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Allele-specific loss and transcription of the miR-15a/16-1 cluster in chronic lymphocytic leukemia.

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Deregulation of the miR-15a/16-1 cluster has a key role in the pathogenesis of chronic lymphocytic leukemia (CLL), a clinically heterogeneous disease with indolent and aggressive forms. The miR-15a/16-1 locus is located at 13q14, the most frequently deleted region in CLL. Starting from functional investigations of a rare SNP upstream the miR cluster, we identified a novel allele-specific mechanism that exploits a cryptic activator region to recruit the RNA polymerase III for miR-15a/16-1 transcription. This regulation of the miR-15a/16-1 locus is independent of the DLEU2 host gene, which is often transcribed monoallelically by RPII. We found that normally one allele of miR-15a/16-1 is transcribed by RPII, the other one by RPIII. In our subset of CLL patients harboring 13q14 deletions, exclusive RNA polymerase III (RPIII)-driven transcription of the miR-15a/16-1 was the consequence of loss of the RPII-regulated allele and correlated with high expression of the poor prognostic marker ZAP70 (P = 0.019). Thus, our findings point to a novel biological process, characterized by double allele-specific transcriptional regulation of the miR-15a/16-1 locus by alternative mechanisms. Differential usage of these mechanisms may distinguish at onset aggressive from indolent forms of CLL. This provides a basis for the clinical heterogeneity of the CLL patients carrying 13q14 deletions.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in the Western world,1 is characterized by a heterogeneous clinical course, essentially divided into indolent and aggressive forms.2 Deletion of 13q14 is the most frequent genetic aberration associated with CLL3–6 and represents an early disease-associated event.7–9 Although generally considered a favorable prognostic factor,10,11 CLL patients bearing this deletion show heterogeneous clinical outcomes, that can be partially stratified according to 13q deletion subtype.12,13

In vitro and in vivo studies14–17 conclusively demonstrate that the miR-15a/16-1 cluster, located at 13q14, controls cell cycle and apoptosis in B cells and that deregulation of these miRNAs contributes to the pathogenesis of CLL. The 13q14 deletion is one of the main causes of the downregulation of the miR-15a and miR-16-1 in CLL, although it should be considered that miR-15a/16-1 expression shows allelic imbalances,18,19 and that TP53 mutations or deletions affect the transcription and/or maturation of the primiR.20 This cluster is located within the DLEU2 gene, whose expression is controlled by epigenetic mechanisms entailing a monoallelic expression in lymphoid cells from both CLL patients and healthy controls.21 This occurs for the majority of the genes located at this region, suggesting that the miR-15a/16-1 cluster could be regulated in the same way.

In the present work, we studied the region immediately upstream the miR-15a/16-1 cluster. We first investigated a functional SNP, rs115069827, whose minor allele abrogates the maturation of primiR-15a/16-1. We then demonstrated that the region surrounding the SNP functions as an activator of transcription by both RNA polymerase II and RPII. The RPII-driven transcription of primiR-15a/16-1 from this cryptic activator is allele-specific and, in our CLL patients, results dominant in the cases with 13q14 deletions that are characterized by high expression of ZAP70, a predictor of poor prognosis.

MATERIALS AND METHODS

Primary cells, cell lines and cell culture

CLL samples were obtained from patients enrolled in the CLL Research Consortium and ‘Policlinico Agostino Gemelli’, ‘Università Cattolica del Sacro Cuore’, Rome, on written informed consent in accordance with the Declaration of Helsinki. The study protocols were approved by the Institutional Review Boards of The Ohio State University and the ‘Policlinico Agostino Gemelli’. The participating institutions provided the clinical data associated with each patient at the time of sample collection. Among patients, there was a rare case of monozygotic twin sisters that share a germline single nucleotide variation in ARLT5.22 The samples were analyzed to determine expression of ZAP70, immunoglobulin heavy chain variable mutational status and karyotype, as previously described.22 Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Ficoll-PaquePlus (Amersham Biosciences, Uppsala, Sweden). Cell lines WaC3CDs (WAC),23 MEC-2, MEC-1,24 HEK293T, K562 (from ATCC) and CLL cells were cultured in RPMI1640, supplemented with 10% fetal bovine serum of Australian origin, 2 mmol/l l-glutamine, 100 units/ml of penicillin and 100 units/ml streptomycin in a humidified incubator containing 5% CO2 at 37°C. All the cell lines were

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authenticated by short tandem repeat analysis (Promega, Fitchburg, WI, USA, PowerPlex16HS) comparing the results with the on-line databases (www.lgstandards-atcc.org/en/STR_Database.aspx?slp=1; www.dsmz.de/泌/cgi-bin/str.html; http://strdb.cogcell.org/) (Supplementary Table S5). The STR profile of WaC3CD5 was not in the databases. MEC-1 and MEC-2 cell lines, which originate from the same patient and share the same STR profile, were authenticated by karyotype analysis (Supplementary Table S5). The densities of cultured cells were maintained between 1 and 3 million/mL. Epstein–Barr virus infection of CLL cells was conducted as described by Doyle et al.25

Cell treatments, transfection and luciferase assay
The RNAiP inhibitor, 2-aminantin (A2263 Sigma, St Louis, MO, USA) was dissolved in di-distilled sterile water and filtered by 0.2 μm filter. The experiments were performed by using growing doses of a-aminatin, that is, 1, 2, 3, 5, 10 μg/mL on each point of 2000.000 cells/ml or 2 and 10 μg/mL on each point of 500.000–1000.000 cells/ml. After 16 h, the cells were lysed in 700 μl of QiZol (Qiagen, Venlo, Netherlands) for the RNA extraction.

Transfection of DNA vectors (200–500 ng/50,000 cells) in HEK293T was performed with Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s procedures, whereas transfection of MEC-1, MEC-2, WaC3CD5 and K562 was performed using the Amaxa nucleofector (Lonza, Basel, Switzerland) according to the manufacturer’s protocols (plasmids:3000 ng/1,000,000 cells; small interfering RNA 40 pmol/1,000,000 cells). siPol3RNA (sc-90684), siPol3RG (sc-43507) and small interfering RNA -A as control (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The efficiency of transfection was determined by cytofluorometry, messenger RNA expression and/or fluorescence microscopy. After transfection, the luciferase reporter vectors (pGL3Ren-187_A and pGL3Ren-187_G), the luciferase assay was conducted according to manufacturer’s dual luciferase assay protocol (Promega).

Cell staining for flow cytometry
Cell staining was performed as already reported.26,27 Briefly, 5 × 106 cells per sample were washed, resuspended in 100 μl of surface antibody mix (1 μl sample of anti CDS-PE and 1 μl/sample of anti CD19-PECy5) and incubated for 30 min at 4 °C in the dark until acquisition. Phycoerythrin-conjugated anti-CD5 (CD5-PE) and 1 μl of surface antibody mix (1 μl/sample of anti-CD19-PECy5) were purchased from BioLegend (San Diego, CA, USA). Cell staining for flow cytometry was performed as already reported.26,27 Briefly, 5 × 106 cells per sample were washed, resuspended in 100 μl of surface antibody mix (1 μl sample of anti CDS-PE and 1 μl/sample of anti CD19-PECy5) and incubated for 30 min at 4 °C in the dark until acquisition. Phycoerythrin-conjugated anti-CD5 (CD5-PE) and PE-cyanin 5-conjugated anti-CD19 (CD19-PECy5) were purchased from BioLegend (San Diego, CA, USA).

DNA extraction, sequencing and copy number variation
Genomic DNA was isolated from cell lines and PBMC from CLL samples by standard treatment with Het’s solution in the presence of 200 μg/ml of proteinase K, followed by phenol/chloroform/soamyl extraction and ethanol precipitation. The DNA regions of interest were amplified with primers detailed in Supplementary Table S4 and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Total RNA (50–200 ng) was retrotranscribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies). The primiR exogenous expression was assessed with the SYBR green technology (Qiagen), while the endogenous expression of the primiR was performed with TaqMan assay (Life Technologies). To test DNAse treatment efficiency, cDNA synthesis was performed without the enzyme reverse transcriptase (nORT-PCR).

MicroRNas quantization was performed using stem loop RTPR primers designed28 with a modification to include the UPL #21 sequence binding site.29 UPL probe #21 was from UPL database (Roche Diagnostics, Basel, Switzerland). Total RNA (50 ng) was retrotranscribed with TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). Reactions were incubated 30 min at 16 °C, followed by pulsed RT of 60 cycles at 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s.30 Real-time PCRs were performed in an Applied Biosystems 7900 instrument. MiRNA and messenger RNA levels were measured using Ct (threshold cycle). Target amount, normalized to an endogenous references 18S/RNU44 and relative to a calibrator, is given by 2−ΔΔCt and/or 2−ΔCt (Life Technologies).

RESULTS
A single nucleotide variation upstream the mir-15a-16-1 cluster in CLL patients
Because of the importance of the mir-15a-16-1 cluster in CLL, we sequenced ~1000 nucleotides around this genomic region in 249 DNA samples from PBMCs of CLL patients. We found A–G heterozygosis (rs115069827) in DNA from two CLL patients, CLL479 and CLL585 (Table 1, Supplementary Figure S1a). This heterozygosis (rs115069827) in DNA from two CLL patients, was on a median average of 8.15. Gene sequences were from NCBI (http://www.ncbi.nlm.nih.gov/), RNA sequences for miR-15a-16-1 were measured using Ct (threshold cycle). Target amount, normalized to an endogenous references 18S/RNU44 and relative to a calibrator, is given by 2−ΔΔCt and/or 2−ΔCt (Life Technologies).
of miR-15a (Figure 1). To complete the genetic analysis of these samples, we confirmed germline heterozygosity of CLL479 and CLL585 by analyzing DNA from buccal swab of the two patients.

The genomic region upstream the miR-15a/16-1 cluster acts as its transcriptional activator and is not affected by the SNP rs115069827 to determine whether the A–G SNP affected miR-15a/16-1 expression, we studied the genomic region encompassing the variant allele. At first we speculated that this region could act as transcriptional activator of the candidate miRNAs and that the G and A variants differently influence miRNAs expression. To test this, PCR-amplified fragments (187 bp), comprising either the A or the G variants, were obtained from CLL479 and subcloned into a modified version of the pGL3 basic vector including Renilla luciferase as internal control for transfection efficiency. HEK293 and MEC-02 cells were transiently transfected with the generated constructs (pGL3Ren-187_A and pGL3Ren-187_G). The results indicated strong promoter activity of the subcloned fragments, with no differences between the two variants (Figure 2a). To assess the primiR expression, we then cloned a broad region (1070 bp), including the entire cluster and the putative promoter with either the A or the G variant (Figure 2b), in the lentiviral expression vector lacking the constitutive promoter (pCDF1070_A and pCDF1070_G). The vectors were transiently transfected into K562 and WAC cells. We noted a threefold induction of exogenous primiR-15a/16-1 expression compared with the controls (empty vector), irrespective of the variant. The results were more convincing in the WAC cell line, in which only exogenous primiR was detectable, as these cells lack both alleles of the miR-15a/16-1 cluster locus. The primiR was normalized on GFP expression, to exclude differences in transfection efficiency, and the effective DNase treatment was tested by PCR in the RNA samples (noRT, Supplementary Figure S2).

These findings suggest that the regulation of the primiR-15a/16-1 can occur independently of its host gene DLEU2.

To corroborate these results, we correlated the expression of miR-15a and miR-16 with that of DLEU2 in two subgroups of patients with different CLL karyotype and prognosis.
and 11q deletion).31 Respective Spearman correlation factors (r) for miR-15a and miR-16 were 0.55 (P = 0.0095) and 0.47 (P = 0.031) for all the CLL cases; 0.83 (P = 0.0008) and 0.86 (P = 0.0003), for the CLLs with the 13q deletion; conversely, 0.166 (P = 0.67) and 0.3 (P = 0.43) for the CLLs with 11q deletion (Supplementary Figure S3). This evidence further suggests that the miR-15a/16-1 has its own transcriptional activator in the immediate 5’ flanking region and that, according to CLL karyotype, its regulation is related or unrelated to DLEU2 expression.

The SNP rs115069827 affects the maturation of miR-15a
As the SNP rs115069827 does not affect the strength of the newly identified transcriptional activator, we investigated its role in the processing of the miR-15a. We evaluated the expression of the primiR and the miR-15a in PBMCs from CLL479 and CLL585 (both heterozygous for rs115069827 SNP), from 5 CLL cases with normal karyotype and from five healthy donors. Among these samples, CLL479 showed the highest primiR expression level, whereas the expressions of the mature forms of miR-15a and miR-16 were significantly downregulated, in line with that of the other CLLs (Figure 3a), according to the published data.4 In matched samples, the median ratios between primiR and miR-15a expression in the CLLs and in the normal samples were respectively 0.002 and 0.028 (P = 0.017) for miR-15a, versus 1.72 and 8.34 for miR-16 (Figure 3b, Supplementary Table S1); hinting at a strong relevance of impaired miR-15a/16-1 regulation in CLL. The ratio for miR-15a was even lower (0.00047) in CLL479, suggesting that in this specific case, defective maturation was the main cause of miR-15a downregulation. In this analysis, the expression of miR-16 was not considered, as it is also transcribed by a different genomic locus. Of note, CLL585, also positive for the SNP, showed a miR-15a/primiR ratio similar to that of CLL479 (0.00076) (Supplementary Table S1). CLL585 carries a homozygous deletion of the miR-15a/16-1 locus in B cells and hence, the detected miR and primiR must have originated from non-B cells. Taken together, these data support the involvement of rs115069827SNP in the maturation steps of miR-15a, in both leukemic and healthy cells. To investigate this, we evaluated the expression of the mature form...
of the miR-15a in K562 and WAC cells transfected with either pCDF-1070_A or _G. In both cell lines, the expression of the miR-15a from the vector with the G variant was lower than that from the vector with the A variant (Figure 3c), in spite of the allele type independent mode of expression of the exogenous primiR-15a/16-1 (Figure 2b). For miR-16-1, similar results were obtained in B cells derived from the transgenic CLL mouse model Eµ-TCL132 and infected with lentivirus particles transducing the 1070 bp region with either the A or the G allele (Supplementary Figure S4). SNPs in the flanking non-structured RNA sequences of microRNAs can change the hairpin structures of the primiR, interfering with the binding of proteins necessary for their processing to the mature forms.41 Given that in mice the DEAD-box helicase subunits of the Drosha complex (p68 and p72) are required for the processing of primiR-15a/16-1 to mature miR-16-1,38 we evaluated the efficiency of the interaction between the DROSHA complex and the primiR-15a/16-1 in K562 cells transfected with the pCDF-1070_A or _G vectors. By RIP analysis, the G allele showed reduced DROSHA binding capacity when compared with the A allele (Figure 3d).

RPIII is involved in the transcription of the primiR-15a/16-1

During the course of our experiments, we found that the 107 bp region immediately upstream the microRNAs stem loops acts as transcriptional activator of miR-15a/16-1 locus and is transcribed as well. This is explainable considering either RPIII occupancy at this region or a mechanism of transcription independent of the RPI/RPII/RPIII, as recently described.29 We first investigated the involvement of RPIII. We tested the primiR-15a/16-1 expression in K562 cells transfected with either the pCDF_1070A/G vectors or the control after treatment with the RPII inhibitor α-amanitin. The left side of Figure 4a shows that the RPII takes part in the transcription of the primiR-15a/16-1 derived from the pCDF_1070A/G vectors. However, as these pCDF vectors hold an RPII promoter (EF1, upstream the GFP reporter gene) we could not exclude its influence at this region and/or a cooperation between RPII and RPIII transcription.40,41 Therefore, we performed a similar experiment after subcloning the 1070 bp fragments within the pCR2.1 vector, to obtain pPCR2.1-1070_A/G vectors. We chose this plasmid because it does not contain the exogenous promoters and/or regulatory sequence. Treatment with α-amanitin significantly reduced the endogenous ACTB pre-messenger RNA used as control in the transfected cells (Supplementary Figure S5a), but did not reduce the exogenous primiR-15a/16-1 expression (Figure 4a), suggesting the existence of an RPII-independent mechanism of transcription. Controls show that plasmid contamination, although not completely removed, was very low and irrelevant to the experimental results (Supplementary Figure S5b).

Similar results were observed by measuring the expression of the miR-15a (Supplementary Figure S5c and d). To further support RPIII involvement in primiR-15a/16-1 transcription, we silenced two polymerase III subunits (POLR3A and POLR3G) in K562 cells, to inhibit the RPIII activity. POLR3G is a specific subunit of the RPIII complex, but its silencing does not prevent RPIII elongation,42 while POLR3A is specifically implicated in the elongation of the RPIII transcripts.43 At 48 h from silencing, the cells were transfected with the pCR2.1_1070_A vector to induce primiR transcription by RPIII. Silencing of the POLR3A gene reduced the expression of the primiR and of trRNA tyr (transcribed by RPIII), while the expression of the ACTB pre-messenger RNA, transcribed by RPII, was not affected; (Figure 4b). Moreover, by ChIP analysis, we also observed slight binding of RPIII to the new transcription activator region, apparently independent of the SNP allele (Figure 4c). Finally, we performed ChIP analysis with the RPIII RPC32 subunit on the MEC-1 B-CLL cell line24 transfected with the pCDF_1070_A vector and treated or not with α-amanitin. Given that the pCDF_1070_A vectors drive primiR-15a/16-1 expression by RPII (Figure 4a), we tested RPII occupancy after inhibition of RPII transcription. We registered twofold primiR-15a/16-1 DNA enrichment only in cells treated with α-amanitin (Figure 4d). A similar experiment was conducted in K562 cells by measuring the endogenous primiR-15a/16-1 expression. K562 cells harbor a monoallelic 13q deletion and show RPII-dependent transcription of primiR-15a/16-1 (Supplementary Figure S5d). The ChIP analysis shows a primiR-15a/16-1 DNA enrichment only in K562 cells treated with α-amanitin (Figure 4e). These results confirm RPIII involvement in a different cell line and support the participation of RPIII in primiR-15a/16-1 transcription only when RPII activity is prevented or inhibited.

Allele-specific transcription of primiR-15a/16-1

To assess the existence of an RPII-independent mechanism at the candidate region in CLL patients, we evaluated the expression of the primiR-15a/16-1 in PBMCs from CLL patients upon α-amanitin treatment. To evaluate one allele at the time, we chose a subset of 31 cases (48% of the total analyzed patients) in which the 13q region showed macroleodizations (by FISH) and/or microdeletions (by copy number variation analysis of the DLEU2/miR-15a/16-1 region, Supplementary Table S2, Supplementary Figure S1b). Regulation of primiR-15a/16-1 was respectively scored as RPII dependent, in the samples that upon treatment shared a trend similar to that of preACTB and/or preGAPDH and opposite to that of the trNA tyr gene, or RPIII-dependent, in the samples where primiR expression was either unchanged or increased after α-amanitin treatment (Supplementary Figure S6). Twenty out of 31 samples were informative on the basis of technical controls. The results showed that in 8/20 cases (40%), the regulation of primiR-15a/16-1 was RPII-dependent, while in the other 12 cases (60%) was driven by RPII (Table 2), suggesting that the two alleles of this locus are differentially regulated. As we could not distinguish whether the RPIII involvement arises from pathologic and/or physiologic mechanisms, we decided to test PBMCs from the patient CLL585 with α-amanitin. This patient has a bi-allelic deletion of the miR-15a/16-1 locus in B-CLL cells, but it still shows heterozygosis of the SNP rs115069827 at both DNA and RNA levels, likely arisen from the non-B cells. Therefore, α-amanitin is predicted to affect only the ‘normal’ non-B PBMC population. By sequencing, we demonstrated selective and progressive inhibition of the expression of the A allele in an α-amanitin dose-dependent manner, while the expression of the G allele was unchanged (Figure 5). Overall, these data support allele-specific regulation of the primiR-15a/16-1: one allele is mostly controlled by the DLEU2 promoter/s, whereas transcription of the other allele occurs through the recruitment of RPII to the newly identified transcription activator region.

Allele-specific transcription of primiR-15a/16-1 is associated with ZAP70 expression in CLL

In the tested cases, we noted that the newly identified RPII-dependent mechanism predominated in CLLs with high expression of ZAP70 (Fisher’s exact test P = 0.019). Indeed, most of the samples with high ZAP70 (6 out of 8) showed transcription of the primiR-15a/16-1 by RPII, conversely in the cases negative for ZAP70, primiR-15a/16-1 transcription was mostly regulated by RPII (10 out of 12 CLL patients) (Table 2, Supplementary Figure S6). Notably, we had informative monozygotic twins with different clinical course of the disease: CLL3tw, aggressive, and CLL4tw, indolent (Supplementary Table S3). CLL cells from these twins were immortalized and treated with α-amanitin. Upon treatment, we noted transcription of the primiR-15a/16-1 respectively mediated by RPII in cells from CLL3tw (aggressive CLL) and by RPII in cells from CLL4tw (indolent CLL) (Supplementary Figure S6b, Table 2). To evaluate at molecular level the differences between B-CLL cells in which the mir-15a/16-1 cluster is transcribed by RPII versus RPIII, we measured the levels of
miR-15a, primiR-15a/16-1 as well as TP53, BCL2 and Cyclin E proteins, direct targets of miR-15a, in our B-purified CLL samples. Although we did not find changes in the expression of primiR-15a/16-1 and/or miR-15a (data not shown), we registered differences in the protein levels of BCL2, TP53 and Cyclin E with decreasing trend in the CLL samples in which the primiR-15a/16-1 was transcribed by RNPIII (Supplementary Figure S7); suggesting that RNPIII/RNPII regulation of miR-15a/16-1 affects the target genes. Considering ZAP70 status, a similar trend was observed only for TP53, while for BCL2 the protein levels were differently distributed between the two groups (F-test, $P = 0.0014$).

In our survey (training set $n = 36$, validation set $n = 18$) there were samples with 13q deletions that did not include the miR-15a/16-1 locus. Almost all of these cases showed high expression of ZAP70 (Fisher’s exact test: training set $P < 0.001$; validation set $P = 0.006$; Supplementary Table S2) and, in those whose cells were available, despite the presence of both alleles of miR-15a/16-1, we detected a RNPIII-dependent primiR regulation. Therefore, we performed copy number

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**Figure 4.** RNPIII is involved in the transcription of primiR-15a/16-1. (a) PrimiR-15a/16-1 expression in K562 cells transfected with the pCDF1070_A/G (left side) and pCR2.1_1070_A/G vectors (right side) with and without α-amanitin treatment (2 μg/ml). (b) PrimiR-15a/16-1, preACTB, tRNA tyr, POLR3A and POLR3G relative expression (normalized on 18S expression) in K562 cells silenced for polymerase III subunits (POLR3A, POLR3G) and then transfected with the pCR2.1_1070_A vector. (c) ChIP analysis for RNPIII occupancy in the primiR-15a/16-1 and tRNA tyr (as control) DNA regions by qPCR in K562 cells transfected with pCR2.1_1070_A/G vectors. Controls are represented by the pCR2.1 Empty vector (e) and the non-specific unconjugated rabbit polyclonal antibody immunoglobulin G IP. Pre 15-16 and Pri 15-16 are two different genomic DNA fragments of the miR-15a/16-1 cluster region (Supplementary Table S4). (d) ChIP analysis for RNPIII occupancy in the primiR-15a/16-1 DNA region by qPCR in MEC-1 cells transfected with pCDF_1070_A vector with and without α-amanitin treatment. Controls are represented by the non-specific unconjugated rabbit polyclonal antibody immunoglobulin G IP and as there is not primiR-15a/16-1 DNA enrichment in the non α-amanitin-treated MEC-1 cells, the amplification of the DNA IP with RNPIII could be considered a specific signal. (e) ChIP analysis for RNPIII occupancy in the primiR-15a/16-1 and tRNA tyr (as control) DNA regions by PCR in nontransfected K562 cell line. $P$ value $> 0.05$ was considered not significant, between 0.01 to 0.05 (*) significant, between 0.001 to 0.01 (**) very significant and < 0.001 (***) extremely significant.
variation analysis of a more telomeric DNA region (~30 kb, U59 DLEU2 region), that encompasses the second DLEU2 promoter. We found monoallelic deletion of this region, resulting in the loss of the primiR regulation by the RPII-dependent DLEU2 promoter.

The molecular model of the DLEU2/miR-15a/16-1 locus we propose is recapitulated in Figure 6, that illustrates the genetic characteristics and their associations with ZAP70 status.

### DISCUSSION

The minimal deleted region of 13q14 starts about 20 kb upstream to the cluster encompassing the second DLEU2 promoter and most genes localized in this critical region are downregulated by a factor 4 in the CLLs with monoallelic 13q14 deletions. The miR-15a/16-1 cluster is located within the DLEU2 gene and, monoallelic DLEU2 expression has so far been demonstrated in 20–40% of B and T cells from healthy donors, suggesting that there could normally be monoallelic expression of the miR cluster as well. As miR-15a and miR-16-1 are strongly implicated in the pathogenesis of the disease, we aim to clarify the transcriptional regulation of the miR-15a/16-1 cluster in CLL. Here, we demonstrate that the transcription of the cluster is regulated simultaneously by alternative mechanisms that use either RPII or RPIII in allele-specific manner. The loss of the transcriptional regulation of primiR-15a/16-1 by RPII is associated with high expression of ZAP70, a poor prognostic feature of CLL patients.

We reached these results through characterization of the SNP rs115069827, identified in two CLL patients at about 100 bp upstream the miR-15a stem loop, able to abrogate the maturation of the primiR-15a/16-1. This SNP is epidemiologically irrelevant for
CLL, because of its rarity, but allowed us to demonstrate the existence of allele-specific effects on the transcription and, consequently, the expression of miR-15a. In fact, the chromosome region immediately upstream the miR cluster, once isolated from other eukaryotic regulatory elements, was able to promote RNA transcription by an RPII-independent, allele-exclusive and RPIII-engaging mechanism. This novel mechanism is consistent with the results of an independent study demonstrating that the primiR-15a/16-1 RNA may or may not present the characteristic RPII 5' cap structure.\(^{25}\) RPIII-dependent transcription of the miR-15a/16-1 cluster and defective miR processing are unmasked in an allele-specific manner by the 13q14 deletion.

RPII and RPIII share several transcriptional elements,\(^{40,46,47}\) but it is well known that there are important differences in their transcriptional regulation.\(^{46}\) Increase in RPIII activity has been observed in cancers.\(^{49}\) Moreover, RPIII activity is linked to the transcription factors MYC, P53 and RB1, 50–52 whose deregulation have been associated with poor prognosis in several cancers, including CLL.\(^{53–55,12}\) These genes control cell cycle and apoptosis in concert with miR-15a/miR-16-1.\(^{50–52,56,57}\) Therefore, we sought to determine the clinical correlations associated with RPII versus RPIII regulation of the miR cluster in our case series. Although the follow up did not allow direct prognostic correlations, we identified a significant association between RPIII-dependent regulation of primiR-15a/16-1 and high expression of ZAP70, a predictor of poor prognosis in CLL.\(^{22}\) Notably, we analyzed a rare case of familial CLL in monozygotic twins that differed in ZAP70 status and clinical features (aggressive versus indolent disease).\(^{22}\) Transcription of primiR-15a/16-1 was driven by RPIII in the aggressive ZAP70-positive CLL patient, but by RPII in the indolent and ZAP70-negative case. We also identified a significant correlation between type of genomic deletion and ZAP70 expression status: almost all the CLLs cases with 13q deletions that retained two copies of the miR-15a/16-1 cluster, but lacked a more telomeric region, showed high ZAP70 expression. Higher miR-15a expression has previously correlated with ZAP70 positivity in CLLs.\(^{5}\) We found RPIII-dependent regulation of the primiR in almost all ZAP70-positive cells, but no difference in miR-15a expression between the RPII- and RPIII-dependent groups was observed, probably because the majority of the patients in our subset had an indolent disease at the time of analysis. However, differences in the levels of P53, BCL2 and Cyclin E proteins, key targets of miR-15a and miR-16-1, were observed between CLL cells with RPII- versus RPIII-mediated transcription of the primiR-15a/16-1.

In conclusion, both alleles of the miR-15a/16-1 cluster are normally transcribed, one by RPII together with DLEU2, which is often expressed monoallelically, the other one by RPIII, independently of the host gene. Being the DLEU2/miR-15a/miR-16-1 locus finely regulated by cell cycle-related transcription factors,\(^{15,44,58,59}\) loss of regulation by RPII due to 13q14 deletions should alter the expression of miR-15a/miR-16-1 and, consequently, cell cycle and apoptosis. Evidences shows that co-expression of DLEU2 and of miR-15a/miR-16-1 by RPII is repressed by CMYC and activated by E2F1 and P53.\(^{15,44,58,59}\) In our model, this applies only to the RPII-dependent allele, while the transcription of the primiR-15a/16-1 from the RPIII-dependent allele could be activated by CMYC and repressed by P53.\(^{47}\) Exclusive RPIII regulation of miR-15a/16-1 could affect P53/miR-34/ZAP70 signaling,\(^{11}\) resulting, over time, in ZAP70-positive B-CLL cells. Two genetic characteristics associate with high ZAP70: (i), primiR expression driven by RPIII; (ii), type of 13q deletion that retains both alleles of the miR-15a/16-1 locus.

**Figure 6.** Model of transcriptional regulation of miR-15a/16-1 cluster. On the left, transcriptional regulation of miR-15a/16-1 in normal cells is modeled. The miR-15a/miR-16-1 expression is driven by two different mechanisms specific for the two alleles. Allele A shows the transcription of DLEU2 gene and therefore, the miR-15a/16-1 cluster by an RPII-dependent mechanism. Conversely, the allele B shows DLEU2 expression epigenetically repressed and consequently the miR-15a/16-1 expression is driven by an RPII-independent mechanism. Immediately upstream, the miR-15a, the newly identified transcriptional activator region engages the RPIII to start the transcription of the miR-15a/16-1 cluster. 13q deletions in CLL patients (right side) lead to the expression of miR-15a/16-1 by RPII and/or RPIII, depending on the deleted allele. Deletion of the region encompassing the miR stem loops on allele A occurs in ZAP70- and ZAP70+ CLL, whereas the deletion of allele B mostly occurs in CLL patients with low ZAP70 expression. In our model, the expression of miR-15a/16-1 cluster in ZAP70+ CLLs is driven by RPII by two different deletions of the allele A: deletion of the stem loop cluster transcribed by RPI or deletion of the regulatory elements of the active DLEU2 gene.
The double allele-specific regulation of a miR-15a/16-1 driven by either RIP1 or RIP3 may provide the basis for understanding the clinical heterogeneity of the CLL patients carrying 13q14 deletions, and exclusive RIP3-driven transcription of this miR cluster may differentiate at the onset aggressiveness from indolent forms of CLL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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