INSIGHTS INTO MIR-29 DYSREGULATION IN BURKITT LYMPHOMA: FROM MIR-29 SILENCING BY DNA METHYLATION TO CELL MIGRATION

Luciano Mazzoccoli[1], **Marcela C. Robaina**[1], **Sheila Coelho**[2], **Claudete E. Klumb**[1]

[1] Programa de Pesquisa em Hemato-Oncologia Molecular, Instituto Nacional de Câncer, Rio de Janeiro, Brasil

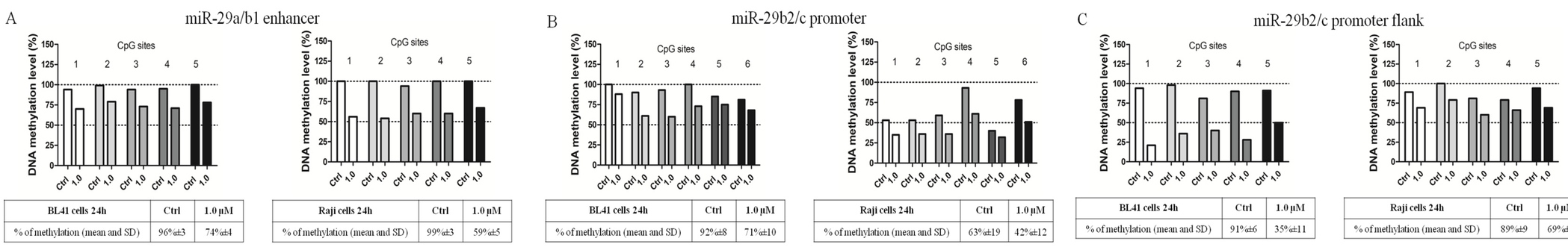
[2] Programa de Carcinogênese Molecular, Coordenação de Pesquisa, Instituto Nacional de Câncer, Rio de Janeiro, Brasil

Abstract

Introduction: Aberrant microRNA (miR) expression has been described in several cancers. Recently, we described that miR-29a/b/c expression levels are decreased in Burkitt lymphoma (BL) tumors and the ectopic expression of miR-29 inhibited proteins of MYC pathway and PI3K-AKT axis both involved in BL pathogenesis. The PI3K/Akt pathway has been reported to contribute to BL through proliferation, growth and survival. The Rho GTPase proteins plays an important role on regulating morphology and actin cytoskeleton organization in a positive feedback loop with PI3K pathway by maintaining cell polarity at the leading edge during cell migration. Given the miR-29 involvement in PI3K-AKT axis, and the decrease of miR-29 levels in BL cells, we sought to investigate the relationship between miR-29 dysregulation in BL cell and the migration of these cells to extranodal sites that is an unknown event in BL pathogenesis. Extranodal disease sites such as bone marrow, testicles and central nervous are associated with poor prognosis in BL patients. Little is know about miR-29 regulation in BL cells. Epigenetic mechanisms such as DNA methylation by DNA methyltransferases (DNMT) contribute to miR control expression. We have previously shown that, along with downregulation of miR-29a/b/c expression, BL tumors showed DNMT1 and 3B overexpression. In this study, we investigated the relevance of miR-29 dysregulation in cell migration and methylation of miR-29 as an additional mechanism for its silencing in BL cells.

Methods and Results: Mature sequences of miR-29 (mimetic) were transfected to BL cell lines and evaluated after 24 and 48h for Cdc42, ITGB1, RhoB and VEGF protein expression by western blot. A decrease of all protein expression levels was observed. In addition, BL cells were treated with PI3K-LY inhibitor for 24 and 48h. After that, a reduction of phosphorylated AKT along with a reduction of DNMT1 and 3B protein expression was detected, but no effect on cell viability was observed. Next, BL cell lines were transfected with miR-29 mimetic and treated with or without the inhibitor LY. After treatments, the transwell assays showed a reduction on cell migration at 24 and 48h. Finally, to investigate the cell migration differences among the lineages, cell cycle analysis were performed. The cell lines less prone to migrate showed more cells in G1 phase of the cycle in comparison to the counterpart. Regarding miR-29 regulation, BL cell lines and tumor samples showed miR-29a/b1 and miR-29b2/c genes methylated in CpG sites located in both promoter and enhancer regions when evaluated by pyrosequencing assay. Furthermore, the treatment of BL cells with the DNMT inhibitor (decitabine) reduced methylation, induced miR-29s expression and downregulated MYC protein levels in a dose-dependent manner.

Conclusion: Our findings suggest a relevant role of miR-29 in BL cell migration and propose an interplay among MYC/miR-29/DNMT1 and 3B, pointing out to miR-29 methylation as a shut-off mechanism that results from MYC overexpression in BL cells.



BL41 cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	96%±3	74%±4

Raji cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	99%±3	59%±5

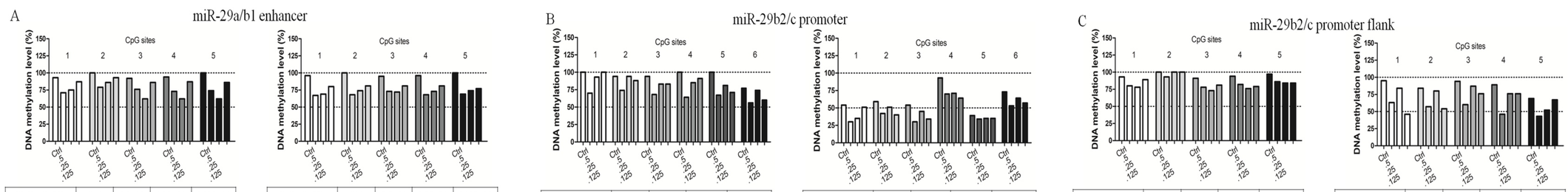
BL41 cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	92%±8	71%±10

Raji cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	63%±19	42%±12

BL41 cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	91%±6	35%±11

Raji cells 24h	Ctrl	1.0 µM
% of methylation (mean and SD)	89%±9	69%±7

Figure 1. MiR-29a/b1 and miR-29b2/c genes are silenced in BL cells by methylation at promoter and enhancer regions. The methylation percentage of each CpG site is depicted in the bar plots followed by the mean and standard deviation (SD) of each CpG sites shown in the box at the bottom of each graph. The % levels were compared between the control (untreated) and decitabine (1 µM) groups after 24 h in BL41 and Raji cells. (A) Five CpG sites on the miR-29a/b1 enhancer region, (B) six CpG sites on the miR-29b2/c promoter, and (C) five CpG sites flanking the miR-29b2c promoter.



BL41 cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	96%±4	75%±3	69%±11	88%±3

Raji cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	97%±2	69%±2	72%±2	80%±2

BL41 cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	94%±9	67%±6	85%±8	82%±14

Raji cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	62%±19	43%±16	50%±15	47%±12

BL41 cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	95%±4	84%±6	82%±11	87%±8

Raji cells 72h	Ctrl	0.5 µM	0.25 µM	0.125 µM
% of methylation (mean and SD)	92%±6	58%±7	85%±9	66%±15

Figure 2. Treatment with low doses of decitabine causes miR-29 demethylation in BL cells. Methylation analysis after treatment with low doses of decitabine. The methylation percentage of each CpG site is depicted in the bar plots followed by the mean and standard deviation (SD) of the CpG sites, as shown in the box at the bottom of each graph relative to each decitabine concentration (0.5, 0.25 and 0.125 µM). The % levels were compared between the control (untreated) vs decitabine groups after 72 h in BL41 and Raji cells. (A) Five CpG sites in the miR-29a/b1 enhancer region, (B) six CpG sites in the miR-29b2/c promoter, and (C) five CpG sites flanking miR-29b2c promoter.

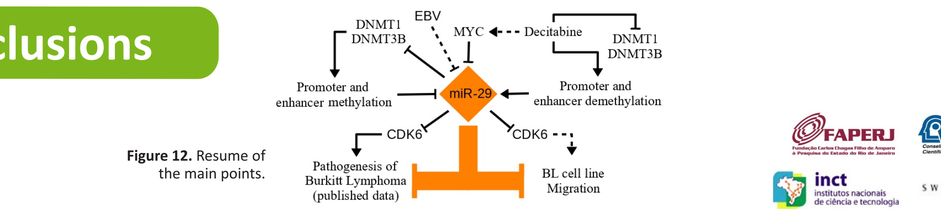


Figure 3. The expression of miR-29s in BL cells is upregulated by decitabine treatment. Quantitative PCR of miR-29a/b1 and miR-29b2/c in the BL41 and Raji cell lines. The miR-29 expression levels were analysed after treatment with low doses of decitabine. BL41 (A) and Raji (B) cells were treated with decitabine (0.5, 0.25 and 0.125 µM) and evaluated after 72 h for miR-29a/b/c expression, along with CDKN2A (p16) mRNA expression. The data represent the mean of three independent experiments and respective standard deviation, whereas the dotted line represents the mimetic control. * p<0.05 Mann-Whitney test.

Figure 4. Evaluation of miR-29a/b/c expression from BL tumour samples and inhibition of EBV-miR-BART6-5p. From immunohistochemical analysis of previous published data, tumour samples were revisited and analyzed according to the percent of cells expressing DNMT3B protein (10-50% vs >50%), along with the corresponding miR-29 expression. Left, (A) miR-29a (n=3 vs n=11); (B) miR-29b (n=3 vs n=10), (C) miR-29c (n=3 vs n=12) expression. After assembling the samples positive to DNMT3B protein expression from immunohistochemical analysis (10-50 and >50%), the tumour samples were placed according to EBV status: positive (+) or negative (-). (D) miR-29a (n=10 vs n=10); (E) miR-29b (n=11 vs n=8), (F) miR-29c (n=12 vs n=9). miR-29 expression was evaluated by real-time quantitative PCR in BL41 and Raji cells. Right, (A) Raji cells were transfected with the antagomir for BART6-5p, followed by decitabine (1.0 µM) and harvested 72 h later. Data from two independent experiments. Right, (B) The relative levels of miR-29a/b/c expression between untreated BL41 and untreated Raji cells. The results show the fold increases in the miR-29a/b/c expression levels in BL41 cells compared to Raji cells. The mean of five independent experiments plus the standard deviation. *p<0.05 Mann-Whitney test.

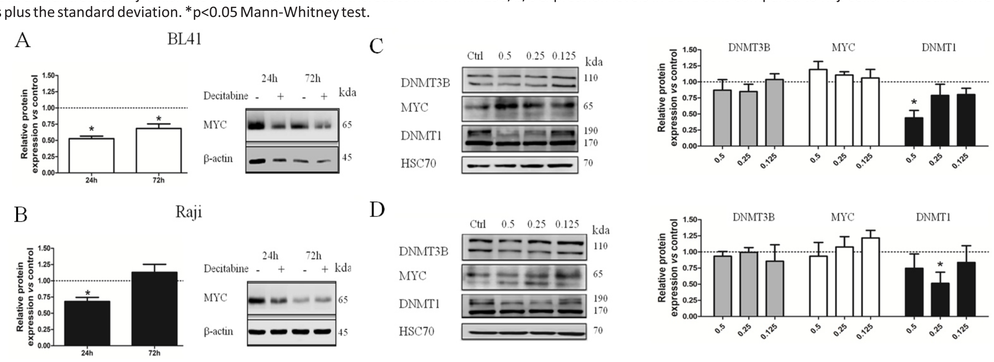
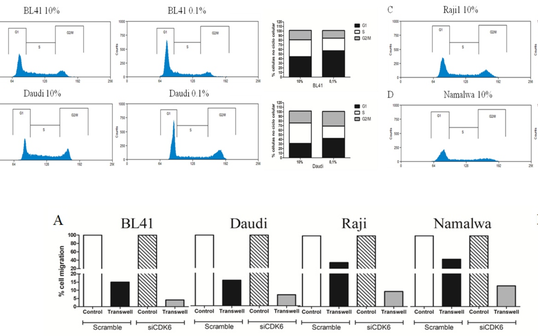


Figure 5. MYC protein expression is modulated by decitabine and lower doses downregulates DNMT1 treatment in BL cell lines. Western blot analysis of MYC protein expression after decitabine treatment. Band intensities were measured by densitometry analysis (left), and a representative Western blot image is shown on the right. (A) BL41 and (B) Raji cells were evaluated at 24 and 72 h after decitabine treatment (1.0 µM). The dotted line represents the untreated control. β-actin was used as the endogenous control. DNMT1, DNMT3B and MYC protein expression were evaluated by Western blot in BL cell lines (C) BL41 and (D) Raji after treatment with low doses of decitabine (0.5, 0.25 and 0.125 µM) for 72 h. HSC70 was used as the endogenous control. Band intensities were measured by densitometry (right). The dotted line represents the untreated control. The mean of three independent experiments plus the standard deviation. *p<0.05 Mann-Whitney test.

Conclusions

Figure 12. Resume of the main points.



BL tumour samples (10/10)	CpG1	CpG2	CpG3	CpG4	CpG5
% of methylation (mean and SD)	97.1% ±0.73	97.9% ±2.28	91.3% ±1.25	95.3% ±1.7	92.9% ±1.19

BL tumour samples (7/10)	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
% of methylation (mean and SD)	47.7% ±3.8	52.2.7% ±1.6	40.1% ±6.8	91.4% ±4.3	49% ±3.6	80.4% ±2.9

Figure 6. Quantitative pyrosequencing analysis reveals methylation on miR-29a/b1 enhancer and miR-29b2/c promoter regions in BL tumour samples The genomic locations of pyrosequencing assays and representative pyrograms in BL tumour samples are depicted. A) miR-29a/b1 enhancer region, (B) miR-29b2/c promoter. The percentage of methylation at each CpG site is represented in shaded areas. Mean and standard deviation are shown in the box at the bottom of each pyrogram.

Figure 7. Modulation of PI3K in BL. BL41 and Raji cells were trated for 48h with PI3K inhibitor LY (5 uM) and evaluated for DNMT1, DNMT3B, AKT and p-AKT protein expression (left). The densitometry is depicted on the right from two different experiments.

Figure 8. Effect of miR-29 on Integrin B1, CDC42, RhoB and VEGF protein expression. Mature sequences of miR-29c were transfected to BL41 and Raji cells and evaluated for Integrin B1, CDC42, RhoB and VEGF protein expression (left). Depicted on the right, the corresponding densitometry from two independent experiments. The dot line at position 1 represents the scramble control.

Figure 9. Effect of miR-29 alone and in association with LY on cell migration inhibition. Mature sequences of miR-29c were transfected to BL cells and evaluated 24h (white bars) and 48h (black bars) later for cell migration on transwell system. The PI3K inhibitor LY (5 uM) was used alone or in combination with miR-29c. The (%) of migration was calculated regarding the counterpart on non-transwell system. The medium used on the bottom of the plate was CXCL12 supplemented. Three different experiments are depicted.

Figure 10. Figure 10 Effect of medium supplementation on cell migration. In order to understand the differences about cell migration on different BL cells, the cell lines were supplemented with 10% of Serum Fetal Bovine or 0.1% and 24h later evaluated by flow cytometry for their cell cycle status with propidium iodide. Depicted on the right side, the (%) of cells found at the respective G1, S or G2/M phase of the cycle.

Figure 11. Effect of CDK6 inhibition on cell migration. BL cells were left with 0.1% Serum Fetal bovine (A) or 10% (B) for 24h prior siCDK6 or scramble (100 nM) transfection. BL cells were seeded on transwell assay after siCDK6 or scramble transfection and evaluated for (%) of cell migration in comparison to the counterpart on a non-trasnwell system. Data from one experiment.

Projeto Gráfico: Área de edição e produção de materiais técnico-científicos / INCA