Expression of *miR-15a* and *miR-16-1* in patients with chronic lymphocytic leukemia

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Introduction. MicroRNAs (miRNAs) are small non-coding single-stranded RNA molecules that regulate gene expression at the post-transcriptional level. In the pathogenesis of chronic lymphocytic leukemia (CLL), *miR-15a* and *miR-16-1* play an important role. These miRNAs are located on chromosome 13 in the 13q14.3 region, which is deleted in more than 55% of CLL patients. This aberration affects expression of miRNAs.

Objectives. The study aimed at performing a molecular genetic analysis of *miR-15a* and *miR-16-1* expression in a group of 39 patients diagnosed with CLL and determining the association between the expression of the two miRNAs and types of deletions in the 13q14 region.

Methods. We used fluorescence in situ hybridization (FISH) for determination of mono- or biallelic deletion 13q and quantitative polymerase chain reaction (Q-RT-PCR) to revealed expression *miR-15a* and *miR-16-1* in 39 patients suffering from CLL.

Results. The analysis comprised 19 patients with monoallelic 13q14 deletion, 3 patients with biallelic deletion, 9 patients with both monoallelic and biallelic deletions, and 8 patients without 13q14 deletion serving as controls. The results showed different levels of miRNA expression in individual patients. Significantly higher normalized levels of *miR-15a* expression were found in the control group and patients with monoallelic 13q14 expression compared with patients with biallelic deletion. There was a significantly decreased expression of both miRNAs in patients with biallelic deletion of the 13q14 region but only when deletions were present in 77% or more of cells, as detected by fluorescent *in situ* hybridization (FISH).

Key words: chronic lymphocytic leukemia, microRNA, miRNA expression, fluorescence in situ hybridization, 13q14 deletion, monoallelic deletion, biallelic deletion

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of hematological malignancy diagnosed in adults in the Western hemisphere. This condition is characterized by the accumulation of mature-looking B cells with CD19+/CD20+/CD23+ immunophenotypes and aberrant expression of the CD5 marker. Currently, little is still known about the molecular mechanisms responsible for the expansion of leukemic clones and the development of CLL. Defects in both apoptosis and the proliferation of B cells are involved in the pathogenesis of CLL (ref.¹⁻³). The clinical manifestations of the disease are heterogeneous, ranging from indolent to aggressive, rapidly progressing forms. The prognosis of patients with CLL is determined by various biological parameters, such as immunoglobulin heavy chain variable region (IgVH) mutation status^{4,5}, 70 kDa zeta-associated protein (ZAP-70) expression^{6,7}, and cytogenetic changes⁸.

MicroRNAs (miRNAs) are a large group of short non-coding single-stranded RNA molecules that affect numerous significant processes in cells by acting at the

post-transcriptional level^{9,10}. The process of miRNA biogenesis begins with the transcription of long precursors that are subsequently processed, giving rise to mature functional miRNA molecules that are 18 to 25 nucleotides long. These mature miRNA molecules are incorporated into the RNA-induced silencing complex (RISC) (ref.¹¹). The mechanism of miRNA action is based on the modulation of gene expression at the post-transcriptional level by RNA interference, during which the translation of target mRNA is inhibited¹². A single miRNA may regulate the expression of tens to hundreds of genes by the direct recognition of target mRNA based on complementarity with the three-prime untranslated region (3' UTR) of approximately 7 nucleotides of this target mRNA (ref.¹³), with the complementarity not necessarily reaching 100 percent. *In silico* predictive models suggest that miRNAs are responsible for the regulation of more than 30% of the human genome¹⁴.

In 2002, Calin et al.¹⁵ discovered two miRNA genes that played a role in CLL pathogenesis, *miR-15a* and *miR-16-1*. Both are located in the 13q14.3 region between two exons of the *DLEU2* gene. In patients diagnosed with

CLL, 13q14 deletion is the most frequent chromosomal abnormality, and it is observed in more than half of cases. Although deletion of the 13q14 region is mostly monoallelic (~76% of CLL cases), it is also detected in the biallelic form (~24% of CLL cases) (ref.^{8,16}).

The target gene for *miR-15a* and *miR-16-1* is *BCL2*, as identified by Cimmino et al.¹⁷. Using heterologous cell lines, these authors confirmed that the expression of *miR-15a* and *miR-16-1* in CLL is inversely correlated with expression of the *BCL2* gene and that these miRNAs negatively regulate *BCL2* expression at the post-transcriptional level. The inactivation of *miR-15a* and *miR-16-1* expression and subsequent increased expression of the *BCL2* gene contribute to CLL pathogenesis. However, this theory was refuted by some later studies¹⁸⁻²⁰, which showed that the expansion of B cells is primarily controlled by the modulation of proliferation rather than influencing cell survival through the regulation of *BCL2* expression. Another model suggests that the *miR-16* family triggers the accumulation of cells in the G0/G1 phase through simultaneously silencing multiple genes of the cell cycle²¹.

This study aimed at determining the relative levels of *miR-15a* and *miR-16-1* expression in a group of CLL patients with deletion of the 13q14 region detected by FISH and second, at assessing potential changes in *miR-15a* and *miR-16-1* expression with respect to the type of 13q14 deletion present and other prognostically significant parameters.

PATIENTS AND METHODS

The group comprised 39 patients diagnosed with CLL in the Department of Hemato-Oncology of the University Hospital Olomouc. There were 29 men and 10 women (median age, 58.9 years; range, 34-74 years). Of these, 12 patients were examined at the time of diagnosis, 8 in the phase of stable disease and 16 during disease progression. In one patient, two biological samples were collected: one at the time of diagnosis (CLL12) and the other at the time of progression (CLL12*). Treatment was initiated in 32 patients with a median survival of 28.9 months. Median survival was assessed from overall survival, defined as the time from first treatment to the date of last follow-up examination or the date of death from any cause.

Following patients' informed consent, samples were taken as part of routine diagnostic procedures and used anonymously for research purposes with approval from the University Hospital ethics committee.

The patient cohort and clinical parameters are characterized in Table 1.

Fluorescence in situ hybridization (FISH)

All patients from the cohort were examined using a routine CLL FISH panel involving probes for detecting deletions of chromosomes 13q, 11q and 17p and trisomy of chromosome 12. Deletion of the 13q14 region was detected by FISH using the LSI D13S319 locus-specific probe (Abbott Molecular, IL, USA) of ~130 kb contain-

ing the 13q14.3 region. In each patient, at least 300 interphase nuclei were analyzed, and the results were assessed according to the ISCN 2009 (ref.²²).

Quantitative RT-PCR

RNA isolation

The starting biological material for verification of miRNA expression was RNA obtained from the peripheral blood of patients diagnosed with CLL. Mononuclear cells were isolated by separation with Histopaque®-1077 density gradient medium (Sigma Aldrich, St. Louis, USA). Total cellular RNA was isolated with TRIzol® reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

Reverse transcription (RT)

Complementary DNA (cDNA) for measuring the expression of *miR-15a* and *miR-16-1* was obtained from 20 ng of total RNA. Reverse transcription was performed according to the manufacturer's instructions using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) and specific stem-loop RT primers contained in TaqMan® MicroRNA Assays (Life Technologies).

qRT-PCR

The expression of *miR-15a* and *miR-16-1* was determined with a quantitative system based on TaqMan® probe technology (Life Technologies). The assay was performed in a total reaction volume of 20 µL and contained 7.67 µL of nuclease-free water, 10 µL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies), 1 µL of a TaqMan® MicroRNA Assays specific primer (Life Technologies), and 1.33 µL of cDNA generated by reverse transcription. The expression of *miR-15a* and *miR-16-1* was measured with a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) under the following conditions: 95 °C for 10 min, 40 cycles 95 °C for 15 s and 60 °C for 1 min, and 40 °C for 1 h. In a single experiment, each patient's sample was measured three times for the calculation of a normalized expression value (see below).

miRNA expression assay

The relative expression of *miR-15a* and *miR-16-1* was determined by the $\Delta\Delta C_t$ method²³. The reference (housekeeping) gene was small nuclear RNA (snRNA) U6 (RNU6B). As a calibrator, a mixed sample of blood collected from 10 healthy donors was used. Normalized *miR-15a* and *miR-16-1* expression values for individual patients were calculated as follows: $\Delta C_t = C_t$ (target miRNA) - C_t (RNU6B); $\Delta\Delta C_t = \Delta C_t$ (patient) - ΔC_t (calibrator); and normalized expression value = $2^{-\Delta\Delta C_t}$. The final normalized *miR-15a* and *miR-16-1* expression values for individual patients were expressed as the mean values calculated from three technical repeats (MS Excel 2007, Microsoft, Redmond, USA).

Table 1. Clinical data and parameters of the patient cohort.

Case	Gender ¹	Age at diagnosis ²	Binet stage	Survival ³	Clinical phase ⁴	Percentage of pathological clones ⁵	ZAP-70 ⁶	Mutation status of IgVH ⁷
CLL1	M	34.6	C	37.1	DG	90%	P	UM
CLL2	M	71.6	A	51.5	SD	80%	N	M
CLL3	M	73.3	B	18.6	PD	90%	P	UM
CLL4	M	50.9	B	49.1	SD	70%	P	M
CLL5	F	61.0	B	70.6	PD	45%	P	M
CLL6	M	46.3	B	25.0	PD	70%	P	UM
CLL7	M	70.1	A	54.8	SD	70%	N	UM
CLL8	M	60.8	C	29.0	DG	70%	P	UM
CLL9	M	70.3	C	28.9	PD	80%	n/a	UM
CLL10	F	41.0	A	115.7	SD	90%	N	M
CLL11	M	56.7	C	143.1	PD	90%	P	UM
CLL12	M	49.5	A	69.1	DG	60%	n/a	UM
CLL12*	M	49.5	A	69.1	PD	60%	n/a	UM
CLL13	M	57.2	A	50.8	DG	60%	P	M
CLL14	M	55.9	B	90.1	PD	90%	P	UM/M
CLL15	M	58.6	A	12.8	SD	60%	N	UM
CLL16	F	63.2	B	21.6	PD	80%	P	UM
CLL17	F	56.7	C	77.2	PD	70%	P	UM
CLL18	M	52.4	B	20.7	SD	90%	P	UM
CLL19	M	68.5	B	16.7	PD	90%	n/a	UM
CLL20	M	59.3	C	68.1	PD	n/a	n/a	UM
CLL21	M	58.3	B	26.6	PD	90%	P	UM
CLL22	F	51.9	B	201.0	SD	80%	P	M
CLL23	M	63.2	C	96.3	PD	n/a	n/a	M
CLL24	M	73.8	A	25.1	SD	40%	P	M
CLL25	M	59.9	C	79.8	PD	70%	P	UM
CLL26	F	58.9	C	89.6	PD	80%	P	UM/M
CLL27	M	74.0	A	24.6	SD	60%	P	UM
CLL28	M	60.6	A	12.0	DG	60%	N	UM
CLL29	M	46.4	B	11.1	DG	70%	N	UM
CLL30	M	43.6	B	54.6	PD	70%	N	UM
CLL31	M	49.3	A	23.6	DG	85%	P	UM
CLL32	M	65.2	C	17.0	PD	70%	N	UM
CLL33	M	58.8	C	22.1	DG	73%	P	UM
CLL34	F	71.0	A	21.7	DG	60%	N	UM
CLL35	F	69.3	C	88.2	PD	80%	n/a	M
CLL36	M	52.4	A	23.4	DG	75%	P	UM
CLL37	M	66.5	A	24.1	DG	50%	n/a	UM
CLL38	F	61.6	A	21.7	DG	80%	P	M
CLL39	F	50.0	B	24.0	DG	50%	P	UM

Footnotes: ¹gender; M = male, F = female²patients age at the time of diagnosis in years³overall survival in months⁴phase of clinical examination in which the analysis was performed; DG = time of diagnosis, SD = stable disease, PD = disease progression⁵percentage of pathological clone CD5+/CD19+/CD23+, n/a - not available⁶ZAP-70; P = positive, N = negativ, n/a - not available⁷mutation status of IgVH; M = mutated, UM = unmutated, UM/M = intermediate homology

Statistical analysis

Statistical analyses were carried out with the SPSS v. 15 software (SPSS Inc., Chicago, USA). Fisher's exact test was used to analyze categorical parameters. Quantitative parameters were analyzed using the following non-parametric methods: the Mann-Whitney U test, the Kruskal-Wallis ANOVA and Spearman's rank correlation analysis. The Shapiro-Wilk test was used to test the normal distribution of analyzed data. The statistical analyses were performed at a 5% significance level.

RESULTS

FISH

Of a total of 39 CLL patients, deletion of the 13q14 region was found in 31 patients (32 biological samples) (Table 2). In this group, 13q14 deletion as a single aberration was observed in 11 patients, with ATM deletion in 12 patients, with TP53 deletion in 5 patients, and with trisomy 12 in one patient; three patients had a complex karyotype. The group of 8 controls with CLL comprised 2 patients with a normal karyotype and positive FISH findings, two patients with ATM deletion, one patient with TP53 deletion, two patients with trisomy 12, and one patient with 6q deletion.

In 20 out of 32 (62.5%) biological samples, only monoallelic 13q14 deletion was observed. In 8 (25%) samples, both monoallelic and biallelic 13q14 deletion were observed. In the remaining 4 (12.5%) samples, only biallelic deletion was noted (Table 2). In one case, two biological samples (CLL12 and CLL12*) were collected from a single patient. In the sample collected at the time of diagnosis, monoallelic 13q14 deletion was observed in 93% of cells (Fig.1A), whereas biallelic 13q14 deletion was observed in 93% of cells in the sample taken during the disease progression (Fig.1B). The group of 8 patients

without 13q14 deletion served as controls for measuring the expression of *miR-15a* and *miR-16-1*.

qRT-PCR – CLL patients

Quantitative RT-PCR was used to determine the levels of *miR-15a* and *miR-16-1* expression in the group of 39 patients diagnosed with CLL (Table 2).

The values of miRNA expression were related to the following patient individual clinical parameters: sex, age at diagnosis, clinical stage of the disease according to Binet's system, overall survival, expansion of the pathological clone, presence of the ZAP-70 marker, and IgVH mutation status. Statistical analysis revealed a moderate negative correlation between normalized *miR-15a* expression values and patient age at diagnosis and a weak negative correlation between normalized *miR-16-1* expression values and patient age at diagnosis (data not shown). The other diagnostic parameters did not show a significant relationship with the levels of expression of either miRNA (unpublished results).

The levels of *miR-15a* and *miR-16-1* expression were different among individual patients from the cohort, despite both miRNAs being colocalized in the 13q14.3 region. The control group of 8 patients without 13q14 deletion (as revealed by FISH) showed marked variability in the expression of *miR-15a* and *miR-16-1* (Fig. 2). The normalized values of expression of both miRNAs ranged from 0.818 to 3.864. A high variability in *miR-15a* and *miR-16-1* expression was also observed in the group of patients with monoallelic 13q14 deletion (Fig. 3). Significantly higher normalized levels of *miR-15a* expression were found in the controls and patients with monoallelic 13q14 deletion compared with patients with biallelic deletion ($P = 0.045$). There was no significant relationship between normalized levels of expression of the two miRNAs and monoallelic deletion of the 13q14 region. Similarly, no significant difference in normalized levels of *miR-15a* and *miR-16-1*

Fig. 1. Example of 13q14 deletion using FISH in Patient CLL12. **A.** Monoallelic 13q14 deletion at the time of diagnosis (CLL12); probe LSI D13S319 (red) /13q34 (blue)/ CEP12 (green) (see Material and Methods). Monoallelic 13q14 deletion was observed in an interphase cell (single red signal marked by an arrow). **B.** Biallelic 13q14 deletion (red signal is missing) was observed in metaphase chromosomes in disease progression (CLL12*). Region 13q34 was translocated onto another chromosome (marked by an arrow).

Table 2. Results of qRT-PCR, FISH and conventional cytogenetic examination.

Case	<i>miR-15a</i> ¹	<i>miR-16-1</i> ²	Deletion of 13q14 ³		11q ⁴	17p ⁵	+12 ⁶	Conventional cytogenetics ⁷
			monoallelic	biallelic				
CLL1	1.165	1.347	97%		96%			UNS
CLL2	0.895	1.301	64%	28%				NK
CLL3	2.346	2.297	93%			93%		NK
CLL4	1.021	0.432	75%	6%				UNS
CLL5	0.196	0.582		96%	13%			CK
CLL6	1.932	0.742	86%		88%			UNS
CLL7	0.163	0.570		96%	25%			UNS
CLL8	1.741	0.933	81%	7%	88%			CK
CLL9	0.551	1.262	91%		94%			UNS
CLL10	1.537	2.657	91%	3%				NK
CLL11	0.790	1.042	96%		95%			NK
CLL12	1.102	1.346	93%			93%		UNS
CLL12*	0.227	0.218		93%		95%		CK
CLL13	2.868	3.837	48%					NK
CLL14	2.990	1.602	63%	35%	85%			CK
CLL15	4.056	3.630	76%		91%			CCH
CLL16	0.979	0.758	87%			97%		ND
CLL17	2.433	2.056	69%					NK
CLL18	1.580	1.729	77%	17%	47%			NK
CLL19	1.682	1.815	97%			55%		UNS
CLL20	0.117	0.297	4%	77%	60%			CCH
CLL21	1.591	5.657	88%					NK
CLL22	0.908	1.014	30%	62%				CCH
CLL23	0.559	1.197	92%					CK
CLL24	1.125	1.214	43%				43%	NK
CLL25	3.784	0.395	84%					CK
CLL26	0.129	0.395		95%				CK
CLL27	0.758	1.753	66%					NK
CLL28	1.945	3.010	82%					NK
CLL29	2.828	2.129	81%					CCH
CLL30	4.659	3.227	39%					UNS
CLL31	4.891	3.706	53%		92%			UNS
CLL32	3.249	3.095						NK
CLL33	0.818	2.603				85%		CK
CLL34	0.920	1.625			90%			CCH
CLL35	1.042	1.474					79%	CCH
CLL36	2.378	3.864			78%			UNS
CLL37	0.835	2.549					73%	CCH
CLL38	1.189	2.071						UNS
CLL39	1.165	1.705						CCH

Footnotes: ¹normalized values of *miR-15a* expression²normalized values of *miR-16-1* expression³detection of 13q14 deletion using FISH (probe LSI D13S319/13q34/CEP12)⁴detection of 11q deletion using FISH (LSI ATM probe)⁵detection of 17p deletion using FISH (LSI p53 probe)⁶detection of trisomy of chromosome 12 using FISH (CEP 12 probe)⁷the karyotype examination using conventional cytogenetics

UNS – unsuccessful

NK – normal karyotype

CCH – chromosomal changes

CK – complex karyotype

Fig. 2. Expression of *miR-15a* (yellow) and *miR-16-1* (green) in a group of patients without 13q14 deletion (control group).

Fig. 3. Expression of *miR-15a* (yellow) and *miR-16-1* (green) in a group of patients with monoallelic 13q14 deletion.

Fig. 4. Expression of *miR-15a* (yellow) and *miR-16-1* (green) in a group of patients with biallelic 13q14 deletion.

Fig. 5. Change of *miR-15a* expression in Patient CLL12. Amplification curves and C_p values at the time of diagnosis (green) and in disease progression (red). The bar chart shows *miR-15a* transcript content and its dependence on the type of 13q14 deletion at the time of diagnosis (green) and in disease progression.

Fig. 6. Change of *miR-16-1* expression in Patient CLL12. Amplification curves and C_p values at the time of diagnosis (green) and in disease progression (red). The bar chart shows *miR-16-1* transcript content and its dependence on the type of 13q14 deletion at the time of diagnosis (green) and in disease progression.

I expression was found between patients without 13q14 deletion and those with monoallelic 13q14 deletion.

In patients with biallelic 13q14 deletion, there was a significant decrease in *miR-15a* and *miR-16-1* expression in individuals in whom FISH revealed biallelic deletion in at least 77% of cells (Fig. 4). There was a weak negative correlation between normalized levels of *miR-16-1* expression and biallelic deletion of the 13q14 region (unpublished results).

qRT-PCR – Patient CLL12

In Patient CLL12 (male, aged 50 years at the time of diagnosis), the levels of *miR-15a* and *miR-16-1* expression were determined in two biological samples taken at the

time of diagnosis and during progression of the disease. At the time of diagnosis, FISH revealed monoallelic 13q14 deletion in 93% of the patient's cells (CLL12). Over 67 months, the patient's disease progressed, and FISH confirmed the presence of biallelic 13q14 deletion in 93% of cells (CLL12*). Using qRT-PCR, the levels of *miR-15a* and *miR-16-1* expression were determined.

In the sample obtained at diagnosis (93% of cells with monoallelic 13q14 deletion), the normalized values of *miR-15a* and *miR-16-1* expression were 1.102 and 1.346, respectively. In the progression sample (93% of cells with biallelic 13q14 deletion), the expression of both miRNAs was significantly decreased. For *miR-15a*, the level of expression was 4.85-fold lower compared with

the sample taken at diagnosis (normalized value of *miR-15a* expression = 0.227) (Fig. 5). For *miR-16-1*, 6.17-fold lower expression was observed compared with the sample obtained at diagnosis (normalized value of *miR-16-1* expression = 0.218) (Fig. 6).

DISCUSSION

The deletion of chromosome 13q14 is the most frequent chromosomal aberration found in patients with CLL. FISH is a routine method for its identification, which is of prognostic significance. CLL patients with a normal karyotype or with 13q14 deletion as the only genetic abnormality have a better prognosis than CLL patients with complex karyotypes or deletions of the 11q23 or 17p13 regions^{8,24-26}. Isolated deletion of the 13q14 region is associated with a lower level of expression of ZAP-70 and the IgVH gene mutation status²⁴. Although 13q14 deletion is considered to be associated with a favorable prognosis, some studies have shown that this is not true for some patients. It has been confirmed that heterogeneity of the 13q14 deleted region and the presence of biallelic deletion may significantly contribute to the course of the disease¹⁹. In our cohort, deletion of this region was detected in 31 patients (32 biological samples). One patient (CLL12) was investigated both at the time of diagnosis and during disease progression. Monoallelic 13q14 deletion was found in 62.5% of 32 biological samples and with biallelic 13q14 deletion in 25% of cases. In 12.5% of 32 biological samples, biallelic 13q14 deletion was found. As 13q14 deletion is not present in all CLL patients, it is obvious that this abnormality is not primarily responsible for the development of the condition; however, its significance as a prognostic factor has been confirmed^{8,24}. According to Oscier et al.²⁵, deletion of the 13q14 region is associated with the IgVH gene mutation status. In our group, however, there was no significant association between IgVH gene mutation status and 13q14 deletion. Deletion of the 13q14 region was not the only genetic abnormality. In most patients, deletion was observed with other changes (11q-, 17p-, +12 and others). However, the identified aberrations were not directly associated with the presence, type or frequency of 13q14 deletion.

This study aimed at determining the levels of expression of *miR-15a* and *miR-16-1* in a group of CLL patients divided into three subgroups: patients without deletion, patients with monoallelic deletion and patients with biallelic deletion of the 13q14 region. The expression of miRNAs was normalized to healthy controls. The levels of *miR-15a* and *miR-16-1* expression were different in individual patients, despite both miRNAs being colocalized in the 13q14.3 region. This result is suggestive of the different regulation of expression of both miRNAs and is likely to be indicative of their functional independence.

Variable expression was also observed in both patients without 13q14 deletion and the group of patients with monoallelic 13q14 expression. No significant decrease in expression was observed in either of the studied miRNAs

depending on the percentage of cells with monoallelic 13q14 deletion. As recently reported, the decreased expression of *miR-15a* and *miR-16-1* in cases without 13q14 deletion may also result from epigenetic changes. Sampath et al.²⁷ found that the genes for *miR-15a* and *miR-16-1* are silenced through histone deacetylases that are highly expressed in CLL. Moreover, the extent of potential deletion of the 13q14 region, where *miR-15a* and *miR-16-1* are located, can be variable¹⁹, and this may affect the heterogeneous profile of expression of both miRNAs. It was also observed that the variability in expression may be due to the presence of homologous gene loci *miR-15b/miR-16-2* in the 3q25-26.1 region and *miR-497/miR-195* in the 17q21 region. Given their high sequence homology, *miR-15a* and *miR-16-1* may not be distinguishable from redundant miRNAs using qRT-PCR primers. This may significantly influence the results of analyses²⁸⁻³⁰. It is also likely that if there is at least one functional allele in a cell, this allele will substitute for the other non-functional deleted allele by increasing its expression. Thus, no significant decrease in the levels of *miR-15a* and *miR-16-1* would be observed.

In the group of patients with biallelic 13q14 deletion, significant variability in the measured levels of *miR-15a* and *miR-16-1* expression was noted, with the exception of patients with biallelic deletion in 77% or more of the cells. These patients were found to have a significantly lower expression of both miRNAs. The relationship between levels of *miR-15a* and *miR-16-1* expression and other clinical parameters studied in the whole group showed no statistical significance (unpublished results), which may be due to small sample size.

Investigations revealing the significance of miRNAs in tumorigenesis involved molecular biology studies of patients with CLL (ref.^{15,31,32}). An analysis of an approximately 30-kb minimal deleted region (MDR) in 13q14 led to the discovery of the *miR-15a* and *miR-16-1* genes and the finding that these genes are deleted or down-regulated in ~ 68% of CLL cases¹⁵. In another study, a total of 13 miRNAs were selected, and their expression profiles enabled the stratification of CLL patients into prognostic groups. These miRNAs included *miR-15a* and *miR-16-1* (ref.³³). It is generally assumed that approximately 50% of genes encoding miRNAs are located in regions that are frequently susceptible to breaks, deletions and mutations during tumorigenesis^{11,31}. These regions include both oncogenes and tumor suppressors³⁴, which thus play an important role in the initiation and progression of human tumors.

Earlier studies reported decreased expression of *miR-15a* and *miR-16-1* in most CLL cases^{15,17,31-33}. More recent studies, however, have not confirmed any a correlation between the levels of expression of these two miRNAs and the presence of 13q14 deletion^{19,35}. Our study showed a significant decrease in expression of both miRNAs in a small group of patients (12.8% of the entire cohort). These data correspond to the results of Fulci et al.¹⁸, who showed a markedly decreased expression of *miR-15a* and *miR-16-1* in patients diagnosed with CLL. Similar to our study, they observed biallelic 13q14 deletion in a high proportion of CLL cells (63% - 85%) of these patients.

As mentioned above, the 13q14 region is deleted in patients⁸. The location of *miR-15a* and *miR-16-1* in this region suggests that the role of these miRNAs in the pathogenesis of CLL is not negligible¹⁵. Based on our own research, we assume that both miRNAs act as tumor suppressors, as confirmed by Patient CLL12 whose disease progression was associated with newly present biallelic 13q14 deletion and a related significant decrease in *miR-15a* and *miR-16-1* expression.

At present, studies of the expression of selected miRNAs are a useful tool for determining the prognosis, prediction of treatment response and development of the disease in patients with lymphoproliferative disease³⁶. Determining the levels of miRNA expression is beneficial for not only distinguishing healthy and malignant tissues but also the classification of tumor types and degree of disease progression. The deregulation of miRNA expression is likely to be one of the critical points in the pathogenesis of lymphoproliferative diseases. However, molecular mechanisms of regulation and functions of this group of RNA must be carefully analyzed. More detailed studies aimed at understanding the roles of selected miRNAs in the development and progression of diseases and the identification of new miRNA genes could lead to more accurate diagnoses, prognoses and therapeutic applications in the treatment of lymphoproliferative diseases.

CONCLUSION

This study aimed at determining the relative levels of *miR-15a* and *miR-16-1* expression in a group of CLL patients with deletion of the 13q14 region detected by FISH and second, at assessing potential changes in *miR-15a* and *miR-16-1* expression with respect to the type of 13q14 deletion present. The results showed different levels of miRNA expression in individual patients. Significantly higher normalized levels of *miR-15a* expression were found in the control group and patients with monoallelic 13q14 expression compared with patients with biallelic deletion. The significantly decreased expression of both miRNAs was detected in patients with biallelic deletion of the 13q14 region but only when deletions were present in 77% or more of cells, as detected by fluorescent *in situ* hybridization (FISH).

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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