

Brief report

MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia

Marek Mraz,^{1,2} Dasa Dolezalova,³ Karla Plevova,^{1,2} Katerina Stano Kozubik,^{1,2} Veronika Mayerova,^{1,2} Katerina Cerna,^{1,2} Katerina Musilova,^{1,2} Boris Tichy,^{1,2} Sarka Pavlova,^{1,2} Marek Borsky,² Jan Verner,^{1,2} Michael Doubek,^{1,2} Yvona Brychtova,² Martin Trbusek,^{1,2} Ales Hampel,³ Jiri Mayer,^{1,2} and Sarka Pospisilova^{1,2}

¹Center of Molecular Medicine, Central European Institute of Technology, Masaryk University, Brno, Czech Republic; ²Center of Molecular Biology and Gene Therapy, Department of Internal Medicine—Hematology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic; and ³Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

MicroRNAs (miRNAs) play a key role in chronic lymphocytic leukemia as well as in normal B cells. Notably, miRNA gene encoding miR-650 and its homologs overlap with several variable (V) subgenes coding for lambda immunoglobulin (IgL). Recent studies describe the role of miR-650 in solid tumors, but its role in chronic lymphocytic leukemia (CLL) has not yet

been studied. Our experiments demonstrate that miR-650 expression is regulated by coupled expression with its host gene for IgL. This coupling provides a unique yet unobserved mechanism for microRNA gene regulation. We determine that higher expression of miR-650 is associated with a favorable CLL prognosis and influences the proliferation capacity

of B cells. We also establish that in B cells, miR-650 targets proteins important in cell proliferation and survival: cyclin dependent kinase 1 (CDK1), inhibitor of growth 4 (ING4), and early B-cell factor 3 (EBF3). This study underscores the importance of miR-650 in CLL biology and normal B-cell physiology. (*Blood*. 2012;119(9): 2110-2113)

Introduction

Chronic lymphocytic leukemia (CLL) originates from antigen experienced B-cells, and its biology is largely influenced by antigen stimulation, immunoglobulin structure, and BCR-signaling.¹ Interestingly, these processes were recently shown to involve regulation by microRNAs (miRNAs).²⁻⁴ Typical feature of such miRNA-mediated control is a cell type-specific regulation of target protein(s) over a mostly modest range by inhibiting mRNA translation and/or stability.⁴ Importantly, our group and others have previously described the role of miRNAs in CLL pathogenesis, prognosis, and progression.⁵⁻⁹ Considering the significant functions of immunoglobulin genes (and microRNAs) in normal and malignant B cells, it is notable that the human locus for immunoglobulin genes encodes a miRNA. The miR-650 gene and its homologs are localized in the leader exon (exon 1) of λ light chain (IgL) variable subgenes of the V2 family (see Figure 1A). This is the only microRNA gene known to overlap with immunoglobulin genes.

Evolutionary analysis indicated that miR-650 genes are present in multiple copies and overlap in the same transcription orientation with the leader exon of primate IgL genes.¹⁰ This miRNA was initially identified by a miRAGE cloning approach in colorectal cells,¹¹ and it seemed to be transcribed independently of the immunoglobulin host gene.^{10,12-14} Remarkably, recent studies describe the role of miR-650 in the biology of colorectal cancer, breast cancer, gastric cancer, and melanoma.¹²⁻¹⁵ Here we have determined that miR-650 expression is associated with CLL prognosis, influences B-cell proliferation, and we identify its targets. Moreover, we show that miR-650 expression is regulated by coupled expression with its host gene for IgL.

Methods

Samples of peripheral blood from CLL patients (n = 80) were separated by RosetteSep B Cell Enrichment Cocktail (purity \geq 95% of CD5 + 19+ cells; StemCell Technologies). The expression of immunoglobulin light chain on B cells was determined by flow cytometry (mouse Anti-IgL/IgL κ ; Invitrogen). The used immunoglobulin light chain (kappa vs lambda) and the exact IgL variable (V) segment were determined from cDNA using BIOMED-2 protocol.¹⁶ Total RNA was isolated by TRI-Reagent (Sigma-Aldrich),¹⁷ and miR-650 expression was analyzed by commercially available TaqMan Assay (Applied Biosystems). The study was approved by the institutional review board of University Hospital Brno.

B-cell line NALM-6 was cultured under standard conditions (5% CO₂, 37°C) in RPMI-1640 with 10% FBS. To study miR-650 target genes, NALM-6 cells were electroporated (Neon Transfection System, Invitrogen; buffer R, Program 2) with a short artificial miR-650 (miRIDIAN microRNA Mimic, 100nM; Dharmacon) or a control short RNA (miRIDIAN microRNA Mimic Negative Control 1, 100nM). This cell line was used for transfection experiments, as it expresses miR-650 very weakly (amplification curve close to background as determined by TaqMan Assay). NALM-6 cells can be efficiently transfected (efficiency \sim 75%; see supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) when compared with CLL cells, and with a small effect on cell viability (viability 85%). Cells were harvested 48 hours and 72 hours after transfection, lysed in lysis buffer, and proteins were immunodetected using primary antibody and HRP-conjugated secondary antibody. The following primary antibodies were used: EBF3, ING4 (Sigma-Aldrich), CDK1 (Neomarkers), and α -tubulin (Exbio; Vestec).

Statistical analyses were done by GraphPad Prism Version 5.0 software, and MedCalc Version 12.1.1 software for multivariate analysis by Cox proportional hazards model. *P* values < .05 were considered significant.

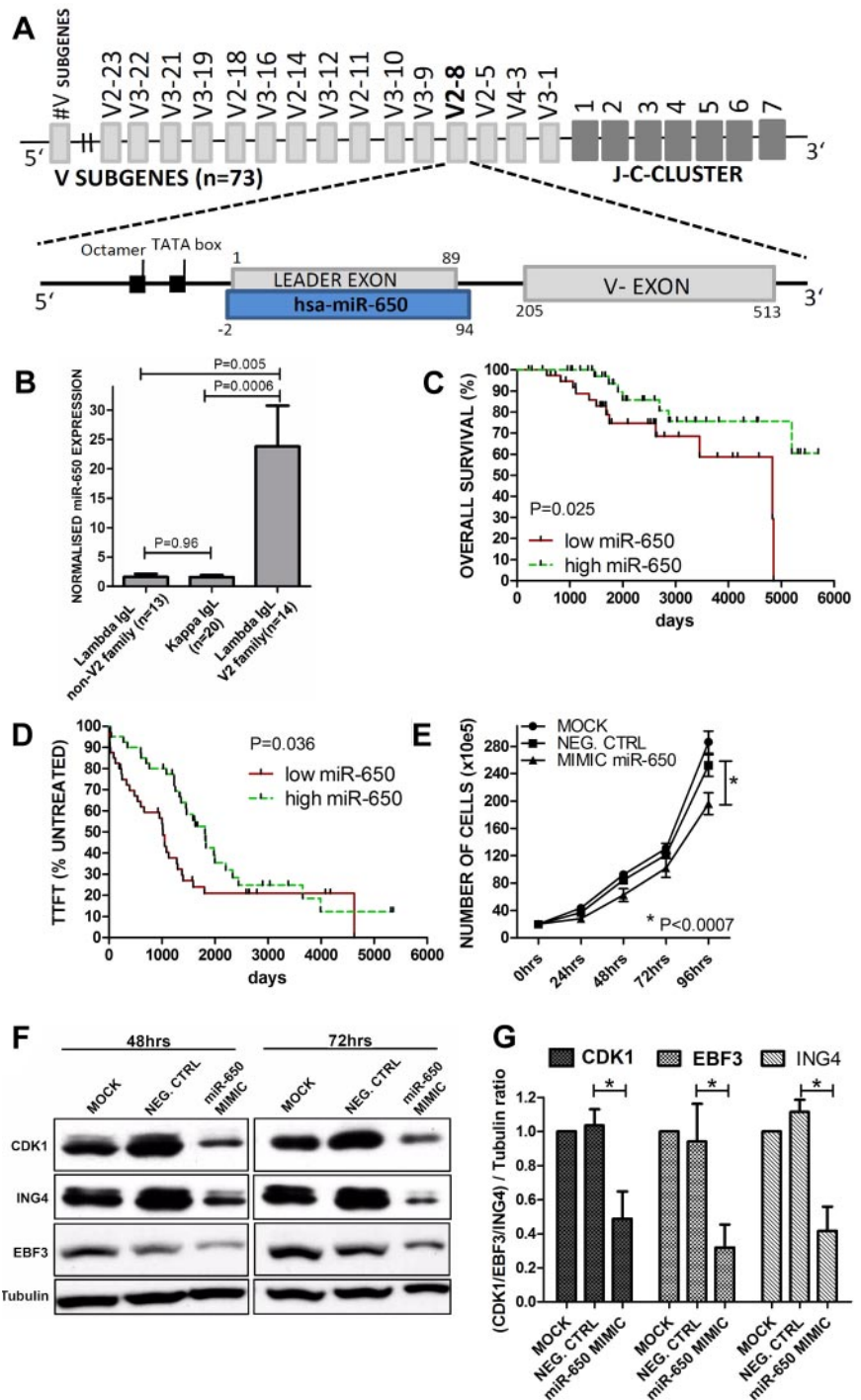
Submitted November 25, 2011; accepted December 28, 2011. Prepublished online as *Blood* First Edition paper, January 10, 2012; DOI 10.1182/blood-2011-11-394874.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2012 by The American Society of Hematology

Figure 1. The regulation, targets, and prognostic significance of miR-650. (A) Genomic organization of the IgL λ locus (chr. 22q11) and the relation of IGVL gene and J-C cluster to miR-650 (modified from Das¹⁰). All members of family 2 of variable (V) subgenes (V2-8, V2-14, V2-28, V2-11, V2-5, V-34, V2-33, V2-18, V2-23) for λ light chain include homologs of miR-650 (here shown in detail for V2-8). The miR-650 overlaps with the first exon of IGVL. The position of hsa-miR-650 gene (MI0003665), leader exon of IGVL, V-exon, J-C cluster, TATA box, and octamer are indicated. (B) The relation of IgL usage and the expression of miR-650. The expression of miR-650 was analyzed (TaqMan assay for mature miR-650) in CLL samples using V2 subgenes for IgL λ (n = 14: V2-14 [8 \times], V2-8 [2 \times], V2-23 [2 \times], V2-5 [1 \times], V2-18 [1 \times]) and compared with CLL samples using different IgL λ V family (n = 13: V3-21 [5 \times], V1-51 [2 \times], V1-44 [2 \times], V3-27 [1 \times], V3-19 [1 \times], V5-45 [1 \times], V1-96 [1 \times]) or expressing κ IgL (n = 20). The IgL usage (kappa vs lambda) determined by sequencing (BIOMED-2 protocol) was verified by flow cytometry and in all cases, but one bclonal case, the surface IgL expression corresponded to the PCR result. Statistical differences were tested by 2-tailed *t* test. Error bars represent SEM. (C-D) Survival analysis and the time to first treatment (TTFT) analysis in 80 CLL patients. Patients were divided in 2 groups (low miR-650, n = 40; high miR-650, n = 40) based on the median of miR-650 expression (median expression was used as a cut-off). Cohort characteristics are included in Table 1. Survival and TTFT analysis were done using the Kaplan-Meier survival estimator. Median survival, median TTFT, and differences between the curves were evaluated by the Log-rank test. (E) The effect of miR-650 on cell proliferation. NALM-6 cells were electroporated with a short artificial miR-650 (MIMIC miR-650) or a control short RNA (NEG. CTRL) and plated in fresh cultivation media. Cell numbers were calculated 24 hours, 48 hours, 72 hours, and 96 hours after transfection by direct cell count (Countess Automatic Cell Counter; Invitrogen). Five independent experiments were performed and statistically analyzed by 2-way ANOVA. NALM-6 cells were transfected with the efficiency of ~75% (supplemental Figure 1). MOCK stands for cells treated only with the electroporation pulsed. Error bars represent SEM. (F) The identification of miR-650 targets. B-cell line NALM-6 was electroporated with a short RNA mimicking miR-650 (miR-650 MIMIC), or control short RNA (NEG. CTRL) and harvested 48 hours and 72 hours after transfection. NALM-6 cells were transfected with the efficiency of ~75% (supplemental Figure 1). For Western blot analysis mouse-monoclonal antibodies against EBF3, ING4, CDK1 and α -tubulin (as a loading control) were used. A representative example from at least 3 transfection experiments is shown. MOCK stands for cells treated only with the electroporation pulsed. (G) The quantification of EBF3, CDK1, and ING4 protein levels after transfection with RNA mimicking miR-650 (harvested 72 hours after transfection). Transfection experiments were performed as at least 3 independent experiments (as described in panel F). Blot images were quantified with ImageJ 1.42q (National Institutes of Health), and the quantity of proteins is visualized as Target protein/Tubulin ratio and normalized to MOCK. miR-650 MIMIC (short RNA mimicking miR-650), NEG. CTRL (control short RNA), MOCK (cells treated only with the electroporation pulsed). **P* < .05 (2-tailed *t* test). Error bars represent SEM.



Results and discussion

The overlap of miR-650 gene with IgL λ led us to hypothesize that miR-650 can be activated by the expression of its host gene for IgL (after a productive IgL rearrangement using IgL λ V2). We analyzed the miR-650 expression and the exact variable segment used by CLL cells in 47 patients. Our analysis revealed that cells using subgenes from V2 family for IgL λ (n = 14) had ~14-fold higher expression of miR-650 (*P* < .005) when compared with samples using different λ V family (n = 13) or κ IgL (n = 20; Figure 1B).

Thus, the monoclonal nature of CLL enabled us to demonstrate that miR-650 expression is likely naturally activated by the promoter for IgL λ and is coupled with the expression of its V2 family members. This observation is surprising because previously published data suggested that miR-650 is expressed independently of immunoglobulin genes.¹²⁻¹⁵

We subsequently performed a bioinformatical analysis of the upstream region (500 nt) of the miR-650 and IgL λ leader exon by search for Polymerase II promoter sequence (PROSCAN¹⁸) and transcription elements (TESS System¹⁹). This confirmed that the IgL λ and miR-650 gene likely use the same major promoter region

Table 1. Cohort characteristics

	Low miR-650*	High miR-650*	P
Number	n = 40	n = 40	
Median survival, mo	161.2	Not reached	.025†
Median time to treatment, mo	33.9	60.4	.036†
Median age, y (range)	67.5 (46-85)	69 (40-85)	.557‡
F/M sex ratio	25/15	23/17	.857§
Disease stage at the time of sample analysis			
Low/intermediated stage of the disease (Rai 0, I, II), n (%)	23 (57.5)	32 (80.0)	.384§
Advanced stage of the disease (Rai III, IV), n (%)	14 (34.0)	6 (15.0)	.136§
Not determined, n (%)	3 (7.5)	2 (5.0)	
Treatment prior to sample analysis			
Previously treated, n (%)	21 (52.5)	16 (40.0)	.554§
Previously untreated, n (%)	16 (40.0)	24 (60.0)	.336§
Not determined, n (%)	3 (7.5)	0 (0.0)	
IgVH status 			
Unmutated IgVH, n (%)	28 (70.0)	19 (47.5)	.358§
Mutated IgVH, n (%)	11 (27.5)	20 (50.0)	.205§
Biclonal sample, n (%)	1 (2.5)	1 (2.5)	1.00§
Hierarchical cytogenetics (I-FISH) 			
Deletion 17p13, n (%)	7 (17.5)	4 (10.0)	.525§
Deletion 11q23, n (%)	8 (20.0)	5 (12.5)	.554§
Trisomy 12, n (%)	1 (2.5)	2 (5.0)	1.00§
Deletion 13q14, n (%)	13 (32.5)	17 (42.5)	.67§
Normal, n (%)	10 (25.0)	12 (30.0)	.811§
Not determined, n (%)	1 (2.5)	0 (0.0)	

*Median expression of miR-650 was used as a cut-off to divide patients in 2 groups.

†Log-rank test.

‡t test (2-tailed).

§Fisher exact test (2-tailed).

||IgVH mutation status analyses, FISH, and hierarchical cytogenetics were performed as described previously.²⁵

(immunoglobulin promoter) for their transcription (data not shown). However, expression of miR-650 is clearly not strictly dependent on IgLλ promoter, since it is expressed in all CLL cells and also in other cell types.¹²⁻¹⁵ This suggests that IgLλ promoter rather serves as a potent enhancer element for miR-650. It is known that, similarly to miR-650, a fraction of microRNAs is located in exons of protein-coding genes,²⁰ but the data about the regulation of their expression is limited. Our observation provides a unique yet unobserved mechanism for the coupled expression of the immunoglobulin gene and a microRNA gene.

Recently, the expression of miR-650 was associated with the aggressiveness of colorectal cancer cells, breast cancer, gastric cancer cells, and melanoma in vitro and in vivo.¹²⁻¹⁵ This prompted us to study the potential role of miR-650 for CLL and B cells. Initially we tested the relation of miR-650 levels to CLL prognosis, which at least partially reflects the biology of CLL B cells. This revealed that the expression of miR-650 is associated with prognosis in the original cohort (n = 47; data not shown) as well as in an extended cohort of 80 CLL patients (Figure 1C-D). Patients with higher expression of miR-650 had significantly ($P < .05$) longer overall survival (not-reached vs 161 months, hazard ratio [HR] 0.34, 95% confidence interval [CI]: 0.13-0.87) and time to first treatment (60 vs 34 months, HR 0.57, 95% CI: 0.34-0.96) in a univariate Cox proportional hazard analysis (Figure 1C-D). These 2 groups of patients did not differ in any of the other cohort characteristics (Table 1). The distribution of miR-650 expression in the cohort is shown in supplemental Figure 2. The multivariate analysis by Cox proportional hazards model (including IgVH status, deletion 17p, sex, age, and miR-650) demonstrated that higher expression of miR-650 is a significant ($P < .05$) independent predictor of survival (HR: 0.36, 95% CI 0.13-0.97) and time to

first treatment (HR 0.37, 95% CI: 0.2-0.67). This reveals that miR-650 likely belongs to the several microRNAs (like miR-34a, miR-155, miR-29c, miR-223, and miR-21)^{7,9,21,22} that are related to CLL prognosis and mainly indicates its potential biologic importance in CLL and B cells.

The favorable disease course for CLL patients with higher expression of miR-650 corresponds with the results demonstrating that miR-650 inhibits cell cycle progression by regulating p16INK4-mediated pathway.¹³ This is also in concordance with our observation that transfection with miR-650 results in a reduction in the proliferative capacity of B cells (Figure 1E).

Altogether, ~ 50% of predicted targets of miR-650 (TargetScan, www.targetscan.org) are expressed in B cells with numerous being important in the B-cell physiology (Gene Ontology and Functional analysis by DAVID tool²³). To validate and identify miR-650 targets specifically in B cells, we transfected the B-cell line with an artificial miR-650. The levels of 2 already identified targets (CDK1, ING4) and a predicted target (EBF3) were assessed after transfection. It was shown that miR-650 regulates the cyclin dependent kinase 1 (CDK1) in breast cancer cells¹³ and inhibitor of growth 4 (ING4) in gastric cancer cells.¹⁴ The EBF3 protein was selected as a putative target with the highest score as predicted by TargetScan. Significantly, EBF3 is a member of the "Early B-cell Factor" family, which is recognized as an important transcription regulator of survival and development of B-lineage cells.²⁴ The transfection of B cells with miR-650 resulted in down-regulation ($P < .05$) of protein levels of CDK1, ING4, and EBF3 by 53%, 64%, and 67%, respectively (Figure 1F-G). This confirms the relevance of CDK1 and ING4 as miR-650 targets in B cells and identifies EBF3 as a miR-650-regulated gene.

In summary, we have identified a novel mechanism for the coupled expression of the immunoglobulin gene and miR-650. Moreover, we established that miR-650 has an active role in the regulation of protein levels and proliferation in B cells. This study also points to the necessity for further elucidation of miR-650 involvement in normal B-cell physiology and development.

Acknowledgments

This work is supported by Internal Grant Agency of Ministry of Health Czech Republic (IGA MZCR) NT11218-6/2010, CZ.1.05/1.1.00/02.0068, IGA MZCR NS10439-3/2009, and Ministry of Education, Youth and Sports (MSMT) MSM0021622430.

References

- Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2011;118(16):4313-4320.
- Vigorito E, Perks KL, Abreu-Goodger C, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity*. 2007;27(6):847-859.
- Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132(5):860-874.
- Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell*. 2009;136(1):26-36.
- Calin GA, Croce CM. Chronic lymphocytic leukemia: interplay between noncoding RNAs and protein-coding genes. *Blood*. 2009;114(23):4761-4770.
- Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353(17):1793-1801.
- Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23(6):1159-1163.
- Mraz M, Pospisilova S, Malinova K, Slapak I, Mayer J. MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk Lymphoma*. 2009;50(3):506-509.
- Vargova K, Curik N, Burda P, et al. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. *Blood*. 2011;117(14):3816-3825.
- Das S. Evolutionary origin and genomic organization of micro-RNA genes in immunoglobulin lambda variable region gene family. *Mol Biol Evol*. 2009;26(5):1179-1189.
- Cummins JM, He Y, Leary RJ, et al. The colorectal microRNAome. *Proc Natl Acad Sci U S A*. 2006;103(10):3687-3692.
- Feng L, Xie Y, Zhang H, Wu Y. Down-regulation of NDRG2 gene expression in human colorectal cancer involves promoter methylation and microRNA-650. *Biochem Biophys Res Commun*. 2011;406(4):534-538.
- Chien WW, Domenech C, Catallo R, et al. Cyclin-dependent kinase 1 expression is inhibited by p16(INK4a) at the post-transcriptional level through the microRNA pathway. *Oncogene*. 2011;30(16):1880-1891.
- Zhang X, Zhu W, Zhang J, et al. MicroRNA-650 targets ING4 to promote gastric cancer tumorigenicity. *Biochem Biophys Res Commun*. 2011;395(2):275-280.
- Chan E, Patel R, Nallur S, et al. MicroRNA signatures differentiate melanoma subtypes. *Cell Cycle*. 2011;10(11):1845-1852.
- van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257-2317.
- Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun*. 2009;390(1):1-4.
- Prestridge DS. Predicting Pol II promoter sequences using transcription factor binding sites. *J Mol Biol*. 1995;249(5):923-932.
- Schug J. Using TESS to predict transcription factor binding sites in DNA sequence. *Curr Protoc Bioinformatics*. 2008;Chapter 2:Unit 2.6.
- Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. *Genome Res*. 2004;14(10A):1902-1910.
- Stamatopoulos B, Meuleman N, Haibe-Kains B, et al. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. *Blood*. 2009;113(21):5237-5245.
- Rossi S, Shimizu M, Barbarotto E, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood*. 2010;116(6):945-952.
- Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44-57.
- Hagman J, Lukin K. Early B-cell factor 'pioneers' the way for B-cell development. *Trends Immunol*. 2005;26(9):455-461.
- Trbusek M, Smardova J, Malcikova J, et al. Missense mutations located in structural p53 DNA-binding motifs are associated with extremely poor survival in chronic lymphocytic leukemia. *J Clin Oncol*. 2011;29(19):2703-2708.

Authorship

Contribution: M.M., D.D., and K.P. designed the experiments; M.M. and S. Pospisilova wrote the paper; and all authors performed the experiments.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Sarka Pospisilova, PhD, or Marek Mraz, MD, Center of Molecular Biology and Gene Therapy, University Hospital Brno, Cernoplni 9, 625 00, Brno, Czech Republic; e-mail: sarka.pospisilova@fnbrno.cz or marek.mraz@email.cz.