Brief report

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

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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 (IgLλ). 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 IgLλ. 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 (IgLλ) 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 IgLλ 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 IgLλ.

Methods

Samples of peripheral blood from CLL patients (n = 80) were separated by RosetteSep B Cell Enrichment Cocktail (purity ≥ 95% of CD5 + 19+ cells; StemCell Technologies). The expression of immunoglobulin light chain on B cells was determined by flow cytometry (mouse Anti-IgLλ/IgLκ; 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% CO_2, 37^0C) 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 ~ 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.

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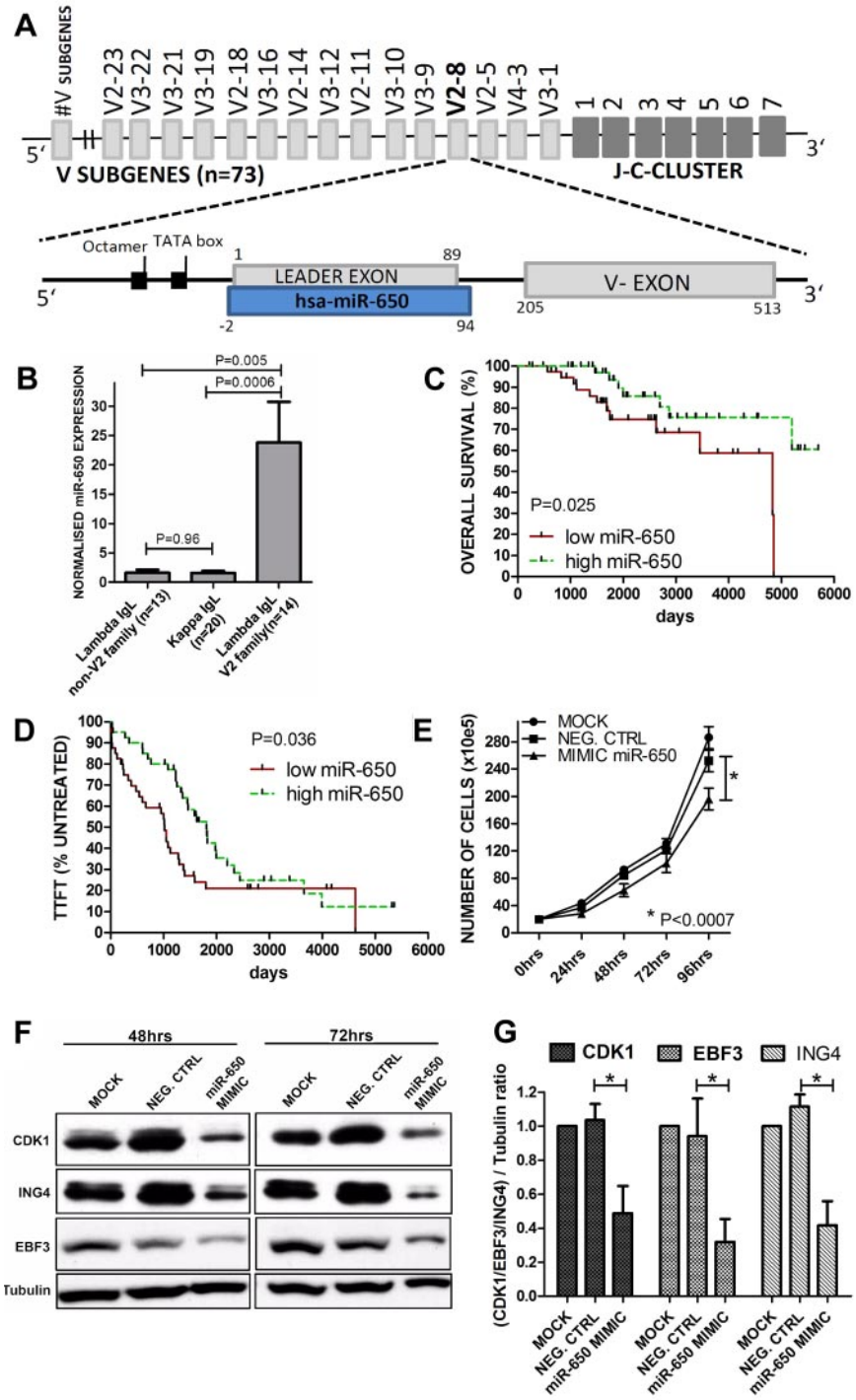

Figure 1. The regulation, targets, and prognostic significance of miR-650. (A) Genomic organization of the IgLλ locus (chr. 22q11) and the relation of IGVL gene and J-C cluster to miR-650 (modified from Das[10]). All members of family 2 of variable (V) subgenes (V2-8, V2-14, V2-28, V2-11, V2-5, V-34, 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 IgLλ (n = 14: V2-14 [8×], V2-8 [2×], V2-23 [2×], V2-5 [1×], V2-18 [1×]) and compared with CLL samples using different IgLλ V family (n = 13: V3-21 [5×], V1-51 [2×], V1-44 [2×], V3-27 [1×], V3-19 [1×], V5-45 [1×], V1-96 [1×]) 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 biclonal 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 puls. 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 puls. (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 puls). *$P < .05$ (2-tailed *t* test). Error bars represent SEM.

Results and discussion

The overlap of miR-650 gene with IgLλ 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 IgLλ 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 IgLλ (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 IgLλ 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 IgLλ leader exon by search for Polymerase II promoter sequence (PROSCAN[18]) and transcription elements (TESS System[19]). This confirmed that the IgLλ 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 tool[23]). 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 cells[13] 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.

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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.

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