Methylation of p53-responsive microRNA genes in tumor tissue of lymphoma

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The antitumor effects of the p53 protein are largely mediated by microRNAs, the expression of which it induces directly, or regulates at the post-transcription level by influencing the processing of these molecules. We were interested in a group of oncosuppressive p53-regulated microRNAs, the expression of which is reduced in lymphomas (Tabl. 1).

Most of the microRNA coding genes are located in CpG-rich regions. For this reason, methylation is considered to be one of the significant mechanisms of regulation of microRNA expression.

Table 1. Brief description of microRNA genes

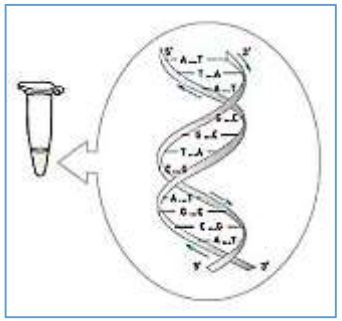
MicroRNA	Gene	Localization	Location
miR-203	***MIR-203***	**14q32.33**	**Intergenic**
miR-129	***MIR-129-2***	**11p11.2**	**Intragenic, host gene *EST***
miR-34a	***MIR-34A***	**1p36.22**	**Intragenic, host gene *EF570048***
miR-34b	***MIR-34B/C***	**11q23.1**	**Intragenic, host gene *BC021736***
miR-34c			

Research objective was to identify the frequency and specificity of p53-responsive oncosuppressive *MIR-34B/C*, *MIR-34A*, *MIR-203* and *MIR-129-2* genes methylation in Diffuse Large B-cell Lymphoma (DLBCL).

Materials and methods. The design of the study is presented below.

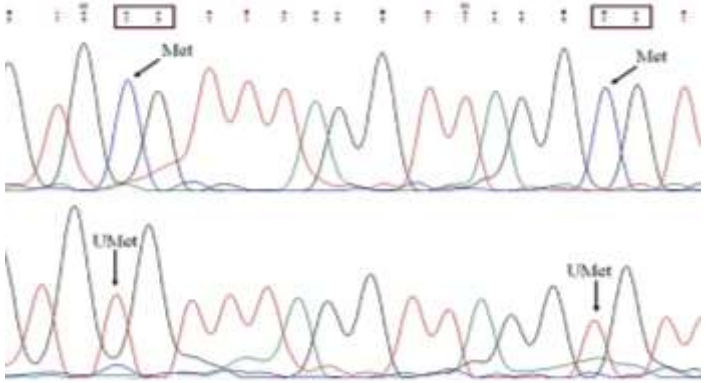
Figure 1. *Design of the study.*

FFPE tissue blocks

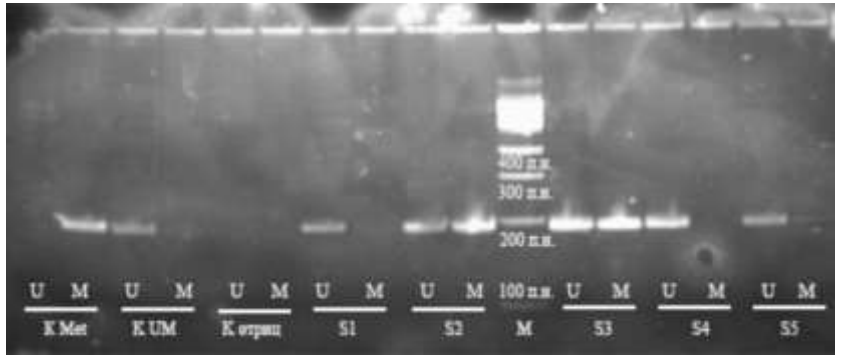


DNA isolation

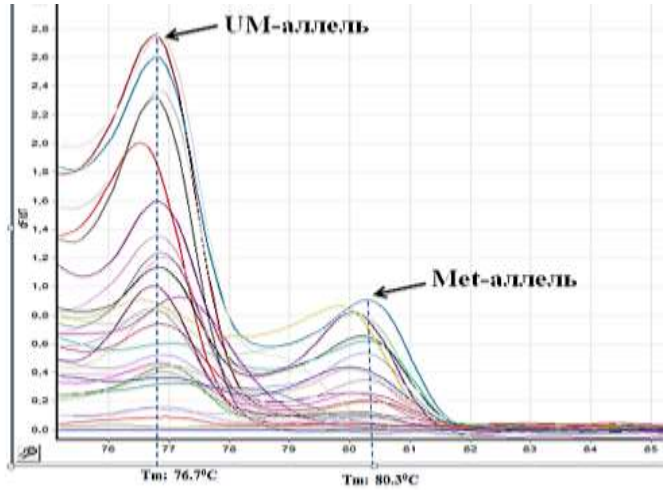
Treatment with sodium bisulfite



Bisulfite sequencing



Results of methyl-specific PCR to determine the methylation status of the *MIR-129-2* gene (electrophoresis in 5% polyacrylamide gel): M – methylated allele, UM – unmethylated allele, K Met – control methylated DNA, K UM – control unmethylated DNA, K otrits – negative control, S1-S5 – samples of patients with DLBCL, M – marker of molecular weight of 100 bp.



Results of methyl-sensitive *HRM* to determine the methylation status of the *MIR-34B/C* gene.

Diagnostic FFPE tissue blocks of 73 patients with DLBCL were taken. Sections of blocks contained at least 50% of tumor cells. Lymph node biopsies with reactive polyclonal B-cell proliferation (n=11) was used to control the tumor-specificity of the detected methylation. DNA was isolated from tissue blocks by phenol-chloroform extraction using guanidine and treated with sodium bisulfite using an EZ DNA methylation-lightning kit with manufacturer's protocols.

Determination of the methylation status of the gene in tumor tissue was carried out by methods of methyl-specific PCR (*MIR-129-2* and *MIR-203*) and methyl-sensitive analysis of high-resolution melting curves (*MIR-34A* and *MIR-34B/C*). We evaluated the combined methylation of genes in pairs using the one-sided Fisher exact criterion (p-value) and multiple testing correction with Benjamin-Hochberg procedure (q-value).

Results. The methylation of *MIR-129-2*, *MIR-203*, *MIR-34A* and *MIR34B/C* in lymphoma occurred with frequency of 67%, 66%, 27% and 62%, respectively (Fig. 1).

It was not detected in the tissue of reactive lymph nodes.

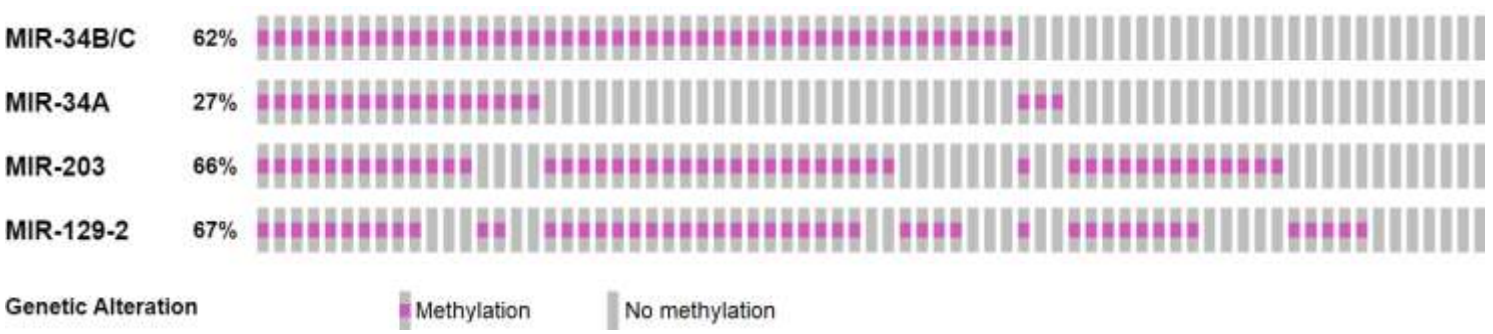


Figure 2. Co-occurrence of microRNA genes methylation.

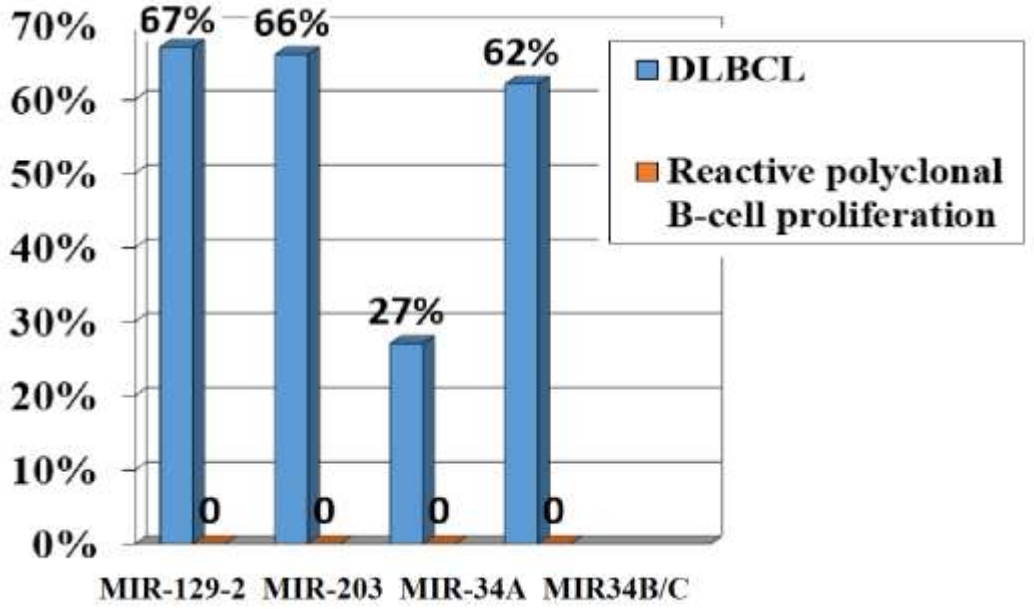


Figure 1. Frequency of the microRNA genes methylation.

Combined methylation of *MIR-203*, *MIR-129-2* and *MIR-34B/C* genes (p<0.013, q<0.020), as well as pair of *MIR-34B/C* and *MIR-34A* genes (p=0.010, q=0.029) was detected (Fig. 2, Table 2).

Table 2. The analysis tested pairs between the *MIR-34A*, *MIR-34B/C*, *MIR-203* and *MIR-129-2* genes methylation

Gene A	Gene B	p-value	q-value	Tendency
MIR-34B/C	*MIR-34A*	<0.001	<0.001	Co-occurrence
MIR-34B/C	*MIR-203*	0.013	0.020	Co-occurrence
MIR-34B/C	*MIR-129-2*	0.014	0.029	Co-occurrence
MIR-129-2	*MIR-203*	0.003	0.018	Co-occurrence

Table 3. Association of *MIR-34A* methylation with clinical and laboratory parameters

Parameter	Met (n=20)	UMet (n=53)	p-value
IPI risk group			
Low and intermediate/low	2/20 (10%)	27/53 (50.9%)	0.002
High and intermediate/high	18/20 (90%)	26/53 (49.1%)	
LDH level in blood serum			
> 450 U/l	15/20 (75%)	27/53 (50.9%)	0.064
Effectiveness of treatment			
Remission frequency	11/20 (55%)	41/53 (77.4%)	0.060
5-year OS, months	40.0	56.6	0.162

An assessment of the relationship between studied microRNA genes methylation and clinical-laboratory features of the DLBCL (Table 3) showed that 18/20 (90%) patients in the subgroup with the *MIR-34A* methylation had a high and intermediate/high risk according to International Prognostic Index against 26/53 (49.1%, p=0.002) in the subgroup of patients without gene methylation. In the subgroup with a methylated status of this gene there was a tendency (p=0.064) towards a higher frequency of detection of increased level of LDH which a marker of high paraclinical activity of the tumor.

Methylation of *MIR-34A* was associated with reduced frequency of remission (p=0.060) and the tendency to decrease of 5-year overall survival (OS) (p=0.162) (Table 3, Fig. 3).

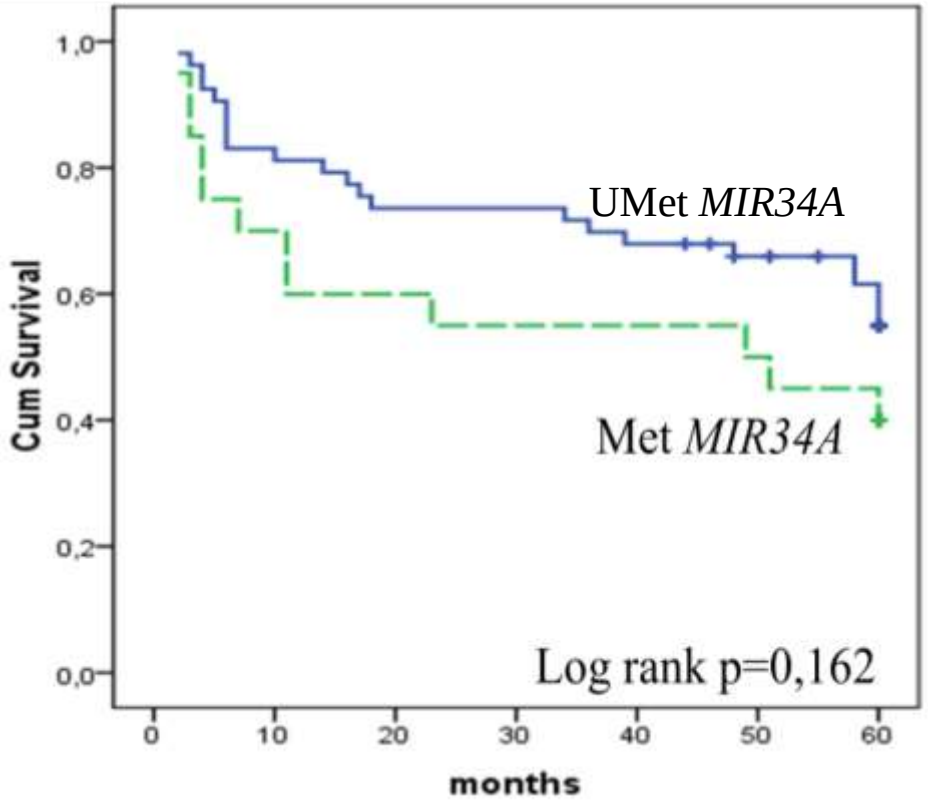


Figure 3. OS of DLBCL patients with methylated and unmethylated *MIR-34A* gene status.

The threshold value of the determined immunohistochemically high expression of Ki-67 was assumed at the level of 45%. In our study group, the association of *MIR-34B/C* and *MIR-203* methylation with high Ki-67 level in tumor tissue was revealed: p=0.026, OR=3,819 (95% CI: 1,139; 12,804) and p=0.011, OR=4,457 (95% CI: 1,372; 14,481), respectively (Table 4).

At the same time no correlation between methylation of *MIR-34B/C*, *MIR-129-2* and *MIR-203* and clinical parameters or effectiveness of therapy in the analyzed group was found.

Table 4. Association of *MIR-34B/C* and *MIR-203* methylation with Ki-67 expression level

Gene	Ki67 expression > 45% cells			p-value	OR	95% CI
MIR-34B/C	Met	25/37	67.57 %	0.026	3.819	(1.139;12.804)
	UMet	6/17	35.29 %			
MIR-203	Met	24/34	70.59 %	0.011	4.457	(1.372;14.481)
	UMet	7/20	35 %			

Conclusions. Tumor-specific methylation of gene promoters can serve as a significant mechanism for reducing the miR-34B/C, miR-34A, miR-203 and miR-129 expression in DLBCL.

In the lymphoma tissue the *MIR-34A*, *MIR-34B/C*, *MIR-129-2* and *MIR-203* methylation is of a combined nature, which may make biological sense. The fact is that these microRNAs have common target oncogenes (Fig. 4).

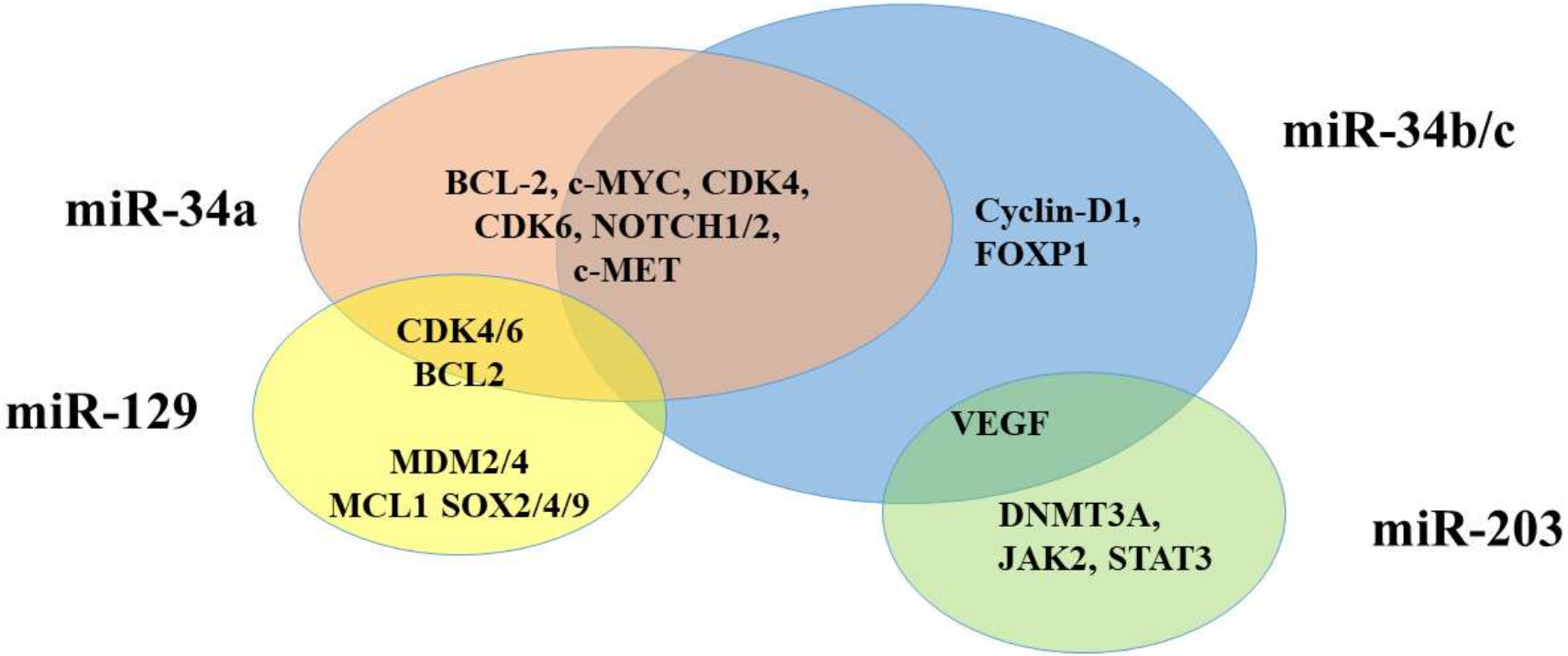


Figure 4. The common targets of miR-34a, miR-34b/c, miR-129 and miR-203.

Aberrant methylation of oncosuppressive microRNA genes associated with underlying p53 signalling pathways is a potentially useful molecular biomarker in the lymphoma diagnosis.

MIR-34A gene methylation is potentially helpful in prognosis and targeted therapy strategy development of DLBCL.

Funding: This work was supported by grant of Russian Science Foundation № 22-25-00222.