miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia

Christian Philipp Pallasch¹, Michaela Patz¹, Yoon Jung Park², Susanne Hagist¹
Daniela Eggle¹, Rainer Claus², Svenja Debey-Pascher³, Alexandra Schulz¹, Lukas P. Frenzel¹, Julia Claasen¹, Nadine Kutsch¹, Günter Krause¹, Christine Mayr⁴, Andreas Rosenwald⁵, Christoph Plass², Joachim L. Schultze³, Michael Hallek¹ and Clemens-Martin Wendtner¹

Department I of Internal Medicine, Center of Integrated Oncology and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany¹; Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center, Heidelberg, Germany²; Institute for Life and Medical Sciences (LIMES), Genomics and Immunoregulation, University of Bonn, Germany³; Whitehead Institute, MIT, Cambridge, MA, USA⁴, Institute of Pathology, University of Würzburg, Germany⁵

To whom correspondence should be addressed:
Clemens-Martin Wendtner, M.D.
University of Cologne
Department I of Internal Medicine
Center for Integrated Oncology Cologne Bonn
D-50924 Cologne
GERMANY
Phone: ++49 221 478 86720
Fax: ++49 221 478 86779
Email: clemens.wendtner@uni-koeln.de

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Abstract

MicroRNAs play a key role in cellular regulation and if deregulated in the development of neoplastic disorders including chronic lymphocytic leukemia (CLL). RNAs from primary cells of 50 treatment-naïve CLL patients and peripheral B-cells of 14 healthy donors were applied to miRNA-expression profiling using bead chip technology. In CLL cells a set of 7 up- and 19 down-regulated miRNAs was identified. Among the miRNAs being downregulated in CLL cells, 6 out of 10 miRNA promoters being examined showed gain of methylation as compared to normal B cell controls. Subsequent target prediction of deregulated miRNAs revealed a highly significant binding prediction at the 3’UTR of the pleomorphic adenoma gene 1 (PLAG1) oncogene. Luciferase reporter assays including site directed mutagenesis of binding sites revealed a significant regulation of PLAG1 by miR-181a, miR-181b, miR-107 and miR-424. While expression of PLAG1 mRNA was not affected, PLAG1 protein expression was shown to be significantly elevated in CLL cells as compared to the levels in healthy donor B cells. In summary, we could demonstrate disruption of miRNA-mediated translational control, partly due to epigenetic transcriptional silencing of miRNAs, with subsequent overexpression of the oncogenic transcription factor PLAG1 as a putative novel mechanism of CLL pathogenesis.
Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemic disorder in the western hemisphere and is characterized by an accumulation of mature B cells in the blood, bone marrow, and secondary lymphoid organs. The clinical heterogeneity in CLL is reflected by various chromosomal aberrations, e.g. deletion of chromosome 17p indicating highly unfavorable prognosis due to loss of p53, and, on the other hand deletion of chromosome 13q14 being associated with favorable prognosis.1 In previous work, the localization of microRNA (miRNA) and non-coding RNA genes on chromosome 13q14 led to the assumption that down-regulation of miR-15 and -16 is mediated by deletion of the locus and subsequently leads to over-expression of BCL2. In independent approaches, miR-155 was identified to be overexpressed in basically all CLL patients, whereas down-regulation of miR-15/16 appeared to be critical only in a subset of patients.2-5

MiRNA-mediated control of messenger-RNA stability and protein translation plays a pivotal role in the fine regulation of central cellular pathways,6 particularly in embryonic development and cell differentiation. In the multistep development of immune cells miRNAs have been shown to be key players in differentiation, in particular the multi-step development of B-cells shows stage specific miRNA signatures.7,8 With regard to pathogenesis of neoplastic diseases, miRNA control of putative oncogenic target genes has been previously shown to be directly involved in malignant transformation.9,10 There is increasing evidence for extensive miRNA deregulations in multiple neoplasias. However, both deregulations of miRNAs as well as the identification of their functional relevant targets and regulatory circuits in CLL pathogenesis are only partly understood and remain to be elucidated. Especially, the underlying mechanisms of predominant
miRNA down-regulation can not be generally explained by genetic alterations or defects in the miRNA processing machinery.

By applying miRNA profiling to CLL B cells derived from a large cohort of previously untreated CLL patients, we identified a novel group of deregulated miRNA besides previously identified alterations in CLL. Based on the predominant down-regulation of miRNAs in this cohort, we investigated genetic and epigenetic alterations as potential underlying mechanisms of the observed miRNA deregulations. As a down-stream result of miRNA deregulation, we attempted to identify miRNA target genes affected by loss of miRNA control. Surprisingly, a strong oncogenic transcription factor, pleomorphic adenoma gene 1 (PLAG1), was predicted to be a target of several deregulated miRNAs. Indeed, over-expression of PLAG1, primarily identified to be involved in tumorigenesis by promoter swapping in pleomorphic salivary gland adenomas, could be verified in CLL. Alterations in the axis of miRNA-mediated oncogene control by epigenetic transcriptional silencing of miRNAs might be a novel oncogenic pathway in CLL pathogenesis.

Material and Methods

Patients and cells

After informed consent, blood was obtained from patients fulfilling diagnostic criteria for CLL. Only patients without prior therapy were included in this study. Fresh CLL samples were enriched by applying B-RosetteSep (Stem Cell Technologies, Vancouver, Canada) and Ficoll-Hypaque (Seromed, Berlin, Germany) density gradient purification resulting in
purity of >98% of CD19+/CD5+ CLL cells. CLL cells were characterized for CD19, CD5, CD23, FMC7, CD38, ZAP70 and sIgM expression on a FACS Canto flow cytometer (BD PharMingen, Heidelberg, Germany). IgV\_H-hypermutational status of CLL patients was analyzed as previously published. Control cells were isolated by positive selection with anti-CD19-MACS-beads (Miltenyi, Bergisch Gladbach, Germany). All cells were immediately lysed by Trizol and cryopreserved at –80°C. This study was approved by the ethics committee of the University of Cologne ( Approval No. 01-163). Blood samples were given with informed consent according to the Declaration of Helsinki protocol.

**miRNA expression analysis**

BeadCHip miRNA expression assay (PAS, Illumina San Diego, CA) was carried out as previously described according to the manufacturer’s protocol\(^\text{11}\). Briefly, 200 ng of total RNA extracted from Trizol lysed samples was first applied by polyadenylation agent (PAS, Illumina). The attached poly(A) tail was used for further priming of cDNA synthesis (CSS, Illumina,) using biotinylated oligo-dT primers containing an additional 5’-universal PCR primer sequence. Biotinylated cDNA was captured to a solid phase by streptavidin binding and further hybridized with miRNA-specific assay oligonucleotides. Assembled oligonucleotides were extended by DNA polymerase and subsequently eluted for universal PCR-based amplification with fluorescently labeled universal primers. Single stranded PCR products were hybridized to a 96 Sentrix Array Matrix (Illumina). Arrays were scanned using a BeadArray reader and data were exported to Bead Studio v3.2 (Illumina). RT-PCR expression analysis for further validation was carried out using mirCURY LNA (Exiquon, Woburn, MA). SNORD48 and U6 RNA served as endogenous house-keeping controls. Microarray data are available under https://webmail.uni-koeln.de/horde-2.2/util/go.php?url=http%3A%2F%2Fwww.medizin.uni-koeln.de%2Fkliniken%2Finnere1%2Fforschung%2Fillumina_miRNA_CLL_versus_HD-
Methylation analysis

Isolation of genomic DNA was carried out by lysis of cells for 15 min with lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA pH 7.4) at 4°C. Cell nuclei were sedimented by centrifugation, subsequently resuspended by nuclear lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na2-EDTA pH 8.2) and lysed by adding SDS. Proteinase K digestion was carried out overnight at 50°C. Saturated NaCl-solution was added for precipitation of proteins, after centrifugation for 30 min at 4000 rpm solved genomic DNA was isolated from the supernatant, precipitated with isopropanol, washed with 70% ethanol and dissolved in TE buffer for storage. Genomic DNA (1–2 µg) was chemically modified with sodium bisulfite using the EZ methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s instructions. Quantitative DNA methylation analysis at single CpG units (containing one or more CpG dinucleotides) was performed by MassARRAY technique, as previously described, targeting amplicons of up to 600bp size. Regions of analysis were chosen by putative promoter regions of ESTs and/or protein coding genes harboring pre-miRNAs. The supposed promoter activity in some of the selected regions was confirmed by genome-wide miRNA promoter analyses (7 out of 17 analyzed loci). Also, arbitrarily chosen genomic regions approximately 1 kb upstream of the pre-miRNAs were analyzed in order to test whether changes in DNA methylation were specific for promoter regions.

Bisulfite-treated DNA was PCR-amplified (primer sequences given in Supplemental Table 4), in vitro transcribed, base-specifically cleaved by RNase A and subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry. DNA methylation standards (0%, 20%, 40%, 60%, 80% and 100% methylated genomic DNA)
and correction algorithms based on R statistical computing environment were used for data correction and normalization.

**PLAG1 mRNA expression analysis**

Quantification of PLAG1 mRNA was performed by LightCycler Taqman Master (Roche, Penzberg, Germany). Universal probe No. 70 (Roche) with forward primer 5’-GTCCAGCCCCGAAATATGAGA-3’ and reverse primer 5’-CAGCACCAAGGAGGCAACC-3’ was used for PLAG1 amplification. Beta-2-microglobulin was applied as housekeeping gene standard by Universal probe No. 42 (Roche) with forward primer 5’-TTCTGGCCTGGA-GGCTATC-3’ and reverse primer 5’-TCAGGAAATTTGACTTTCCATT-3’. All experiments were performed in replicates and crossing points were determined by second derivative maximum method. Relative quantification analysis was done by Exor3 software package (Roche).

**PLAG1 protein expression analysis**

A PLAG1 specific mouse monoclonal antibody (clone 3B7, Abnova, Taipei, Taiwan) was used for detection of PLAG1 protein. For control of antibody specificity PLAG1-GFP was transfected by calcium phosphate transfection to HEK293 cells with PLAG1 specific 50 nM siRNA, respectively. Cellular lysates were processed with RIPA buffer, sonicated and further blotted onto nitrocellulose membranes. Western blot detection and density measurements were performed on an Odyssey infrared imaging system (Licor, Lincoln, NE).

**miRNA binding assays**

For luciferase reporter experiments, a 2 kb 3’-UTR segment containing predicted
microRNA interaction sites was amplified from human PLAG1 cDNA (ImaGenes, Berlin, Germany) using forward primer 5’-gactagttgcctatttgttgcttgtgc-3’ and reverse primer 5’-ttttcttttgcggccgaagccccactttccattctga-3’ and cloned into pTrcHis2-TOPO (CR972286; Invitrogen, Carlsbad, CA). From this construct a SpeI / NotI fragment was cleaved out and inserted into pIS1 adjacent to the stop codon of the firefly luciferase gene. Site-directed mutagenesis at predicted miRNA binding sites within the PLAG1 3′-UTR was performed using the Quickchange XL mutagenesis kit (Stratagene, La Jolla, CA). The variants (Supplemental Table 3) were confirmed by sequencing.

HeLa cells at a density of 2x10⁴ cells/well were co-transfected in 96-well plates by using Lipofectamine 2000 (Invitrogen) with 45 ng pIS1-PLAG1 constructs containing the Renilla luciferase gene, 5 ng of pISO expressing firefly luciferase as transfection control, and 1 pg microRNA duplex (miRIDIAN, Dharmacon, Chicago, IL). The miRNAs used for co-transfection were miR181a, -181b, -107, -424, -141, and a miRNA mimic negative control. Light emission by firefly and Renilla luciferase activities was measured consecutively by using Dual-Luciferase assays (Promega, Madison, WI) 24 h after transfection using a Glomax luminometer (Promega). Ratios of Renilla versus firefly signals served as a measure for reporter activity normalized for transfection efficiency.

**Bioinformatic and statistical analysis**

The array intensity data were imported into BeadStudio v3.2 (Illumina) and a quantile normalization procedure was applied. Further analysis of differential expression was carried out using R software package and dCHIP. DNA methylation data was normalized and corrected by R software package and analyzed with Wilcoxon test by JMP (8.0, SAS inc).

For target prediction Target-Scan 4.1 (www.targetscan.org), PicTar (http://pictar.mdc-berlin.de), MiRDB (http://mirdb.org) and miRanda (http://www.microrna.org)
algorithms were applied. GeneTrail database (http://genetrail.bioinf.uni-sb.de) was accessed for functional annotation of predicted target genes. SPSS, Excel, and Sigma-Plot were applied for statistical analysis of data.

Results

Identification of a novel set of miRNAs reduced in CLL

To assess deregulation of global miRNA expression we profiled human miRNAs using a beadchip-based miRNA expression profiling platform. Purified B-cells from either CLL patients (n=50) or healthy controls (n=14) were assessed. All CLL patients were treatment naïve. The majority of patients presented with Binet stage A disease and showed a favorable risk profile as assessed by clinical and molecular features (Table 1). The most frequent aberration was a deletion 13q14 while only 6 of 45 patients were documented to have a homozygous deletion in this area. Comparing the total number of miRNA being expressed a significantly lower number of miRNA was detected in CLL compared to normal B cells (Figure 1A). The predominance of down-regulated miRNAs in CLL cells was accompanied by highly significantly lower total number of miRNAs expressed above the detection threshold in CLL patients (19.8 % vs 23.5 %; p<10^-6). Applying a threshold of a median fold change larger than 2, a minimal absolute difference of signal intensity defined by the detection threshold level and a p-value minor than 0.001 in CLL patient cells, 7 miRNAs appeared to be up-regulated, whereas 19 miRNA were shown to be significantly down-regulated (Table 2, Figure 1B and supplemental data). Applying Bonferroni’s correction due to multiple testing of miRNA
probe sets underlined significant deregulation of all identified miRNAs except miR-449 and miR-565.

Additionally, for miR-21 previously demonstrated to be up-regulated in CLL, we could detect the highest absolute expression values of all assessed miRNAs. However, in healthy controls the miR-21 expression was not significantly lower.

Due to the clinical heterogeneity of CLL we analyzed for differential expression of miRNAs in CLL patient subsets defined by cytogenetics and prognostic markers. We could not identify significant differentially expressed miRNA in cytogenetic defined subgroups, in particular we could not detect significant deregulation of miRNAs in patients harboring del13q14. However in CD38 positive versus negative CLL cells we could detect significantly reduced expression of miR-660. In IgVH unmutated cases a significantly reduced expression of miR-146b could be observed (supplemental data).

With regard to previous miRNA screening approaches assessing either spotted miRNA-arrays, Solexa-sequencing, miRNA-cloning or RT-PCR based assays, we confirmed up-regulation of miR-155 and down-regulation of miR-181a/b. We also detected heterogeneous up-regulation of miR-34a, previously being identified heterogeneously expressed in CLL and being down-regulated in CLL patients harboring del17p facilitating chemotherapy resistance. We could not identify significant down-regulation of miR-15 and miR-16 except in one patient harboring a homozygous deletion of chromosome 13q14. However, the previous up-regulation of miR-155, a key regulator of B-cell ontogenesis, appeared to be the most prominent up-regulated miRNA in our cohort.

Here we identified so far unknown down-regulation of a set of miRNAs in CLL such as miR-107, -424, -125a, -126 and -326.

For validation of the microarray based data set, we performed quantitative RT-PCR-based relative expression of selected miRNAs. No down-regulation of miR-16 was seen in CLL cells (n=13) compared to healthy donor B-cells (n=10), while miR-107 and -181b
were proven to be significantly downregulated in CLL (p=0.009 and 0.00002, respectively). In summary, we identified a set of predominantly down-regulated miRNAs in CLL including several hitherto unknown deregulated miRNAs.

**Deregulation of miRNAs is not associated with genomic alterations but with epigenetic transcriptional silencing**

With respect to genomic localization of miRNA genes deregulated in CLL, no obvious association to known breakpoints in CLL, frequent deletions and other cytogenetic alterations was seen. At chromosome 11q, four miRNA loci (miR-130, -139a, -143, -326) were identified, however, genomic localizations are widely distributed apart from ATM locus. In addition, only three of the CLL patients in this cohort harbored a del11q deletion, thus rendering major cytogenetic breaks unlikely as the underlying mechanism in general miRNA suppression in CLL cells.

As an alternative mechanism of miRNA downregulation, we hypothesized that epigenetic transcriptional silencing could be involved. DNA hypermethylation at promoter regions is a well known transcriptional silencing mechanism. In order to investigate whether altered DNA methylation leads to aberrant expression of miRNAs in CLL, we analyzed the DNA methylation status at known or predicted transcriptional start sites (TSSs) of pri-miRNAs. DNA methylation was measured in 70 CLL samples (including 40 of 50 patient samples applied for miRNA expression profiling) and was compared to peripheral blood CD19 positive B cells and tonsilar CD19 positive B cells from healthy donors. Among 23 miRNA loci covering all deregulated miRNAs, three miRNAs either not overlapping with known ESTs (miR199a-1 and miR369-3p) or situated in an EST, harboring a CpG poor TSS sequence (miR-181a1/b1) were not further analyzed. MiR-424 and miR-660
promoters were excluded from analysis, because their DNA methylation patterns showed bimordial distribution in a sex-dependent manner due to X-chromosome inactivation.

In most of the miRNAs downregulated in CLL (10 out of 12 loci), promoter regions were unmethylated in normal B cells consistent with transcriptionally active TSSs in general. Intriguingly, in miRNA promoters associated with reduced miRNA expression, many CLL samples showed gain of methylation, compared to normal B cells. Especially, promoters of mir-139 and miR582 showed significant gain of methylation in CLL, whereas promoters of the other miRNAs did not show statistically significant difference (Figure 2).

In contrast, upregulated miRNAs showed either loss of methylation or no change in their promoters that were hypermethylated or hypomethylated in normal B cells, respectively (Supplemental Figure 2.A.). As an evidence for specificity of changes of DNA methylation within miR promoter regions, we also analyzed DNA methylation in arbitrarily chosen regions about 1 kb upstream of pre-miRNAs. Most of these loci showed significant loss of methylation in CLL B cells compared to normal B cells, irrespective of down- or up-regulated aberrant expression of miRNAs (Supplemental Figure 2.B.). This is likely to reflect a consequence of global loss of methylation in CLL, as previously shown.22 Taken together, the gain of methylation was specific to the promoter regions for downregulated miRNAs, indicating that transcriptional silencing of pri-miRNA by DNA methylation, at least in part, leads to downregulation of miRNAs during CLL pathogenesis.
Identification of the transcription factor PLAG1 as a prime target gene for de-regulated miRNAs in CLL

miRNAs are hypothesized as a major control mechanism in cell differentiation and control of oncogenes. In order to identify novel oncogenes evading miRNA control due to the tremendous deregulations of miRNAs in CLL, we assessed target binding prediction algorithms for deregulated miRNAs. All miRNAs classified here for being specifically deregulated in CLL were subjected for prediction by the TargetScan algorithm (www.targetscan.org, version 4.1). All candidate genes predicted by TargetScan were extracted. The initial screen of putative miRNA targets in CLL contained 7760 putative interactions and 3700 candidate genes. Due to the extensive amount of candidate genes, eliminating false positive predictions is required in order to further focus on highly probable targets. Binding-scores calculated by TargetScan to validate the predicted stringency of the miRNA-mRNA binding were assessed to an additive quantification of cumulative putative binding. First, each binding-score of predicted binding sites was added to a cumulative binding-score of each candidate gene. Second, the total number of predicted binding sites for the CLL-specific miRNAs was assessed by additive counting. Third, conservation of binding sites was included in ranking of target candidates. Forth, we focused on genes being affected by down-regulated miRNAs. Applying these filter criteria we ranked all genes for single filter criteria and a total rank was calculated. We focused on the 167 genes ranked in the group of 5% highest total rank for further analysis of functional association. GeneTrail data base for functional annotation was assessed to the high-rank group of 167 genes revealing a highly significant over-representation of transcription factors and DNA-binding proteins.
(Supplemental Table 2). In order to functionally focus on genes involved in malignant transformation we evaluated the functional context of the 167 predicted target genes regarding oncogenesis, proliferation, anti-apoptotic properties and cell cycle control revealing a list of 48 genes. (Supplemental Table 1)

Here, the strong oncogenic transcription factor pleomorphic adenoma gene 1 (PLAG1) was identified as the top-ranked most significant gene. 9 putative evolutionary highly conserved (including chicken) and additional 8 less conserved putative binding sites for deregulated miRNAs in CLL were identified in the 3’UTR of the PLAG1 mRNA. Here we primarily assessed the TargetScan algorithm for first prediction target genes. To verify our hypotheses with an independent target prediction algorithm we performed miRNA–binding prediction of PLAG1 3’UTR in several other bioinformatic approaches. By PicTar approach miR-107, -141, -181a, 181b as for the miR-15/16/195/424/497 family were analogously predicted. MiRDB also predicted miR-141, -424, -181a and -181b. Finally, miRanda algorithms identified miR-107, -141, -424, -181a and 181b.

In conclusion, the oncogene PLAG1 showed up as a key candidate to evade miRNA control especially of the under-expressed miRNAs miR-181a, -181b, -107 and 424 in CLL leading to disturbed oncogene control in leukemia cells.

PLAG1 is under the control of miRNAs being de-regulated in CLL

To experimentally address miRNA target interaction identified above, we co-transfected the individual miRNAs (miRNA-181a, -181b, -107, -424 and -141) together with a Renilla luciferase reporter construct containing a PLAG1 3’-UTR mRNA fragment into HeLa cells. After 24 hours luciferase activities in transfected HeLa cells with and without the specific miRNAs were compared. Relative to unspecific control miRNA, co-transfection with miR-181a significantly suppressed activity of the PLAG1-3’UTR-luciferase construct
by 43.6% (p=0.03, n=5) and in miR-181b by 48.4%, respectively (p=0.03, n=5) (Figure 4A). PLAG1-3´UTR-reporter construct was also significantly suppressed with 28.6% activity reduction by miR-107 (p=0.01, n=5) and 42.7% activity reduction by miR-424 (p=0.005, n=5) (Figure 4B). In contrast, by transfection of miR-141, being up-regulated in CLL, no significant activity reduction was seen. When combining all miRNAs simultaneously to reporter assays no additive effect outranging the sole application of miR-181a/b was seen (data not shown).

In order to examine whether the observed repression of a reporter gene by miRNAs was specifically due to the presence of predicted binding sites in the PLAG1 3´-UTR fragment, these sites were disrupted by site directed mutagenesis (Supplemental Table 3). For interrupting a perfect seed pairing, two nucleotides were substituted in each predicted binding site. Since there are two predicted highly conserved binding sites for miR-181a/b in the PLAG1 3´UTR, three variants were generated, in which one respectively both of these sites were mutated. For assessing the role of the predicted binding sites, the PLAG1 3´-UTR constructs with wild type (wt) or mutated binding sites were co-transfected with the corresponding miRNAs and subjected to reporter assays. Significantly reversed binding of miRNA-181a was demonstrated in miR-181-binding site mutants PLAG1-3´UTR-mut4390 (p=0.04), -mut3500 (p=0.02) and –mut4390/3500 (p=0.0002). Similarly for miR-181b significantly reversed binding by site directed mutagenesis of predicted binding sites PLAG1-3´UTR-mut4390 (p=0.039), -mut3500 (p=0.011) and –mut4390/3500 (p=0.011) was shown (Figure 4A). Moreover, significantly reversed miRNA-mediated suppression of activity was also seen by miR-107-binding site mutant PLAG1-3´UTR-mut4437 (p=0.006) and miR-424-binding site mutant PLAG1-3´UTR-mut4437 (p=0.018) (Figure 4B). Altogether miR-181a, -181b, -107 and –424 significantly bind PLAG1-3´UTR and mutating predicted binding sites completely abrogates miRNA mediated suppression.
Reduced miRNA-based repression is associated with PLAG1 over-expression in CLL

Based on the reduced expression of multiple miRNAs in CLL that bind to the 3'UTR of the PLAG1 mRNA, one would postulate that PLAG1 mRNA and/or protein expression is enhanced in CLL cells. We therefore assessed PLAG1 expression in CLL patients compared to healthy controls. Quantification of PLAG1 mRNA by RT-PCR revealed a slightly elevated level of PLAG1 mRNA in CLL cells (n=11) versus healthy donor control B-cells (n=8) although the difference did not reach statistical significance (Figure 5A). These findings are in line with the current concept that most mammalian miRNA target genes are mainly regulated by repression of protein translation rather than mRNA destruction. We therefore addressed PLAG1 protein expression by immunoblotting of lysates derived from purified CLL cells and healthy donor peripheral B-cells, respectively. Specificity of the applied antibody was confirmed by controls including recombinantly overexpressed PLAG1 and co-transfection controls with PLAG1-specific siRNA, respectively (Figure 5D). Immunoblotting of primary CLL cells (n=29) versus healthy donor controls (n=22) demonstrated a significantly elevated PLAG1 protein expression in CLL cells. Almost no detectable expression was seen in healthy donor control B-cells. Applying immunohistochemistry, PLAG1 could not be detected in CLL lymph nodes (data not shown). This finding is in line with the low absolute expression of PLAG1 in CLL cells. However, applying Odyssey densitometry of specific immunoblotting, signal intensities revealed a significantly elevated mean of PLAG1 specific fluorescence intensity rate, 10.31 (95% confidence interval 4.4 – 16.2, p=0.014) in CLL cells compared to 0.85 (95% confidence interval 0.48 – 1.22) in healthy donor B-cells (Figure 5B). While PLAG1 expression in some patients (CLL2, CLL4) was indistinguishable compared to healthy controls, three patients (CLL1, CLL3, CLL5)
showed high expression of PLAG1 protein expression, however no obvious clinical correlation with methylation patterns of miRNA loci, disease stage or prognostic factors could be identified (Figure 5C).

In conclusion, the oncogene PLAG1 shows a strong aberrant expression in CLL which is significantly elevated in comparison to healthy B-cells harboring no detectable PLAG1.

Discussion

Here we describe a novel set of 26 miRNAs significantly deregulated in CLL. In addition to miR-181a and b a total set of 19 downregulated genes was identified. Strikingly, the majority of downregulated miRNA shows hypermethylation in the respective putative transcriptional starting sites within the promoter sequence strongly suggesting epigenetic rather than mutational regulation of miRNA in CLL. In fact, except for one patient, we could not confirm wide distribution of miR-15 and mir-16 downregulation attributed to homozygous deletion of chromosome 13q14. To determine oncogenic events downstream of downregulated miRNA a computational approach revealed the novel finding of the oncogene PLAG1 to be a potential target of miRNA deregulation. In fact, we clearly demonstrate regulation of PLAG1 expression by 6 of the downregulated miRNAs (miR-181a, -181b, -107, -424, -155 and –141). As a consequence of missing miRNA regulation, PLAG1 is overexpressed in CLL.

In total the number of miRNAs detectable was significantly lower in malignant CLL cells. This phenomenon of decreased miRNA levels in malignantly transformed cells has been
previously described. Comparing our data to recently published data we could verify the down-regulation of miR-181a and miR-181b in CLL. We also showed significant up-regulation of miR-155 known to be a key regulator of B-cell maturation and associated with lymphoma development. However, miR-21 that was previously reported to be highly up-regulated in CLL was shown to be only slightly over-expressed compared to healthy donor B-cells in our screening set. This difference could be partially due to different screening approaches: miRNA cloning and quantitative real-time-polymerase chain reaction (qRT-PCR) of mature miRNAs versus bead chip array technology as used by us. Furthermore, we could not detect high frequencies of miR-15 and miR-16 down-regulation as previously described. Nevertheless, this finding is in line with previous independent observations applying both sequencing and hybridization methods for miRNA detection. We observed miR-16 down-regulation only in one patient harboring homozygous deletion of chromosome 13q14. Nevertheless, we identified significant down-regulation of miR-424 belonging to the miR15/16/195/424/497 miRNA-family sharing the same 3’UTR-binding seed sequence.

We demonstrate that gain of methylation was observed especially in the promoter regions of downregulated miRNAs, while non-promoter regions showed loss of methylation irrespective of up- or down-regulation of miRNAs in CLL. This indicates that DNA methylation could be an important mechanism of transcriptional silencing, in this case of pri-miRNA, with subsequent downregulation of mature miRNAs, although we cannot exclude that loss of methylation in some locations still has biological significance in the individual locus.

We identified several novel miRNAs being significantly deregulated in CLL. This novel group of miRNAs was assessed to predict relevant down-stream targets of miRNA in CLL. Due to the high amount of false positives by in silico prediction of binding sites for a single miRNA, we combined the integrated perspective of a deregulated cluster with
rigorous filtering for predicted binding affinity and frequency of predicted binding sites in order to reveal high probability of miRNA-target interaction. The oncogenic zink-finger transcription factor PLAG1 was identified by application of our step-wise algorithm to be targeted by miR-181a, -181b, -107, -424, -155 and –141. So far the PLAG1 oncogene has not been described in CLL pathogenesis. However, the well-described oncogenic potential of this transcription factor on salivary gland adenomas, breast cancer and AML pathogenesis indicate the influence of PLAG1 on malignant transformation. PLAG1 was primarily identified to be involved in tumorigenesis of salivary gland adenomas by translocation and promotor swapping. Although we previously identified chromosomal translocations in CLL as prognostic parameter, no significantly enhanced frequency of translocations involving the PLAG1 locus could be identified in CLL. In previous gene expression profiling attempts PLAG1 was not identified as over-expressed gene in CLL. Due to the low overall mRNA expression level and the demonstrated miRNA control mechanisms the over-expression of this oncogene was undetectable for classical screening attempts. However, we could demonstrate low detectable mRNA expression levels for PLAG1 by modern RT-PCR technique and even aberrant protein expression of PLAG1 in a significant proportion of CLL cases. In contrast, no protein expression of PLAG1 was detectable in healthy donor B cells although mRNA could be shown to be prevalent in healthy B cell control cells. We therefore hypothesized that miRNA mediated control mechanisms and their disruption are crucial for PLAG1 oncogene over-expression in CLL.

We showed inhibitory potential of predicted miRNAs on the PLAG1 3’UTR by luciferase reporter assays. Moreover, site-directed mutagenesis of binding sites could demonstrate specificity of miRNA-3’UTR interaction by abrogation of miRNA regulatory effects. As a consequence for deregulation of miRNAs and disrupted control of PLAG1 3’UTR by the down-regulated miR-107, -424, -181a and 181b, we demonstrated for the first time over-
expression of PLAG1 in CLL cells. Assessing mRNA expression of PLAG1, a non-significant up-regulation of PLAG1 was seen. However, PLAG1 protein is significantly up-regulated in primary CLL cells compared to healthy donor B cells. As the control of target genes by miRNAs is mediated by multiple mechanisms including both mRNA degradation as well as translational inhibition, the significant up-regulation of PLAG1 protein accompanied by a non-significant increase of PLAG1 mRNA in CLL indicates translational regulation of PLAG1 as the predominant mechanism of miRNA control on the PLAG1 oncogene. Here we identified for the first time the up-regulation of PLAG1 in CLL cells, while previous attempts of mRNA gene expression profiling could not detect modulation of PLAG1 in CLL.

The physiological function of this zinc-finger protein is characterized by direct DNA-binding to a defined sequence motif. Expression of PLAG1 is only detectable in early developmental stages but not in differentiated human tissues indicating that PLAG1 is involved in embryogenesis. PLAG1 mediates transcriptional control of the IGF-II mitogenic pathway and on various other target genes such as VEGF, BPGF-II, Bcl-2, MAPK11, TRAF1 and WT1, as a previous study revealed by gene expression profiling of PLAG1 transfected cells. We applied this PLAG1-dependent gene cluster on our previously published mRNA gene expression data set comparing CLL cells versus healthy donor B-cells. By supervised hierarchical cluster analysis the PLAG1 dependent gene cluster separated malignant CLL cells from healthy B-cells thus indicating differential expression of PLAG1 dependent genes in CLL and an effect of the above described overexpression of PLAG1 in vivo (supplemental data). Aberrant expression of VEGF in CLL leading to autostimulatory loops have been previously described and elevated bcl-2 is a key phenomenon of apoptosis resistance in CLL. The over-expression of PLAG1 dependent genes indicates regulatory effects of PLAG1 as oncogenic transcription factor in CLL, however ongoing research efforts using transgenic
mice models will reveal the oncogenic potential of PLAG1 in B cells.

In conclusion we demonstrate (I) predominant down-regulation of miRNAs in CLL, (II) identified novel deregulated miRNAs in CLL, (III) unraveled underlying epigenetic changes in loci of deregulated miRNA, (IV) applied in silico target prediction of miRNA interactions for identification of novel pathogenetic factors, and (V) identified specific interaction of deregulated miRNA with PLAG1 3’UTRs resulting in over-expression of this oncogene in CLL. Therefore, PLAG1 over-expression in CLL cells represents a novel oncogenic mechanism in CLL pathogenesis on the background of deregulation in miRNA-mediated control mechanisms.

**Acknowledgments**

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Authorship


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


Table 1: **Patients’ characteristics and prognostic factors of miRNA screening cohort.**
Clinical and molecular features including cytogenetic analysis by FISH of 55 pts being diagnosed with CLL were assessed before miRNA profiling was performed.

<table>
<thead>
<tr>
<th>Clinical and molecular features including cytogenetic analysis by FISH</th>
<th>Negative for feature (n / %)</th>
<th>Positive for feature (n / %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase (&gt;10 U/l)</td>
<td>34/50 (68.0%)</td>
<td>16/50 (32.0%)</td>
</tr>
<tr>
<td>CD38 (&gt;10% positive CLL cells)</td>
<td>34/50 (68.0%)</td>
<td>16/50 (32.0%)</td>
</tr>
<tr>
<td>ZAP70 (&gt;20% positive CLL cells)</td>
<td>25/50 (50.0%)</td>
<td>25/50 (50.0%)</td>
</tr>
<tr>
<td>IgV&lt;sub&gt;H&lt;/sub&gt; hypermutational status (homology &gt; 98%)</td>
<td>20/31 (64.5%)</td>
<td>11/31 (35.4%)</td>
</tr>
<tr>
<td>Binet Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>33/50 (66.0 %)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>11/50 (22.0%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>6/50 (12.0%)</td>
<td></td>
</tr>
<tr>
<td>Cytogenetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>12/45 (26.6%)</td>
<td></td>
</tr>
<tr>
<td>del13q14</td>
<td>25/45 (55.5%)</td>
<td></td>
</tr>
<tr>
<td>homozygous del13q14</td>
<td>6/45 (13.2%)</td>
<td></td>
</tr>
<tr>
<td>trisomie 12</td>
<td>3/45 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>del11q</td>
<td>3/45 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>del 17p</td>
<td>1/45 (2.2%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Deregulated miRNAs in CLL versus healthy donor B-cells.
BeadChip microarray based miRNA expression values of quantile normalized data comparing purified CLL cells (n=50) to healthy donor peripheral B-cells (n=14). T-test was carried out for determination of differential expression. For rigid correction of multiple testing Bonferroni’s correction was applied and miRNAs remaining significant by this test are indicated by asterisks.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>mean expression of CLL cells</th>
<th>mean expression of healthy B-cells</th>
<th>fold change</th>
<th>p-value</th>
<th>absolute difference of means</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-107</td>
<td>692,53</td>
<td>1764,03</td>
<td>-2,55</td>
<td>10^{-4} *</td>
<td>1071,5</td>
</tr>
<tr>
<td>hsa-miR-125a</td>
<td>418,88</td>
<td>2039,2</td>
<td>-4,87</td>
<td>&lt;10^{-5} *</td>
<td>1620,32</td>
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<tr>
<td>hsa-miR-126</td>
<td>4540,85</td>
<td>15815,12</td>
<td>-3,48</td>
<td>&lt;10^{-5} *</td>
<td>11274,27</td>
</tr>
<tr>
<td>hsa-miR-126*</td>
<td>1983,83</td>
<td>21761,79</td>
<td>-10,97</td>
<td>&lt;10^{-5} *</td>
<td>19777,95</td>
</tr>
<tr>
<td>hsa-miR-130a</td>
<td>1272,19</td>
<td>10530,37</td>
<td>-8,28</td>
<td>&lt;10^{-5} *</td>
<td>9258,18</td>
</tr>
<tr>
<td>hsa-miR-139</td>
<td>1001,53</td>
<td>3095,88</td>
<td>-3,09</td>
<td>&lt;10^{-5} *</td>
<td>2094,35</td>
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<tr>
<td>hsa-miR-141</td>
<td>3871,12</td>
<td>1623,27</td>
<td>2,38</td>
<td>&lt;10^{-5} *</td>
<td>2247,85</td>
</tr>
<tr>
<td>hsa-miR-143</td>
<td>299,48</td>
<td>1506,67</td>
<td>-5,03</td>
<td>10^{-4} *</td>
<td>1207,19</td>
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<tr>
<td>hsa-miR-148a</td>
<td>14042,43</td>
<td>4724,08</td>
<td>2,97</td>
<td>&lt;10^{-5} *</td>
<td>9318,35</td>
</tr>
<tr>
<td>hsa-miR-155</td>
<td>26073,76</td>
<td>9956,07</td>
<td>2,62</td>
<td>&lt;10^{-5} *</td>
<td>16117,69</td>
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<tr>
<td>hsa-miR-181a</td>
<td>1093,34</td>
<td>9251,04</td>
<td>-8,46</td>
<td>&lt;10^{-5} *</td>
<td>8157,7</td>
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<tr>
<td>hsa-miR-181b</td>
<td>316,23</td>
<td>1364,86</td>
<td>-4,32</td>
<td>&lt;10^{-5} *</td>
<td>1048,63</td>
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<tr>
<td>hsa-miR-199a</td>
<td>386,26</td>
<td>4393,66</td>
<td>-11,37</td>
<td>&lt;10^{-5} *</td>
<td>4007,4</td>
</tr>
<tr>
<td>hsa-miR-199a*</td>
<td>1511,02</td>
<td>8537,04</td>
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<tr>
<td>hsa-miR-326</td>
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<td>3376,34</td>
<td>-15,16</td>
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<tr>
<td>hsa-miR-34a</td>
<td>1876,1</td>
<td>444,46</td>
<td>4,22</td>
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<td>1431,64</td>
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<tr>
<td>hsa-miR-368</td>
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<tr>
<td>hsa-miR-369-3p</td>
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<td>1012,99</td>
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<tr>
<td>hsa-miR-424</td>
<td>902,62</td>
<td>2536,33</td>
<td>-2,81</td>
<td>2x10^{-4} *</td>
<td>1633,71</td>
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<tr>
<td>hsa-miR-449</td>
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<td>5159,03</td>
<td>-3,01</td>
<td>0,002</td>
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<tr>
<td>hsa-miR-451</td>
<td>16872,23</td>
<td>5770,64</td>
<td>2,92</td>
<td>&lt;10^{-5} *</td>
<td>11101,59</td>
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<tr>
<td>hsa-miR-565</td>
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<td>0,0025</td>
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<td>hsa-miR-582</td>
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<td>hsa-miR-584</td>
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<tr>
<td>hsa-miR-660</td>
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<td>896,36</td>
<td>2,23</td>
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<td>1104,02</td>
</tr>
</tbody>
</table>
Figure legends:

Figure 1: miRNA expression profiling of CLL patients samples and healthy donor B-cells.

Significant decrease of expressed miRNA in CLL cells (n=50) versus healthy donor B cells (n=14) (p<10^{-6}) shown as percentage of present calls (A). Heat map of microRNA expression patterns in CLL cells vs healthy donor B cells: 26 miRNAs are significantly deregulated in CLL versus healthy control, 19 miRNA show down-regulation in CLL, 7 miRNAs were demonstrated to be upregulated (B).

Figure 2: DNA methylation status at promoters of miRNAs being down-regulated in CLL.

DNA methylation status of promoter regions associated to down-regulated miRNAs (n=10) was examined in CLL samples (black circles) (n=70) and compared to peripheral blood CD19+ B cells (PB, white circles) and tonsilar CD19+ B cells (TS, grey triangles) from healthy donors. The statistical analysis was done using the Wilcoxon test between CLL and PB or CLL and TS. * p<0.05, ** p<0.001. For miR-181a2b2, the mean value of the CLL samples showed lower methylation as compared to PB and TS samples due to the fact that the majority of CLL samples had very low methylation levels, although some patients showed highly methylated promoters.

Figure 3: Schematic representation of predicted miRNA binding sites at the PLAG1 3’UTR.

CLL-specific deregulated miRNAs were applied to TargetScan 4.1 based prediction of miRNA binding sites for identification of putative targets genes. PLAG1 gene was identified to harbor multiple binding sites of CLL-specific deregulated miRNAs. Evolutionary highly conserved (including chicken) miRNA binding sites of 3’UTR are indicated in black, less
conserved binding sites in grey.

**Figure 4: miRNA reporter assay of PLAG1-miRNA interaction.**

PLAG1-3′UTR was cloned to pIS1-vector containing a renilla luciferase construct. PLAG1-3′UTR-construct was co-transfected with pIS0 firefly luciferase control vector into HEK-293 cells (n=5). For detection of miRNA-binding synthetic miRNAs were co-transfected as indicated. MiR-181a, -181b, -424 and -107 are demonstrated to significantly reduce luciferase activity via PLAG1-3′UTR interaction. Specificity of miRNA-PLAG1-interaction was proven by site directed mutagenesis of miRNA-binding sites in PLAG1 3′UTR. Concerning the miR-181 family two binding sites were mutated revealing abrogated inhibitory effects of miR-181a and -181b.

**Figure 5: PLAG1 expression in CLL and healthy donor B cells.**

PLAG1 mRNA expression was assessed in CLL cells (n=11) vs healthy donor control B cells (n=8) by RT-PCR (A). PLAG1 protein expression was detected by immunoblotting of lysates from CLL cells and healthy donor B cells. Specificity of the PLAG1-specific mAb was proven in PLAG1-transfected HeLa cells (Rec.PLAG1) and co-transfection controls with PLAG1-specific siRNA (D). Protein expression in primary CLL cells (n=29) and healthy donor B-cells (n=22) was assessed by immunoblotting of lysates with PLAG1-specific mAb and quantified by Odyssey densitometry. Two representative experiments are shown (B,C).
**Figure 1**

A

![Box plot showing present calls of miRNAs in con B-cells vs CLL-B-cells](image)

- **Y-axis**: Percent present
- **X-axis**: Control vs CLL
- **Legend**: Present calls for con B-cells vs CLL-B-cells

B

![Heatmap showing miRNA expression in CLL and Healthy Donor B-cells](image)

- **Heatmap color gradient**: Red to blue
- **Legend**: miR-449, miR-565, miR-139, miR-582, miR-107, miR-369-3p, miR-424, miR-143, miR-126, miR-368, miR-199a*, miR-130a, miR-326, miR-199a, miR-126*, miR-584, miR-125a, miR-181a, miR-181b, miR-34a, miR-141, miR-598, miR-451, miR-148a, miR-660, miR-155

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Figure 2

DNA methylation vs miRNAs expression in different cell types:

- miR-125a
- miR-126
- miR-130a
- miR-139
- miR-181a2&b2
- miR-326
- miR-582
- miR-107
- miR-143
- miR-449

Legend:
- PB
- TS
- CLL

Statistical significance:
- * p < 0.05
- ** p < 0.01
Figure 3

PLAG1 3’UTR

Conserved miRNA binding sites

miR-155

miR-181 a+b

miR-369-3p

miR-141 a+b

miR-424

miR-107

Poorly conserved miRNA binding sites

miR-34a

miR-181 a+b

miR-565

miR-191

miR-141

miR-139

miR-141

miR-424
Figure 4A

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Figure 4B

**Top Graph**
- Control miRNA
- miR-107
- miR-107

**Middle Graph**
- Control miRNA
- miR-424
- miR-424

**Bottom Graph**
- Control miRNA
- miR-141
- miR-141
Figure 5

A

Healthy B-cells

PLAG1 mRNA

0,0

CLL

0,2 0,4 0,6 0,8 1,0

B

PLAG1 signal intensity ratio

Healthy Donor B-cells

CLL B-cells

p = 0.014

C

CLL1  CLL2  CLL3  CLL4  CLL5  Healthy B-cell 1  Healthy B-cell 2

PLAG1 55 kD

Beta-Act.

CLL6  CLL7  CLL8  CLL9  CLL10  Healthy B-cell 3  Healthy B-cell 4

D

HEK negative

Rec. PLAG1

Rec PLAG1 + PLAG1 siRNA

Rec PLAG1 + control siRNA
miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia

Christian Philipp Pallasch, Michaela Patz, Yoon Jung Park, Susanne Hagist, Daniela Eggle, Rainer Claus, Svenja Debey-Pascher, Alexandra Schulz, Lukas P. Frenzel, Julia Claasen, Nadine Kutsch, Gunter Krause, Christine Mayr, Andreas Rosenwald, Christoph Plass, Joachim L. Schultze, Michael Hallek and Clemens-Martin Wendtner