MiR-29 silencing by DNA methylation in Burkitt lymphoma cells: Crosstalk between MYC and DNMT3B

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ABSTRACT

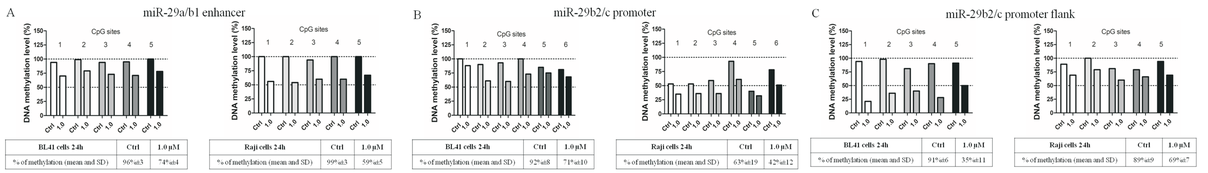
Background: In the last years increased evidences have strongly suggested that altered miRNA expression could play a significant role in the cancer development and progression depending on the tissue type and specific targets. MicroRNAs are small non-coding regulatory RNAs that bind to specific sites of their target genes and regulate post-transcriptional gene expression. MicroRNA genes may be regulated through epigenetic mechanisms, such as specific histone modifications and/or DNA methylation of CpG islands on promoter regions, or on those that are localized next to miRNA genes. MYC plays an important role in microRNA regulation including the miR-29 family of microRNAs. We have previously shown that miR-29a/b/c expression is down-regulated in Burkitt lymphoma (BL) tumor samples and associated to DNA methyltransferase (DNMT) 1 and 3B overexpression. Moreover, the ectopic expression of miR-29a/b/c in BL cell lines inhibited DNMT3B expression.

Objectives: Given the regulatory role of MYC in the miR-29 and DNMT1/ 3B expression, the aim of the study was to investigate the contribution of DNA methylation on miR-29 silencing in BL cells, targeting by both methylation on promoters and enhancers regions.

Methods: We investigated methylation on promoters and enhancers regions of miR-29a/b/c in BL cell lines (BL41 and Raji) using bisulfite pyrosequencing assays. Next, BL cells were treated with 5-aza-2'-deoxicitidine (decitabine) and evaluated miR29 a/b/c expression and methylation status. The expression of MYC, DNMT1 and DNMT3B at varying times and decitabine concentrations were accessed by Western blot.

Results: BL41 and Raji cell line presented methylation in CpG sites located in both promoter and enhancer regions. After 24h of treatment with 1.0 uM decitabine , miR-29s promoter and enhancers regions were demethylated in both BL cell lines. Similar results were observed at 72h even using lower decitabine concentrations. Additionally, the expression of miR-29s was upregulated by decitabine treatment at 24 h (1.0 uM) in both cell lines, and at 72h (0.5, 0.25 and 0.125 uM) in BL41cells. Notably, lower decitabine concentrations (0.5, 0.25 and 0.125 uM) down regulated DNMT1 and DNMT3B protein expression levels, but no effect in the MYC protein was observed.

Conclusion: In summary, the miR-29a/b1 and miR-29b2/c genes have methylated CpG sequences that may contribute to the regulation of miR-29s expression in BL cells. The findings suggest an interplay among MYC/miR-29/ DNMT3B pointing to miR-29 methylation as shut-off mechanism mediated by MYC overexpression in BL pathogenesis.



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

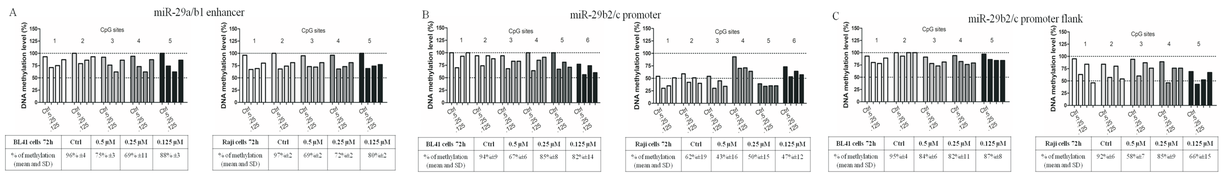
BL41 cells 24h	Ctrl	1.0 μM
% of methylation (mean and SD)	92%±8	71%±10

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 M) 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 M). 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.

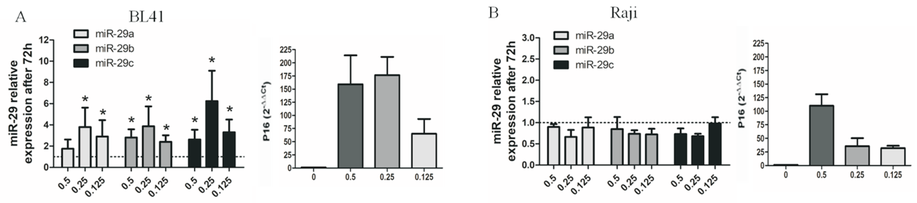


Figure 3 The expression of miR-29s in BL cells is upregulated by decitabine treatment. Quantitative PCR of miR-29a/b1 and miR-29b2/c in the BL41 and Raji cell lines. The miR-29 expression levels were analysed after treatment with low doses of decitabine. BL41 (A) and Raji (B) cells were treated with decitabine (0.5, 0.25 and 0.125 M) and evaluated after 72 h for miR-29a/b/c expression. *P16 (CDKN2A)* mRNA expression was evaluated as decitabine performance due to a well known mietilation of *P16* in BL. 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.

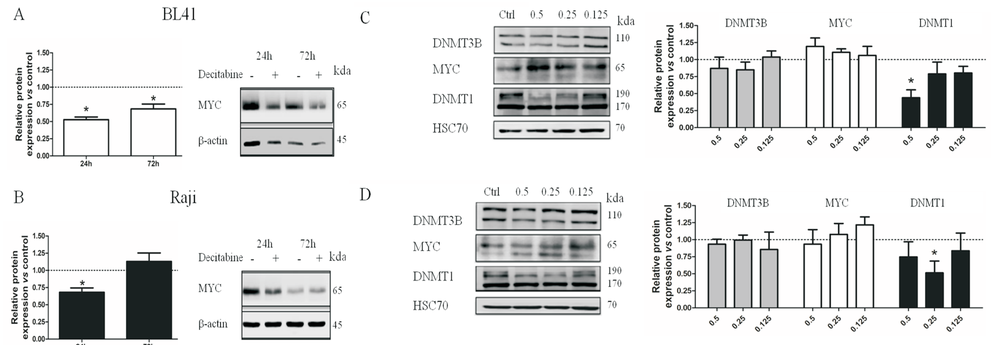
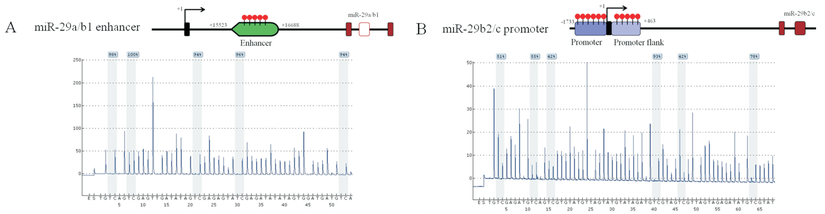


Figure 4 MYC expression is modulated by decitabine and lower doses downregulates DNMT1 treatment in BL cell lines
Western blot analysis of MYC expression after decitabine treatment. Band intensities were measured by densitometry analysis (left), and a representative Western blot image is shown on the right. (A) BL41 and (B) Raji cells were evaluated at 24 and 72 h after decitabine treatment (1.0 M). 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 M) for 72 h. HSC70 was used as the endogenous control (upper panels). Band intensities were measured by densitometry (lower panels). The dotted line represents the untreated control. The mean of three independent experiments plus the standard deviation. *p<0.05 Mann-Whitney test.

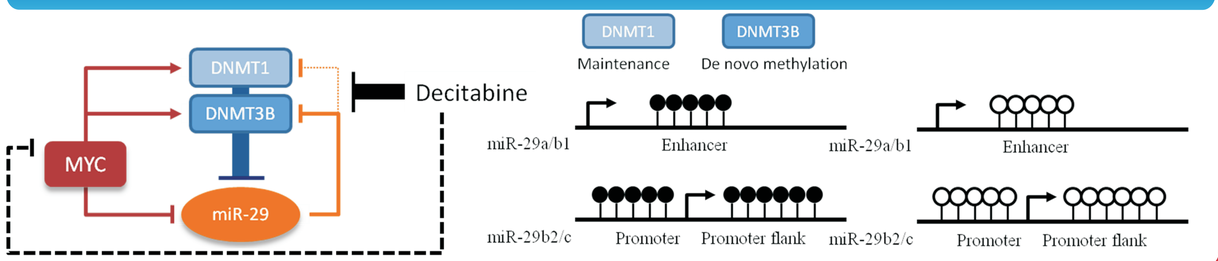


BL tumour samples (10/10)	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5
% of methylation (mean and SD)	97.1% ±0.73	97.9% ±2.28	91.3% ±1.25	95.3% ±1.7	92.9% ±1.19

BL tumour samples (7/10)	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6
% 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 5 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.

CONCLUSIONS



Projeto Gráfico: Setor de Edição e Informação Técnico-Científica / INCA