare, Piscataway, NJ, USA).

Results

Alterations of BRG1 in leukemia cell lines

In silico analysis of the Cancer Cell Line Encyclopedia (CCLE) and canSAR Database were performed to examine whether *BRG1* is mutated in hematological neoplasia. The results showed that *BRG1* is altered in various cell lines (4/17, 23.5%): 2 ALL (1 B cell, and 1 T cell), 1 DLBCL and 1 Burkitt cell lines.

Hypermethylation of BRG1 promoter

The research in MethHC, Methy Cancer, c-BioPortal, and DiseaseMeth databases did not provide any useful results. The literature search generated seven studies although none of them satisfied our inclusion criteria. There are not scientific evidences supporting that *BRG1* gene promoter

hypermethylation may contribute to the development of leukemia.

BRG1 and miR-155 quantitative RT-PCR

We analyzed the expression of *BRG1*, and miR-155 by qPCR in a panel of 23 leukemia cell lines and two normal lymphocyte samples (Fig. 1). The range of *BRG1* expression values was shorter than that observed in miR-155. In addition, it is important to highlight that *BRG1* expression was homogeneous among the different subtypes of leukemias/lymphomas tested, ranging from 0.31 (Karpas 1106) to 0.76 (Riva). In contrast, miR-155 expression levels were more heterogeneous, ranging from 0.12 (MOLT4) to 3.6 (SD-1). *BRG1* expression levels were higher than miR-155 in ten cell lines (DB, Jurkat, MOLT4, MHH-PREB-1, Oci-Ly7, Ramos, RL, RS4;11, SU-DHL4, and Wien 133). However, other ten cell lines (JY, Karpas 1106, Namalwa, NB4, Oci-Ly1, Raji, Riva, SD-1, U2932 and U937) showed higher expression levels of miR-155 than of *BRG1*. Only three cell lines, Daudi, Karpas422, and REH, had the same expression levels of *BRG1* and miR-155. Moreover, it could be seen that miR-155 was abundantly expressed in JY, Karpas 1106, Namalwa, and SD-1; and ten cell lines (JY, Karpas 1106, Namalwa, NB4, Oci-Ly1, Raji, Riva, SD-1, U2932 and U937) expressed, as least, twice more miR-155 than in normal lymphocytes.

According to *BRG1*/miR-155 expression values, we selected for the next assays MOLT4, REH, SD-1, and U937 cell lines. MOLT4 had the highest value of *BRG1*/miR-155 (ratio = 3.753), REH showed the same expression levels (ratio = 0.955), and SD-1 expressed the highest miR-155 values (ratio = 0.117). Finally, we selected U937 cell line because it does not express *BRG1* protein. Next, we analyzed the correlation between *BRG1* and miR-155 defined with the expression levels of mRNA. We observed an inverse correlation between the *BRG1* and miR-155 expression levels in Burkitt's lymphomas ($r = -0.42$; $p = 0.47$) and diffuse large B cell lymphoma ($r = -0.52$; $p = 0.37$). These tumoral cell lines expressing higher levels of the miR-155 displayed a reduced expression of *BRG1*, suggesting that *BRG1* could be post-transcriptionally regulated by miR-155.

Western blot analysis of BRG1

Western blot was performed to determine *BRG1* protein levels. All cell lines, except U937, showed expression of BRG1 protein (Fig. 2). U937 is a particular cell line derived from malignant cells of a pleural effusion of male with diffuse histiocytic lymphoma. According to CCLE and canSAR database, this cell line is *BRG1*-wild type. There was a marked heterogeneity in the level of *BRG1* among the cell
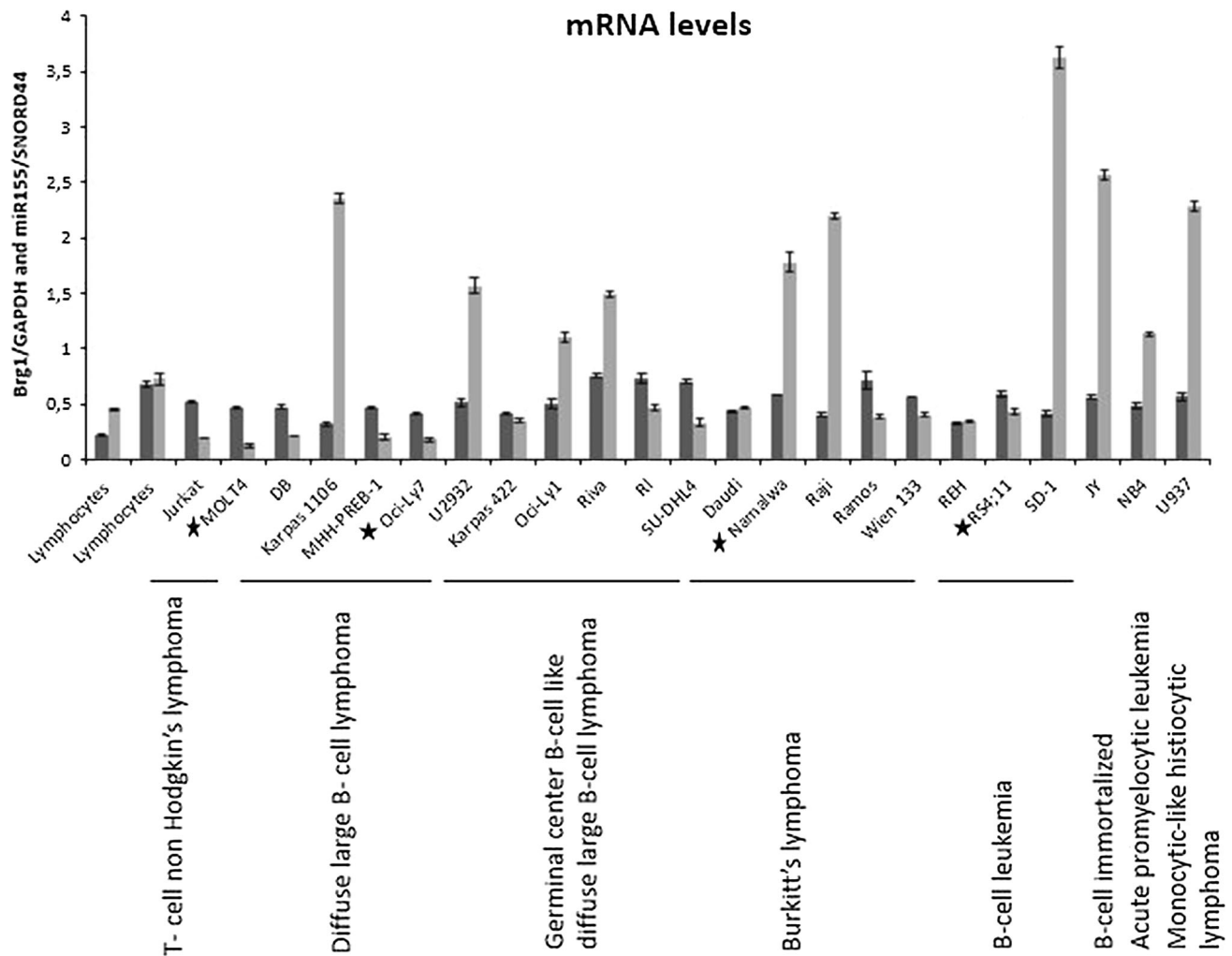
Fig. 1 Distribution of *BRG1/GAPDH* and miR-155/SNORD44 mRNA expression levels in leukemia/lymphoma subtypes. *BRG1* expression levels appears in *black color* and miR-155 are in *gray color*. *BRG1 mutant cell lines* are shown in the cell line panel with a star
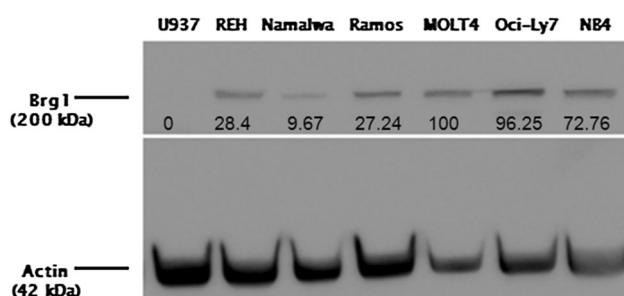

Fig. 2 Detection of BRG1 protein. BRG1 and actin proteins in total cell lysates from leukemia/lymphoma cell lines were determined by western blot analysis with chemiluminescence detection

lines tested. Oci-Ly7 and Ramos showed the highest levels of protein, and Namalwa expressed the lowest. Normal peripheral blood T cell samples expressed BRG1 protein.

Relationship between miR-155 and BRG1 expression

To investigate the effects of BRG1 and miR-155 relationship on leukemia cells, MOLT4, REH, and SD-1 cell lines were transfected with miR-155 and control miRNA. MOLT-4 expressed more BRG1 than miR-155, REH exhibited the same levels, and SD-1 showed the highest miR-155 expression levels. Western blot analysis showed that transfection of miR-155 significantly inhibited the endogenous expression of *BRG1* in MOLT4 cell lines comparing with the control miRNA (Fig. 3), suggesting that BRG1 is under the control of miR-155. In contrast, we found that administration of miR-155 did not decrease expression of BRG1 in REH and SD-1 cell lines. We hypothesized that leukemia cells that express lower levels of miR-155 than BRG1 are more sensitive to miR-155
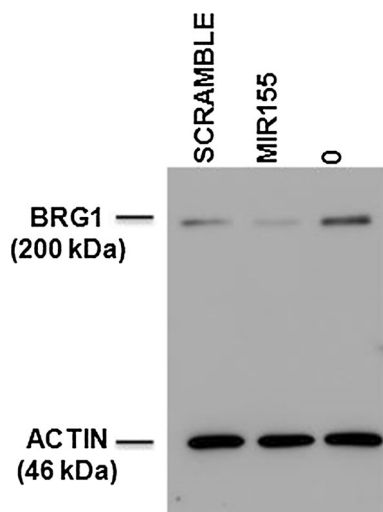
Fig. 3 MOLT4 cells (scramble probe, miR-155–transfected, and untransfected) were harvested at 48 h after transfection. Cell lysates were subjected to western blot with the use of BRG1 and ACTIN antibodies

ectopic expression and the inhibition of BRG1 mediated by miR-155 can be more easily detected.

Determination of LC50 of prednisolone

This investigation was undertaken to determine whether the expression of chromatin-remodeling BRG1 gene and miR-155 differed among cells lines that are more resistant to prednisolone from those that are sensitive. Based on leukemia cell lines showed differences in BRG1 and miR-155 expression levels, we performed prednisolone sensitivity assay in four different cell lines (MOLT4, REH, U937, and SD-1 cell). We have used a measure of glucocorticoid sensitivity based on resazurin assay after 48 and 72 h of continuous drug incubation: LC50 was defined as the point at which cell number was 50% of control untreated cells. There was not a marked variation in antileukemic activity of prednisolone among the cell line

samples. The median lethal concentration (LC50) for prednisolone was 1.11 μM ranging from 0.59 to 1.22 μM. SD-1 had lower LC50 values for prednisolone than the other three cell lines (Table 2).

Discussion

SWI/SNF chromatin-remodeling complex has emerged as a tumor suppressor gene that is altered in several human tumors [16–21]. Recurrent mutations in different subunits of the SWI/SNF complex have been identified in various types of tumors although their consequences remain poorly understood [16, 22]. SWI/SNF function in leukemia oncogenesis remains unclear highlighting the need to further our understanding of the function of BRG1 in these neoplasias. The activity of BRG1 is likely required in leukemia cells to maintain the tumor phenotype and to sustain the occupancy at critical enhancers that regulate *MYC* proto-oncogene expression [7]. In addition, frequent *BRG1* mutations or promoter hypermethylation have not been detected in leukemia, suggesting the oncogenic role of BRG1 and the existence of additional mechanisms that could alter *BRG1* expression. In this respect, a new therapeutic approach for this disease could include the use of proteins, such as *BRG1*, as targets [23]. In this article, the study of a panel of 23 leukemia cell lines for the presence of *BRG1* mutations and the consequent expression of *BRG1* mRNA and protein was carried out. BRG1 mutations in silico were not identified as the predominant underlying mechanism of silencing *BRG1* gene expression. Mutations of *BRG1* were found in leukemia cell lines, however, the expression levels of *BRG1* mRNA were relatively homogeneous among the different subtypes of leukemias/lymphomas tested independently of BRG1 mutations. In addition, the 22 cell lines in which mutations and no mutations had been identified, expressed BRG1 protein. These findings suggest that BRG1 may have not in

Table 2 Determination of LC50 of prednisolone in MOLT4, REH, SD-1, and U937 cell lines

	LC50 (μM)	BRG1/GAPDH	MiR-155/SNORD44	BRG1/miR-155	*BRG1* gene alterations
MOLT4	1.16 (1.13–1.19)	0.47	0.12	3.75	Yes[b]
REH	1.05 (0.78–1.41)	0.33	0.34	0.95	NA[ab]
SD-1	0.59 (0.480–0.73)	0.42	3.62	0.12	NA[ab]
U937	1.22 (0.93–1.61)	0.56	2.29	0.25	No[ab]

NA data no available in data bases

[a] Cancer Cell Line Encyclopedia (CCLE)

https://www.broadinstitute.org/ccle/home

[b] canSAR, https://cansar.icr.ac.uk/cansar/#main_tab_holder:tab_search_main_protein:tab_search_1

leukemia the tumor suppressor roles described previously in solid tumors.

How might *BRG1* become regulated in leukemia cells? Recently, our previous study found that the loss of expression of *BRG1* in lung tumors could be explained by the activity of miRNAs, in particular miR-155 [24]. Carcinogenesis has been extensively studied at a molecular point of view, and it has been enriched with the beginning of the era of non-coding RNA. MicroRNAs (miRNAs) are a type of short non-coding RNAs that regulate gene expression at the post-transcriptional level. It was known that miR-155 is directly implicated in normal hematopoiesis [25], it is highly expressed within lymphocytes (both B and T cells) [26], and it is also related with the leukemogenesis process [27]. In this study, normal lymphocytes samples expressed moderate expression levels of miR-155, compared to some leukemia cell lines. MiR-155 was overexpressed in ten leukemia cell lines (JY, Karpas 1106, Namalwa, NB4, Oci-Ly1, Raji, Riva, SD-1, U2932, and U937), indicating that miR-155 could contribute to leukemia development [14, 15, 24, 28, 29]. Considering the level of endogenous miR-155 in normal lymphocyte samples and our previous data [24], we hypothesized that administration of miR-155 could result in *BRG1* expression inhibition. We have observed that after the ectopic administration of miR-155 into MOLT4 leukemic cells, the expression of BRG1 protein increased at the second day after transfection. These results suggest a significant role for the BRG1/miR-155 ratio in the leukemia cells, as it can have been observed in MOLT4 cell line, which showed the highest value of BRG1/miR-155, and responded to the synthetic miR-155 administration. Future therapies must consider the molecular differences in BRG1 and miR-155 expression levels among the leukemia subtypes.

There is very limited data available regarding to the biological factors that affect the response to treatment in hematological neoplasias. Thus, it is clear that more molecular markers are needed to accurately predict the illness progression and treatment response after chemotherapy. Previous work [8] showed that increased expression of *BRG1* mRNA was statistically significantly associated with prednisolone sensitivity in acute lymphoblastic leukemia. Due to the possible influence of BRG1 and miR-155 expression levels in the therapy outcome, we studied whether the expression of BRG1 and miR-155 could predict the response to prednisolone in leukemia cells. Our study revealed a previously unknown miR-155 heterogeneity that could result in differences in the treatment with miRNAs in our attempt to inhibit BRG1. However, the expression levels of *BRG1* and miR-155, before prednisolone treatment, were not statistically significantly associated with prednisolone sensitive leukemia cells.

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