Extensive Promoter DNA Hypermethylation and Hypomethylation Is Associated with Aberrant MicroRNA Expression in Chronic Lymphocytic Leukemia

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Abstract

Dysregulated microRNA (miRNA) expression contributes to the pathogenesis of hematopoietic malignancies, including chronic lymphocytic leukemia (CLL). However, an understanding of the mechanisms that cause aberrant miRNA transcriptional control is lacking. In this study, we comprehensively investigated the role and extent of miRNA epigenetic regulation in CLL. Genome-wide profiling conducted on 24 CLL and 10 healthy B cell samples revealed global DNA methylation patterns upstream of miRNA sequences that distinguished malignant from healthy cells and identified putative miRNA promoters. Integration of DNA methylation and miRNA promoter data led to the identification of 128 recurrent miRNA targets for aberrant promoter DNA methylation. DNA hypomethylation accounted for more than 60% of all aberrant promoter-associated DNA methylation in CLL, and promoter DNA hypomethylation was restricted to well-defined regions. Individual hyper- and hypomethylated promoters allowed discrimination of CLL samples from healthy controls. Promoter DNA methylation patterns were confirmed in an independent patient cohort, with 11 miRNAs consistently showing an inverse correlation between DNA methylation status and expression level. Together, our findings characterize the role of epigenetic changes in the regulation of miRNA transcription and create a repository of disease-specific promoter regions that may provide additional insights into the pathogenesis of CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of adults in the Western world and is characterized by clonal accumulation of malignant B cells with a low proliferation rate and disrupted apoptotic mechanisms. The frequent deletion of the long arm of chromosome 13 [del(13q14)] harboring the mir-15a/16-1 locus has drawn attention to microRNA (miRNA) involvement in CLL pathogenesis. miRNAs are small noncoding RNAs that contribute to posttranscriptional gene expression control. They are transcribed by RNA-polymerase II as primary miRNA transcript, processed by the RNase Drosha to pre-miRNAs (referenced as mir in this report) and further cleaved by Dicer to short mature miRNAs (miR). Mature miRNAs are loaded into the RNA-induced silencing complex, where base-pairing of the miRNA to the 3′-untranslated regions of target mRNAs leads to mRNA degradation or inhibition of translation. In recent years, several miRNA expression studies found extensive dysregulation of many different miRNAs in CLL and other tumor entities. Dysregulation of miRNAs was shown to affect expression of tumor suppressor or oncogenes and consequently participate in the initiation and progression of malignant phenotypes. For example, miR-15a/16-1 target the apoptosis regulator BCL2 and thereby act as tumor suppressors. Deletion of both miRNAs was identified to promote CLL in humans and to recapitulate the CLL phenotype in mice. An oncogenic function of miRNAs in CLL has been shown by B cell–specific overexpression of miR-29 giving rise to an indolent lymphocytic leukemia after a latency of approximately 2 years.
The mechanisms leading to aberrant expression of miRNAs are not yet completely understood. Indirect evidence for epigenetic regulation of miRNAs stem from DNA methyltransferase (DNMT) deficient cell lines or studies using demethylating drugs to induce transcriptional reactivation of miRNAs (11–13). Selected candidate miRNAs have been associated with DNA hypermethylation in CLL (6) or solid tumors (14, 15). However, a major drawback of previous reports is the missing identification of miRNA promoters. So far, studies focused almost exclusively on upstream CpG islands or regions in direct vicinity of pre-miRNAs (16, 17). miRNA promoters can be identified by the presence of RNA polymerase II (18) or trimethylated histone 3 lysine 4 (H3K4me3; refs. 19 and 20), a well-known chromatin mark of active transcription. However, considering the constant increase of miRNA annotations (21) as well as the tissue specificity of those surrogate markers, these earlier studies have been able to identify promoters only for a limited set of miRNAs. By now, integration of miRNA promoter regions and epigenetic profiling has been successful in healthy mammary tissue (22).

In this study, we present a systematic genome-wide profiling for epigenetic regulation of miRNAs in CLL compared with healthy B cells by simultaneous detection of aberrant DNA methylation and miRNA promoters. We characterize the extent and role of epigenetic alterations in miRNA transcriptional regulation in CLL specimens and report discovery of novel aberrantly regulated miRNAs. In addition, we generate a repository of identified putative promoter regions that are of high interest to further miRNA-related research questions.

Materials and Methods

Patient specimens

Peripheral blood was obtained from healthy donors and patients with CLL seen at the Department I of Internal Medicine, University Hospital of Cologne, Germany, according to Institutional Review Board approved protocols after receipt of written informed consent according to the Declaration of Helsinki (Supplementary Table S1). Blood specimens from patients with CLL and healthy donors were either enriched for B cells with a purity of more than 95% by applying BRosetteSep (StemCell Technologies) or Ficoll-Hypaque (Seromed) density gradient purification and positive magnetic cell sorting for CD19. Granulocytes and T cells were selected by positive magnetic cell sorting for CD15 and CD3 (Miltenyi Biotec GmbH), respectively. Solid tissue samples were obtained from the tissue bank of the National Center of Tumor Diseases Heidelberg. The following tissues were used: transmural tumor-free colon tissue, tumor-free pancreatic parenchyma, normal lymph node, univacular adipose tissue, benign prostate hyperplasia, tumor-free liver tissue, tumor-free renal cortex, and tumor-free lung parenchyma. DNA and RNA were isolated by DNeasy Blood and Tissue Kit (Qiagen), TRIzol (Invitrogen) or AllPrep DNA/RNA Mini Kit (Qiagen) following the manufacturer’s instructions.

Cell lines and 5-aza-2′-deoxycytidine treatment

For identification of H3K4me3 positive regions upstream of miRNA loci, the CLL related cell lines MEC-1, EHEB, GRANTA-519, and the T cell line JURKAT were obtained from the German Collection of Cell Lines and Microorganisms (DSMZ). Authentication was conducted by the DSMZ using short tandem repeat profiles less than 6 months before experiments were conducted or cells were frozen. The myeloid cell lines KASUMI-1 and HL-60 were obtained from DSMZ and authenticated by the Genomics and Proteomics Core Facility at the German Cancer Research Center using multiplex PCR-based amplification of 24 SNP regions. All cell lines were cultured as recommended by the DSMZ (Supplementary Methods) and treated with 1.5 μmol/L (MEC-1, GRANTA-519, and EHEB) or 200 nmol/L (KASUMI-1) 5-aza-2′-deoxycytidine (Sigma-Aldrich) over 7 days (GRANTA-519 over 8 days) by replacing drug and medium every 24 hours. DNA demethylation efficiency was evaluated by quantitative DNA methylation analysis of repetitive elements (Supplementary Table S2). HCT116 and HTT116 DNMT1−/−, DNMT3B−/− were obtained from Johns Hopkins University, Laboratory of Dr. B. Vogelstein and cultured as previously reported (23).

Methyl-CpG immunoprecipitation

Methyl-CpG immunoprecipitation (MCIp) was conducted as described previously (24) with modifications (Supplementary Information).

Chromatin immunoprecipitation

Cells (2 × 10⁶) were formaldehyde cross-linked as previously described (25) directly after purification (primary cells) or after 5-aza-2′-deoxycytidine treatment (cell lines). The cell pellets were resuspended in 1 mL lysis buffer [50 mmol/L Tris, 10 mmol/L EDTA, 0.5% SDS, Protease Inhibitor Cocktails (Roche)] and sonicated with Bioruptor Next Gen (Diagenode). Immunoprecipitation was conducted by the SX-8G IP-Star Automated System using 2 μL of polyclonal antibody against trimethylated histone 3 lysine 4 (H3K4me3, pAb-003-050), the Auto ChIP Kit and the Auto IPure Kit (Diagenode).

Microarray design

Genomic locations of 939 annotated miRNAs from miRBase 15 (21) and control probes were tiled on an Agilent custom-design 244k array. The probe tiling included 35 kb upstream and 5 kb downstream of the pre-miRNAs and ±2 kb regions around transcriptional start sites of miRNA hosting genes. The Array design is available at Agilent’s array platform (A玛ID27633 for human genome assembly hg18, A玛ADID029434 for hg19).

Microarray hybridization and readout

Fluorescent labeling of enriched DNA fragments was conducted using the BioPrime Total Genomic Labeling System (Invitrogen). Hybridization, washing, and scanning of microarrays were conducted following the manufacturer’s protocol for human CpG island microarrays and mammalian
Chromatin immunoprecipitation (ChIP)-on-chip for MCIp and ChIP, respectively. For array scanning, the Agilent G2565BA Microarray Scanner was used. Data from image files were extracted using Agilent’s Feature Extraction Software 10.5.11 (Protocol: ChIP_105_Dec08. Grid: 027633_D_F_20100318, 029434_D_F_20100720). Microarray data are available online from Gene Expression Omnibus (GEO) under accession number GSE33347.

Analysis of MCIp-chip and ChIP-chip data
Analysis of H3K4me3 ChIP data was conducted with the CoCAS ChIP-on-chip analysis suite (26). MCIp-Agilent array data were analyzed using the statistical environment R v2.11, package RJaCGH v2, and the Bioconductor suite. MCIp-array data were background-corrected by the normal-exponential convolution method (27) with offset 50 and within-array normalized by rank-invariant weighted loess regression (28). For each 40 kb tile, Bayesian nonhomogeneous hidden Markov models were fitted to assign posterior probabilities of DNA hypomethylation, hypermethylation, and no change, incorporating model uncertainty by Bayesian model averaging (29). Using these probabilities, the most recurrent regions of DNA methylation differences over all samples were identified by weighted averages across the posterior probabilities of all CLL samples. Regions separated by spaces less than 650 bp were merged. Regions defined by single probe hits (<100 bp) were excluded from further analysis. Further details of statistical processing are described in Supplementary Information.

Quantitative high-resolution DNA methylation analysis
Quantitative DNA methylation was measured using the MassARRAY system as previously described (30). Primers are given in Supplementary Table S2.

miRNA expression microarray analysis
miRNA expression arrays using BeadArray technology and Human v2 miRNA arrays were processed according to the manufacturer's instructions (Illumina). Arrays were scanned using a BeadArray reader and data were analyzed with Illumina’s GenomeStudio Gene Expression Module. Raw data were quantile-normalized. Detailed of statistical analysis are given in Supplementary Information. Microarray data are available online (31).

Quantitative PCR
miRNA expression by quantitative PCR (qPCR) was carried out using the miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit and miScript Primer Assays (Qiagen) according to the manufacturer’s instructions. Relative miRNA expression was calculated by normalizing to the average of the following housekeeping RNAs: SCARNA17, SNORD25, and SNORD73A.

Target gene prediction and pathway analysis
Targets genes of miRNAs were predicted using miRanda (32) and were considered when reaching a mirSVR (33) score below ~0.1. Pathway analysis was conducted using miTALOS (34).

Results
CLL cells exhibit specific distinct DNA methylation profiles upstream of miRNA loci
To identify disease-specific differentially methylated miRNA promoters in CLL, we applied a strategy of combined DNA methylation profiling and miRNA promoter detection (Fig. 1A). Signal detection was based on a custom 2-color tiling array covering ~35 kb/±5 kb of the pre-miRNA locus and the host gene promoter (Fig. 1B). We enriched methylated DNA from 24 patients with CLL (Supplementary Table S1) and from B cells of 10 age-matched healthy donors combined as reference pool. Hybridization of the methylated DNA fractions of individual patients with CLL against the methylated DNA of the healthy B cell reference allowed identification of differentially methylated regions. We detected pronounced DNA methylation changes in individual patients with CLL compared with the healthy control references. The extent of differential DNA methylation considerably exceeded the interindividual differences observed between healthy donors compared with the reference pool (Fig. 1C). By fitting a Bayesian nonhomogeneous hidden Markov model for each individual array, we assigned sample-specific posterior probabilities of hyper- and hypomethylation to regions larger than 100 bp. To identify regions that harbor a high potential of pathologic relevance for CLL, the most recurrent DNA methylation differences were detected by weighted averages across the posterior probabilities of all CLL samples. Excluding the X chromosome, 531 differentially methylated regions (DMR) with a median size of 385 bp were defined upstream of miRNAs and in host gene promoters. DNA methylation differences (as compared with the reference pool) were subjected to unsupervised hierarchical clustering revealing a clear separation of individual healthy B cell samples from CLL samples and a high abundance of DNA hypomethylated in patients with CLL (Fig. 1D). Of all identified DMRs, 408 regions showed loss of DNA methylation in CLL, whereas 123 regions displayed increased DNA methylation.

Next, we aimed at comprehensively identifying miRNA promoters to assign functional relevance to the discovered differentially methylated regions. We enriched chromatin for trimethylated histone 3 lysine 4 (H3K4me3), a hallmark of active promoters, and analyzed the enriched DNA using the custom tiling array platform. To avoid limitations because of tissue-specific expression patterns of miRNAs, we included healthy B cells, CLL cells, and a variety of tumor cell lines including CLL-related cell lines. In addition, CLL-related cell lines were pharmacologically demethylated by 5-aza-2’-deoxycytidine and HCT 116 cells carrying a double knockout for DNMT1 and DNMT3B (23) were used for promoter identification. All H3K4me3 peak data sets were integrated in a comprehensive list and non-miRNA associated hits were removed. In total, we discovered putative promoter regions for 781 miRNAs, which are subsequently designated as promoters (Supplementary Table S3). The validity of this approach was confirmed by a high overlap with complementary promoter-associated features: 77% of the identified regions coincided with start sites of expressed sequence tags, 71%
exhibited highly accessible chromatin as measured by DNase hypersensitivity, and 34% harbored CpG islands.

Next, we focused on those DMRs coinciding with promoters (Fig. 1D, bottom). Individual patients with CLL carried DNA hypermethylation at a median of 40 (range, 16–55) miRNA promoters and DNA hypomethylation at a median of 60 (range, 12–89) miRNA promoters per patient (Fig. 1E). miRNA promoter-associated DMRs differed substantially in their sequence context as 84% of the hypermethylated but only 6% of the hypomethylated promoter sequences colocalized with CpG islands (Fig. 1F).

**Inverse correlation of promoter DNA methylation and expression of mature miRNA**

In total, we identified 128 miRNAs that carry aberrant DNA methylation at a putative promoter (Supplementary Table S4). Some miRNAs harbor multiple differentially methylated H3K4me3-enriched regions and, thus, may possess 2 or more
Epigenetic Regulation of miRNA in CLL

Table 1. Dysregulated miRNAs in patients with CLL versus B cells from healthy donors

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Location</th>
<th>DMR</th>
<th>Expression difference, log2 FC (miRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypermethylated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mir-124-2</td>
<td>AK124256</td>
<td>chr8:65285833–65286806, chr8:65289299–65291897</td>
<td>Literaturea</td>
</tr>
</tbody>
</table>
| mir-129-2 | i | chr11:43601120–43601393 | –0.48 (miR-129-3p); –0.48 (miR-129’)
| mir-551b | EGFM1P | chr3:167967620–167968099 | –0.22 (miR-551b)
| mir-708 | ODZ4 | chr11:79147754–79148140 | –1.23 (miR-708)
| **Hypomethylated** | | | |
| mir-21 | i | chr17:57916274–57916703 | 0.53 (miR-21’)
| mir-29a, mir-29b-1 | anti | chr7:130585935–130586682 | 0.22 (miR-29a’); 0.40 (miR-29b-1’)
| mir-34a | EF570048 | chr1:9222419–9223806 | 0.32 (miR-34a); 0.36 (miR-34a’)
| mir-135a-1 | anti | chr3:52351422–52351797 | 0.46 (miR-135a)
| mir-155 | MIR155HG | chr21:26933508–26934239 | 0.30 (miR-155); 1.017 (miR-155’)
| mir-574 | FAM114A1 | chr3:38869558–38869937 | 0.88 (miR-574-3p); 0.30 (miR574-5p)
| mir-596 | i | chr8:1736148–1736268 | 0.27 (miR-596)
| mir-664 | RAB3GAP2 | chr2:220393518–220393902 | 0.21 (miR-664); 0.36 (miR-664’)
| mir-1204 | PVT | chr8:128808221–128808385 | 0.15 (miR-1204)\(^\text{b}\)

Abbreviations: anti, antisense to overlapping transcript; FC, fold change; i, intergenic; mir, pre-miRNA.
\(^{a}\)miR-9 and miR-124 were confirmed to be epigenetically regulated in solid tumors and hematopoietic malignancies.
\(^{b}\)miR-1204 did not show a log2 fold change larger than 0.2 in this analysis but was included on the basis of recent expression array data (L.P. Frenzel and C.-M. Wendtner, expression array data; ref. 31).

promoters, for example miR-9. To address transcriptional consequences of promoter DNA methylation for the 128 candidates, array-based miRNA expression data was generated from the same samples used for the DNA methylation screening (Table 1 and Supplementary Table S5).

Inversely correlating DNA methylation and expression, we identified 12 miRNAs that were candidates for DNA methylation dependent regulation: mir-129-2, mir-551b, mir-708 and mir-21, mir-34a, mir-135a, mir-155, mir-574, mir-664, mir-1204, and the cluster of mir-29a/29b-1 (Table 1). Furthermore, mir-9 and mir-124-2 were included as they are known to be frequently epigenetically silenced in various tumor entities (35, 36).

In CLL, aberrant promoter DNA hyper- or hypomethylation in those 14 promoters was confirmed and quantified by high-resolution MassARRAY analysis (Fig. 2). Unsupervised hierarchical clustering of the absolute DNA methylation values revealed a clear separation of patients with CLL from healthy B cells. Of note, DNA methylation data of single miRNA promoters (e.g., mir-1204) was already enough to clearly separate CLL and healthy B cells by unsupervised clustering.

Furthermore, we could validate 10 of 11 additional candidates selected from the list of 128 recurrently differentially methylated miRNA promoters (Supplementary Table S4). These miRNAs did not show significant expression changes in our analysis (Supplementary Fig. S1) but were in part previously identified to be differentially expressed in CLL [miR-132, miR-190 (4), miR-451, and miR-598 (6)].

**Epigenetically silenced miRNAs in CLL**

Promoters of the intergenic mir-124-2 and mir-129-2 as well as the intragenic mir-9-2, mir-551b, and mir-708 showed consistent hypermethylation in an independent patient cohort (Supplementary Table S1 and Fig. 3A and B). DNA hypermethylation correlated with significantly reduced expression as assessed by qPCR (Fig. 3C). None of those have been previously described as being aberrantly regulated in CLL. Mir-129-2 exhibited gain of DNA methylation at 2 CpG islands: one covering the sequence of the pre-miRNA and one 2 kb upstream (Fig. 3A and B). In the initial screening, a stronger increase in DNA methylation was detected at the upstream CpG islands. By luciferase promoter assays, we could show promoter activity of the upstream CpG island compared with neighboring regions (Supplementary Fig. S2). Mir-9 is hosted in the transcript LINC00461, a noncoding RNA that has 3 annotated transcript variants. All 3 promoters showed enrichment of H3K4me3 in healthy B cells and increased DNA methylation in patients with CLL (Fig. 3A). We tested DNA methylation in 2 of these promoters by MassARRAY and found significantly increased DNA methylation. This was in concordance with downregulation of mature miR-9 in the majority of patients with CLLs.
The hypomethylated profiles were specific for CLL and clearly distinguished CLL cells from a large panel of different healthy tissues (Supplementary Fig. S4 and Supplementary Table S6 for expression). We also found that the DNA methylation pattern obtained in those 6 miRNA promoters exhibited pronounced tissue-specific DNA methylation differences clearly discriminating normal hematopoietic cells (B and T cells and granulocytes) and healthy solid tissues by unsupervised clustering. This strong separation was not observed for the hypermethylated miRNA promoters (Supplementary Fig. S4).

For mir-21, high promoter activity was identified in CLL cells at a locus in the last intron of the VMP1 gene covering the previously described promoter (ref. 20; Fig. 4A). In the independent patient cohort, we validated both significant DNA hypomethylation in a promoter-associated sequence stretch 2 kb upstream of the mir-21 sequence and a 2-fold upregulation of miR-21 expression (Fig. 4B and C). Mir-34a is located within transcript EF609116 carrying a promoter CpG island (Fig. 4A). We discovered an alternative site 12 kb upstream of the mir-34a sequence characterized by H3K4me3 enrichment and other promoter-associated features (e.g., DNase hypersensitivity). Regionally limited, significantly reduced DNA methylation was found only at this alternative site in both the screening and the validation cohort (Fig. 4A and B). This DNA hypomethylation correlated with upregulation of miR-34a in patients with CLL (Fig. 4C). For mir-155, we found DNA hypomethylation in CLL cells clearly limited to a sequence stretch adjacent to the promoter CpG island of the MIR155 host gene (MIR155HG; Fig. 4A). This was validated in the independent cohort of patients with CLL (Fig. 4B). Although not covering the CpG island, the hypomethylated region is restricted to clearly defined CpG dinucleotides and coincides with numerous transcription factor binding sites including the miR-155 regulator MYB (40) and the miR-155 target PU.1 (ref. 41; Supplementary Fig. S5A). Loss of promoter DNA methylation in this region correlated with increased expression of mature miR-155 (Fig. 4C). The relevance of this region adjacent to the CpG island is further supported by a high correlation of tissue-specific DNA methylation with expression of miR-155 in the respective tissue ($R^2 = 0.7$). The CpG island itself displayed a homogeneously low level of DNA methylation not correlating with expression of miR-155 in the analyzed tissues (Supplementary Fig. S5B).

To evaluate the functional importance of the epigenetically reactivated miRNAs, we combined miRNA target prediction with tissue specific pathway analysis by the miTALOS platform (34). We found significant enrichment for targets involved in apoptosis ($P = 0.005$), a pathway known to be defective in CLL.

**Discussion**

Increasing evidence supports the hypothesis that epigenetic mechanisms are involved in regulating miRNA expression (6, 12, 17, 42, 43). In this study, we used a combined strategy to assign aberrant DNA methylation to putative miRNA promoters. Thereby, we identified extensive disease-specific DNA
methylation patterns. Moreover, we discovered that not only hypermethylation but also hypomethylation at putative miRNA promoters correlates with expression of the mature miRNAs.

Enrichment of H3K4me3 was previously showed to serve as a valid and reliable surrogate for active promoters of protein coding genes and miRNAs (19, 44). To obtain a large diversity of H3K4me3 signals, we used a variety of cell lines derived from different tissues in addition to primary healthy B cell and CLL samples. In total, we were able to identify putative promoter regions for 781 miRNAs. Most of the promoter regions are novel and coappearance with additional promoter characteristics demonstrated the validity of our approach. Of the limited number of previously published promoters (18, 20), an overlap of more than 70% with our data set could be noted.

Profiling of DNA methylation at the identified promoter regions revealed 38 and 90 miRNAs as consistent targets of promoter DNA hypermethylation or hypomethylation, respectively. Thus, our results clearly indicate that differential DNA methylation is frequent at miRNA promoters and thereby might constitute a major mechanism leading to transcriptional miRNA deregulation in CLL. We show that not only aberrantly increased but also decreased DNA methylation levels at miRNA promoters are of functional relevance for the transcriptional control of miRNAs. This finding has so far been underestimated. Particularly, decreased DNA methylation at distinct loci (e.g., mir-1204, see Fig. 4B) generated high contrasts of DNA methylation levels and clearly separated CLL cases from controls. The hypomethylated promoter regions in CLL showed a high degree of tissue-specific methylation in a panel of healthy tissues including hematopoietic lineages as well as solid tissues. This further supports the regulatory importance of these regions (see Supplementary Fig. S4). It is noteworthy that the observed DNA hypomethylation events in CLL were regionally limited with clear boundaries to surrounding sequences.
as seen for mir-29a/29b-1 (see Fig. 4A and B) and mir-155 (see Supplementary Fig. S5). These patterns suggest activity of directed demethylating mechanisms rather than unspecific genome-wide loss of DNA methylation. The frequent appearance of hypomethylated miRNA promoters correlates with the observation that in CLL, more miRNAs are upregulated than downregulated (4, 5), which is in contrast to many other malignancies. Although DNA hypermethylation occurred preferentially in CpG islands, decreased DNA methylation was nearly exclusively found in CpG-poor regions (see Fig. 1F). This is in agreement with recent observations reported for protein-coding genes (45). The focus on CpG islands and the detection of differentially methylated miRNA promoters by pharmacologic DNA demethylation could be one of the reasons why previous studies underestimated the extent of DNA hypomethylation (6, 12, 16).

We focused on 11 miRNAs that were consistently epigenetically deregulated and showed correlation of expression and DNA methylation in an independent validation cohort of patients with CLL thus suggesting general relevance for CLL pathogenesis. However, methylation of distinct miRNA promoters could also recapitulate clinically relevant subgroups as it has already been shown for miRNA expression. For example, expression levels of the miR-29 family or miR-34a

Figure 4. DNA hypomethylation corresponds with increased miRNA expression. Figure systematics correspond to Fig. 3. A, array data obtained for genomic loci upstream of miRNA. Enriched H3K4me3 profiles were obtained from CLL cells or the related cell line EHEB (mir-34a and mir-574). B, quantitative DNA methylation was measured in an independent validation set by MassARRAY and displayed as heatmap. C, expression of the mature miRNA was determined by qPCR; median is shown as black line. Significances of DNA methylation and expression differences were tested by Mann–Whitney U test.
(46) possess prognostic relevance and form subgroups of differential clinical outcome. Interestingly, their promoter methylation pattern is, although generally lower in CLL, not completely homogenous among patients (see Fig. 4B). Follow-up analyses in large CLL study cohorts may allow dissecting prognostically or therapeutically relevant subgroups based on miRNA promoter methylation.

The epigenetically dysregulated miRNAs show enrichment of target genes involved in apoptosis, a defective key pathway in CLL cells (1) and have predicted targets genes, recently identified to carry mutations in functional domains in CLL.

Among the transcriptionally repressed miRNAs, we identified mir-129-2, which was previously detected to be epigenetically silenced in solid tumors where it functions as a tumor suppressor by targeting the mRNA of the oncopgene SOX4 (14). This work, however, focused on increased DNA methylation at the CpG island that directly covers the pre-miRNA sequence stretch. By luciferase reporter constructs, we verified promoter activity at a different significantly hypermethylated CpG island located approximately 2 kb upstream of the pre-mir-129-2. The regulatory relevance of this site is further supported by the start of 2 expressed sequence tags (BR64058, BD120451) that could be identical with the primary miRNA transcript. An alternative epigenetically altered site was also detected for mir-34a, a downstream target of the p53 pathway. DNA hypermethylation at the promoter of the hosting transcript EF69116 has been extensively studied in various tumor entities (47). We detected a potential alternative transcriptional start site approximately 12 kb upstream of the mature miRNA located within the hosting transcript. In CLL, significant DNA hypomethylation accompanied by increased expression could be noted, whereas the hosting transcript promoter did not exhibit differential DNA methylation (see Fig. 4A). Whether this novel regulatory site is also aberrantly methylated in other tumor tissues remains to be determined.

For the epigenetically reactivated miR-155, we identified a regionally restricted significantly hypomethylated region adjacent to the promoter CpG island coinciding with transcription factor binding sites of PU.1, NF-kB, and an MYB consensus sequence (see Supplementary Fig. S5). MYB was recently shown to bind to the mir-155 promoter and thereby to contribute to its regulation in CLL (40). The high correlation of miR-155 expression and DNA methylation in this region as determined in different healthy tissues emphasizes the regulatory function of the region adjacent to the CpG island. Evidence for the importance of miR-155 in CLL is provided by overexpression in a mouse model, which leads to a high-grade B cell malignancy (48).

In addition to miRNAs previously described in the context of CLL pathogenesis, we also identified epigenetic regulation of novel miRNAs (e.g., miR-551b), showing that combined epigenetic profiling and expression screening is an effective strategy of identifying novel aberrantly transcribed miRNAs. Recent miRNA expression studies generated partially inconsistent candidate lists (5, 6, 49). In future studies, combined analyses of expression and epigenetic profiles could increase sensitivity and improve the detection of significantly and constantly deregulated miRNAs. Therefore, the generated repository of 781 putative miRNA promoters is a valuable resource for epigenetic and functional analyses also in other entities.

Remarkably, the number of deregulated miRNAs in the previously published expression profiling studies (5, 6, 49) was much smaller than the number of epigenetically altered miRNA promoters identified in our work. Several reasons might account for this discrepancy. First, many miRNAs are transcribed from different loci in the genome, for example mir-9–1, mir-9–2, and mir-9–3, and share identical sequences of their mature forms. To date, array-based expression analysis does not offer the possibility to discriminate these transcripts and to assign aberrant transcription to altered DNA methylation at distinct promoters. Second, it has been shown for T cells that an altered epigenetic status does not necessarily affect the transcription of a miRNA directly but poises miRNA promoters and creates a permissive state for transcription initiation upon activating signals (50). Thus, in CLL, the distinct identified epigenetic profile could be representative for transcriptional activity upon different pathogenesis relevant stimuli, for example microenvironment contact or cell stress.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Baer, R. Claus, L.P. Frenzel, Y.J. Park, C.P. Pallasch, J.C. Byrd, C.-M. Wendtner, C. Plass
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Baer, R. Claus, L.P. Frenzel, E. Herpel, C.-M. Wendtner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Baer, R. Claus, L.P. Frenzel, M. Zucknick, L. Gu, M. Fischer, C. Plass
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Claus, L.P. Frenzel, E. Herpel, J.C. Byrd
Study supervision: R. Claus, C. Plass

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