

ORIGINAL ARTICLE

A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia

George Adrian Calin, M.D., Ph.D., Manuela Ferracin, Ph.D.,
Amelia Cimmino, M.D., Ph.D., Gianpiero Di Leva, Ph.D.,
Masayoshi Shimizu, B.S., Sylwia E. Wojcik, M.Sc., Marilena V. Iorio, Ph.D.,
Rosa Visone, Ph.D., Nurettin Ilfer Sever, Ph.D., Muller Fabbri, M.D.,
Rodolfo Iuliano, Ph.D., Tiziana Palumbo, Ph.D., Flavia Pichiorri, Ph.D.,
Claudia Roldo, M.D., Ramiro Garzon, M.D., Cinzia Sevignani, Ph.D.,
Laura Rassenti, Ph.D., Hansjuerg Alder, Ph.D., Stefano Volinia, Ph.D.,
Chang-gong Liu, Ph.D., Thomas J. Kipps, M.D., Ph.D.,
Massimo Negrini, Ph.D., and Carlo M. Croce, M.D.

ABSTRACT

BACKGROUND

MicroRNA expression profiles can be used to distinguish normal B cells from malignant B cells in patients with chronic lymphocytic leukemia (CLL). We investigated whether microRNA profiles are associated with known prognostic factors in CLL.

METHODS

We evaluated the microRNA expression profiles of 94 samples of CLL cells for which the level of expression of 70-kD zeta-associated protein (ZAP-70), the mutational status of the rearranged immunoglobulin heavy-chain variable-region (*IgV_H*) gene, and the time from diagnosis to initial treatment were known. We also investigated the genomic sequence of 42 microRNA genes to identify abnormalities.

RESULTS

A unique microRNA expression signature composed of 13 genes (of 190 analyzed) differentiated cases of CLL with low levels of ZAP-70 expression from those with high levels and cases with unmutated *IgV_H* from those with mutated *IgV_H*. The same microRNA signature was also associated with the presence or absence of disease progression. We also identified a germ-line mutation in the *miR-16-1-miR-15a* primary precursor, which caused low levels of microRNA expression in vitro and in vivo and was associated with deletion of the normal allele. Germ-line or somatic mutations were found in 5 of 42 sequenced microRNAs in 11 of 75 patients with CLL, but no such mutations were found in 160 subjects without cancer ($P < 0.001$).

CONCLUSIONS

A unique microRNA signature is associated with prognostic factors and disease progression in CLL. Mutations in microRNA transcripts are common and may have functional importance.

From the Department of Molecular Virology, Immunology, and Medical Genetics and Comprehensive Cancer Center, Ohio State University, Columbus (G.A.C., A.C., G.D.L., M.S., S.E.W., M.V.I., R.V., N.I.S., M.F., R.I., T.P., F.P., C.R., H.A., S.V., C.L., C.M.C.); the Department of Experimental and Diagnostic Medicine, Interdepartment Center for Cancer Research, University of Ferrara, Ferrara, Italy (M.F., M.N.); the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia (R.G., C.S.); and the Department of Medicine, University of California, San Diego, La Jolla (L.R., T.J.K.). Address reprint requests to Dr. Croce at Ohio State University, Comprehensive Cancer Center, Wiseman Hall, Rm. 385K, 400 12th Ave., Columbus, OH 43210, or at carlo.croce@osumc.edu.

N Engl J Med 2005;353:1793-801.

Copyright © 2005 Massachusetts Medical Society.

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL), the most common leukemia among adults in the Western world, arises from a malignant clone of B cells, but little is known regarding its initiation and progression.¹ Nevertheless, several factors that can predict the clinical course have been identified.²⁻⁶ Cases in which the leukemic cells have few or no mutations in the immunoglobulin heavy-chain variable-region (*IgV_H*) gene or a high level of expression of the 70-kD zeta-associated protein (ZAP-70) have an aggressive course, whereas cases involving mutated CLL clones or few ZAP-70 cells have an indolent course.⁷ Genomic aberrations in CLL are also independent predictors of disease progression and survival.⁸ However, the molecular basis of these associations is largely unknown.

The most frequent deletion of genomic DNA in CLL occurs in chromosome 13q13.4. This deletion is evident in about 50 percent of cases and is associated with a long interval between diagnosis and the need for treatment (the treatment-free interval).⁸ The 13q13.4 deletion is frequently the sole abnormality in CLL and other types of cancers,⁹ suggesting a pathogenic role for the deleted gene or genes. We used positional cloning to identify two members of a recently discovered class of small, noncoding RNAs, or microRNAs, *miR-15a* and *miR-16-1*, which are located in the smallest region of the deletion at 13q13.4 and are frequently deleted or down-regulated in CLL cells.¹⁰

MicroRNAs range in size from 19 to 25 nucleotides and are typically excised from a hairpin (fold-back) RNA structure of 60 to 110 nucleotides (named pre-microRNA) that is transcribed from a larger primary transcript (named pri-microRNA).¹¹ MicroRNAs can reduce the levels of many of their target transcripts as well as the amount of protein encoded by these transcripts.¹² The finding that approximately 50 percent of the known human microRNAs are located at cancer-associated regions of the genome¹³ suggests that microRNAs play a role in the pathogenesis of various human cancers.^{10,14-19} Using a microRNA microchip,²⁰ we found that CLL cells (which are CD5+ B cells) have a distinct pattern of expression of microRNA that differs from that of normal CD5+ B cells.²¹

We performed genome-wide expression profiling with the microRNA microchip in a large number of CLL samples from patients with available clinical data to investigate whether the expression of noncoding microRNA genes is associated with

factors that predict the clinical course of CLL. We also screened several microRNAs for mutations in a panel of CLL cells.

METHODS

PATIENTS AND CLINICAL DATABASE

For the expression study, we investigated 94 samples of CLL cells after obtaining written informed consent from patients receiving care at CLL Research Consortium institutions.^{2,10} Clinical and biologic information (sex, age at diagnosis, treatment, time between diagnosis and therapy, the level of ZAP-70 expression, and mutational status of *IgV_H*) was available for all patients (Table 1). A second independent set of 50 samples of CLL cells for which the level of ZAP-70 expression was known was used to validate the predictive power of the microRNA signature.

RNA EXTRACTION, NORTHERN BLOTTING, AND MICRORNA-MICROCHIP EXPERIMENTS

RNA extraction, Northern blotting, and microRNA-microchip procedures were performed as described in detail elsewhere.^{20,21} Briefly, labeled targets from 5 μ g of total RNA were used for hybridization on each microRNA microchip, which contained triplicates of 368 probes, corresponding to 245 human and mouse microRNA genes. We tested 76 microRNAs on the microRNA microchip with two specific synthetic oligomers; one identified the active 22-nucleotide part of the molecule, and the other detected the precursor composed of 60 to 110 nucleotides.²⁰

STATISTICAL ANALYSIS

Raw data were normalized and analyzed with the use of GeneSpring software (version 7.2, Silicon Genetics). Expression data were centered around a median with the use of the GeneSpring normalization option alone and with global-median normalization contained in the Bioconductor package (www.bioconductor.org), with no substantial difference in results. Statistical comparisons were made with the use of both the GeneSpring analysis-of-variance tool and the Significance Analysis of Microarray (SAM) software (available at www-stat.stanford.edu/~tibs/SAM/index.html). MicroRNA predictors were calculated with the use of Prediction Analysis of Microarray (PAM) software (available at www-stat.stanford.edu/~tibs/PAM/index.html); the Support Vector Machine tool of Gene-

Spring was used for cross-validation and prediction of the test set. The Kaplan–Meier plot (survival-analysis portion of the PAM software) was used to identify any association between microRNA expression and the time from diagnosis to the beginning of therapy. MicroRNAs that best differentiated among groups of patients were identified at the same time. All data were submitted to the Array Express database with the use of MIAMExpress (accession numbers E-TABM-41 and E-TABM-42). We validated the microarray data for four microRNAs (*miR-16-1*, *miR-26a*, *miR-206*, and *miR-223*) in 11 CLL samples and normal CD5+ cells by means of solution hybridization detection as described elsewhere.²¹ Furthermore, the expression of *miR-15a* and *miR-16-1* in the patients with a germ-line mutation was confirmed by Northern blotting.

ANALYSIS OF ZAP-70 AND SEQUENCE ANALYSIS OF *IgV_H*

Analysis of ZAP-70 and sequence analysis of *IgV_H* were performed as described previously.² Briefly, ZAP-70 expression was assessed by immunoblotting and flow cytometry, whereas the analysis of expressed *IgV_H* was done by direct sequencing.

DETECTION OF MICRORNA MUTATIONS

The genomic region corresponding to each precursor microRNA from 40 samples of CLL cells and normal mononuclear cells from three control subjects was amplified, including at least 50 bp at the 5' and 3' ends. For the microRNAs located in clusters less than 1 kb apart, the entire corresponding genomic region was amplified and sequenced with the use of the Applied Biosystems DNA sequencing system (model 377, Applied Biosystems). When a deviation from the normal sequence was found, a panel of DNAs from the blood of 160 control subjects was screened to identify polymorphisms, as was an additional panel from the blood of 35 patients with CLL (for a total of 75 patients with leukemia). All subjects were white, as indicated by medical records or information obtained during an interview with control subjects. The personal and family history of cancer was known for 46 patients with CLL.

IN VIVO STUDIES OF THE EFFECTS OF *miR-16-1* MUTANTS

We constructed *miR-16-1* and *miR-15a* expression vectors containing an 832-bp genomic sequence including both *miR-16-1* and *miR-15a*, one wild-

Table 1. Characteristics of the Patients.

Characteristic	Value
Male sex — no. of patients (%)	58 (62)
Age at diagnosis — yr	
Median	57.3
Range	38–78
Therapy begun	
No	
No. of patients	53
Mean time since diagnosis — mo	88
Yes	
No. of patients	41
Mean time from diagnosis to therapy — mo	40
Level of ZAP-70 expression — no. of patients	
≤20%	48
>20%	46
<i>IgV_H</i> status — no. of patients	
Unmutated (≥98% homology)	57
Mutated (<98% homology)	37

type sequence, and one containing the (C→T)+7 substitution, by ligating the relevant open reading frame in a sense orientation into a mammalian expression vector pSR-GFP-Neo (OligoEngine). All sequenced constructs were transfected in 293 cells with the use of Lipofectamine2000 according to the manufacturer's protocol (Invitrogen). The expression of both constructs was assessed by Northern blotting.

RESULTS

MICRORNA SIGNATURE, ZAP-70 EXPRESSION, AND MUTATIONAL STATUS OF *IgV_H*

We investigated whether the microRNA-microchip microarray could reveal a specific molecular signature that is associated with subgroups of CLL with different clinical courses. Using 20 percent as the cutoff for defining ZAP-70 positivity and 98 percent homology as the cutoff for defining a germ-line *IgV_H*, we divided the 94 patients with CLL into four groups: group 1 (expression of ZAP-70 and unmutated *IgV_H*) included 36 patients, group 2 (expression of ZAP-70 and mutated *IgV_H*) included 10 patients, group 3 (no expression of ZAP-70 and unmutated *IgV_H*) included 1 patient, and group 4 (no expression of ZAP-70 and mutated *IgV_H*) included 47 patients. We found, using several algo-

rithms for statistical and prediction analysis (PAM, SAM, and GeneSpring), that a signature composed of 13 mature microRNAs could discriminate ($P < 0.01$) between group 1 and group 4, the two main groups of patients (Table 2; and Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org); the prediction made using Support Vector Machine correctly classified all patients (Table 2 of the Supplementary Appendix). Of 13 microRNAs, 9 were significantly over-expressed in group 1, the group with a poor prognosis (Table 2). The 10 patients in group 2 were equally assigned according to their microRNA signature to groups 1 and 4, suggesting that there are

no microRNAs on the microRNA chip that can identify distinctive characteristics in these patients, that these two groups are not different with respect to microRNA expression, or that this group is too small to be correctly classified.

We applied the Support Vector Machine algorithm to an independent set of 50 samples of CLL cells with known ZAP-70 status (Table 2 of the Supplementary Appendix). Using the microRNA signature consisting of 13 microRNAs, we found that the classification according to ZAP-70 status was correct in all cases. Confirming the previously reported microarray specificity,²⁰ we found that the signature of 13 microRNAs did not include

Table 2. MicroRNA Signature Associated with Prognostic Factors (ZAP-70 Expression and *IgV_H* Mutations) and Disease Progression in Patients with CLL.*

Signature Component	MicroRNA Component	Chromosomal Location	P Value	Level of Expression in Group 1†	Putative Targets‡	Comment§
1	<i>miR-15a</i>	13q14.3	0.02	High	<i>BCL2, CNOT6L, USP15, PAFAH1B1, ESRRG</i>	Cluster <i>miR-15a-miR-16-1</i> Deleted in CLL and prostate carcinoma ¹⁰
2	<i>miR-195</i>	17p13	0.02	High	<i>BCL2, CNOT6L, USP15, PAFAH1B1, ESRRG</i>	Deleted in hepatocellular carcinoma
3	<i>miR-221</i>	Xp11.3	0.01	High	<i>HECTD2, CDKN1B, NOVA1, ZFPM2, PHF2</i>	Cluster <i>miR-221-miR-222</i>
4	<i>miR-23b</i>	9q22.1	0.009	High	<i>FNBP1L, WTAP, PDE4B, SATB1, SEMA6D</i>	Cluster <i>miR-24-1-miR-23b</i> FRA 9D; deleted in urothelial carcinoma ¹³
5	<i>miR-155</i>	21q21	0.009	High	<i>ZNF537, PICALM, RREB1, BDNF, QKI</i>	Amplified in a child with Burkitt's lymphoma ¹⁶
6	<i>miR-223</i>	Xq12-13.3	0.007	Low	<i>PTBP2, SYNCRIP, WTAP, FBXW7, QKI</i>	Expression normally restricted to myeloid lineage ²³
7	<i>miR-29a-2</i>	7q32	0.004	Low	NA	Cluster <i>miR-29a-2-miR-29b-1</i> FRA7H; deleted in prostate carcinoma ¹³
8	<i>miR-24-1</i>	9q22.1	0.003	High	<i>TOP1, FLJ45187, RSBN1L, RAP2C, PRPF4B</i>	Cluster <i>miR-24-1-miR-23b</i> FRA 9D; deleted in urothelial carcinoma ¹³
9	<i>miR-29b-2</i>	1q32.2-32.3	<0.001	Low	NA	
10	<i>miR-146</i>	5q34	<0.001	High	<i>NOVA1, NFE2L1, C1orf16, ABL2, ZFYVE1</i>	
11	<i>miR-16-1</i>	13q14.3	<0.001	High	<i>BCL2, CNOT6L, USP15, PAFAH1B1, ESRRG</i>	Cluster <i>miR-15a-miR-16-1</i> Deleted in CLL and prostate carcinoma ¹⁰
12	<i>miR-16-2</i>	3q26.1	<0.001	High	Same as for <i>miR-16-1</i>	Identical to <i>miR-16-1</i>
13	<i>miR-29c</i>	1q32.2-32.3	<0.001	Low	NA	

* All the members of the signature are mature microRNAs. NA denotes not available, and FRA fragile site.
 † Group 1 includes patients with unmutated *IgV_H* and high expression of ZAP-70, both of which are predictors of poor prognosis.
 ‡ The top five putative targets identified with use of TargetScan at <http://genes.mit.edu/targetscan>²² were included.
 § Specific gene names are available at www.ncbi.nlm.nih.gov/entrez.

very similar members of the same families, such as *miR-23a* (one-base difference from *miR-23b*) and *miR-15b* (four-base difference from *miR-15a*), whereas the identical mature microRNAs *miR-16-1* and *miR-16-2* were both present, indicating that the chip can discriminate between highly similar isoforms of microRNA.

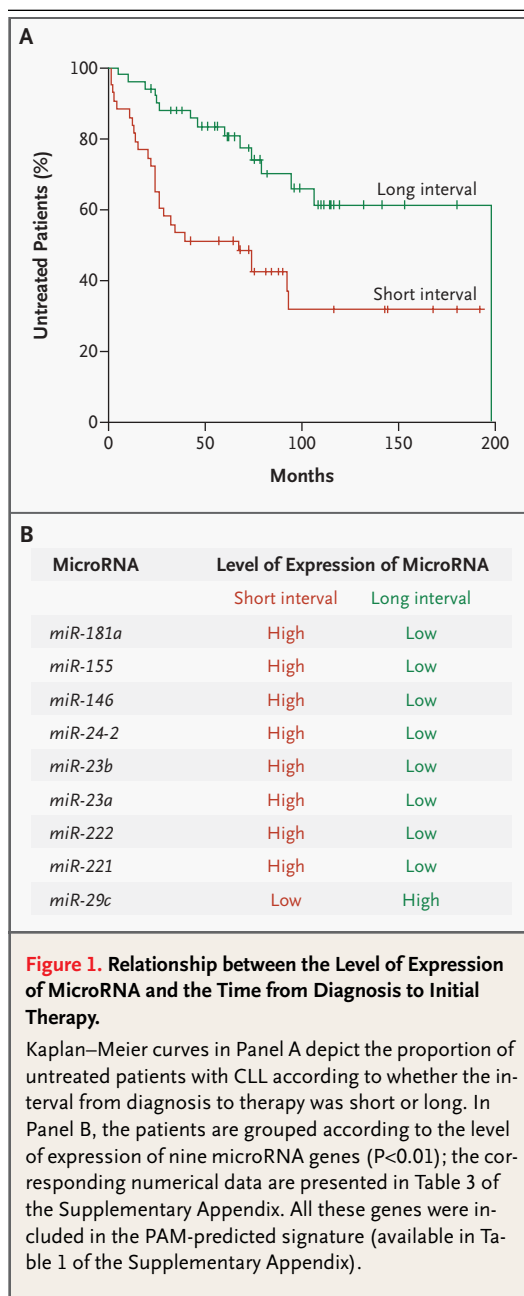
ASSOCIATION BETWEEN MICRORNA EXPRESSION AND THE TIME TO INITIAL THERAPY

The treatment of CLL begins with the development of symptomatic or progressive disease, as defined according to the criteria of the National Cancer Institute Working Group.²⁴ Of the 94 patients we studied, 41 had begun therapy (Table 1).

Using PAM survival analysis, we examined the relationship between the expression of 190 microRNA genes and the time from diagnosis to initial therapy for all 94 patients. We found 9 microRNAs, all members of the 13-member prognostic signature, that best differentiated patients with a short interval from diagnosis to initial therapy (average [\pm SD], 40 \pm 39 months) from patients with a significantly longer interval (average, 88 \pm 42 months; $P < 0.01$) (Fig. 1, and Table 1 of the Supplementary Appendix). The significance of the differences in the Kaplan–Meier curves increased if we restricted the analyses to the 83 patients in the two main groups (groups 1 and 4) (P values decreased from < 0.01 to < 0.005). All nine microRNAs that were associated with the time to initial therapy were over-expressed, except *miR-29c*, in the group with a short interval from diagnosis to initial therapy (Fig. 1).

GENOMIC SEQUENCE ABNORMALITIES OF MICRORNA IN CLL

Abnormally expressed cancer genes are frequently targets for mutations that can activate or inactivate their function. Therefore, we screened 42 microRNAs, including 15 genes that are either components of the expression signature or members of the same genomic clusters as the genes in the expression signature. We identified germ-line or somatic mutations in 11 of 75 CLL samples (15 percent) in 5 of 42 microRNAs (12 percent): *miR-16-1*, *miR-27b*, *miR-29b-2*, *miR-187*, and *miR-206*. None of these mutations were found in 160 persons without cancer ($P < 0.001$) (Table 3). All the abnormalities were in regions that are transcribed as shown by the reverse-transcriptase–polymerase-chain-reaction assay (RT-PCR) (Fig. 2). Of the 11 patients with an abnormal microRNA sequence, 8 (73 percent) had



a known personal or family history of CLL or other hematopoietic or solid tumors (Table 3).

In two patients, we found a C→T homozygous substitution in the *pri-miR-16-1*, 7 bp in the 3' direction after the precursor. This genomic region is strongly conserved in all primates analyzed,²⁵ suggesting an important functional role for *pri-miR-16-1*. RT-PCR showed that the *pri-microRNA* was at least 800 bp long and included the 3' region harboring the base substitution (Fig. 2). MicroRNA-

Table 3. Genetic Variations in the Genomic Sequences of MicroRNAs in Patients with CLL.*

MicroRNA	Location†	Patients with CLL no./total no.	Control Subjects no./total no.	MicroRNA-Microchip Expression	Comment
<i>miR-16-1</i>	Germ-line pri-microRNA (C→T)+7-bp in the 3' direction	2/75	0/160	15 percent and 40 percent of normal levels, respectively	Normal allele deleted in CLL cells in both patients (by FISH and LOH); history of breast cancer in 1 patient; mother died with CLL; sister died with breast cancer
<i>miR-27b</i>	Germ-line pri-microRNA (G→A)+50-bp in the 3' direction	1/75	0/160	Normal	Throat and lung cancer diagnosed in mother at 58 yr; lung cancer diagnosed in father at 57 yr
<i>miR-29b-2</i>	Pri-microRNA (G→A)+212 in the 3' direction	1/75	0/160	75 percent of normal	Breast cancer diagnosed in sister at 88 yr (still living); "some type of blood cancer" diagnosed in brother at 70 yr
<i>miR-29b-2</i>	Pri-microRNAs insertion (+A)+107 in the 3' direction	3/75	0/160	80 percent of normal	Family history of unspecified cancer in 2 patients
<i>miR-187</i>	Pri-microRNA (T→C)+73 in the 3' direction	1/75	0/160	Not available	Unknown
<i>miR-206</i>	Pre-microRNA 49(G→T)	2/75	0/160	25 percent of normal	Prostate cancer diagnosed in 1 patient; esophageal cancer diagnosed in mother; prostate cancer diagnosed in brother; breast cancer diagnosed in sister
<i>miR-206</i>	Somatic pri-microRNA (A→T)-116 in the 5' direction	1/75	0/160	25 percent of normal‡	Aunt had some type of leukemia (dead)
<i>miR-29c</i>	Pri-microRNA (G→A)31 in the 5' direction	2/75	1/160	Not available	Patient's paternal grandmother had CLL; sister had breast cancer
<i>miR-122a</i>	Pre-microRNA 53(C→T)	1/75	2/160	33 percent of normal	Patient's paternal uncle had colon cancer
<i>miR-187</i>	Pre-microRNA 34(G→A)	1/75	1/160	Not available	Patient's grandfather had polycythemia vera; father had a history of cancer but not lymphoma

* For each patient and control more than 12 kb of genomic DNA was sequenced, and in total, we used direct sequencing to screen approximately 627 kb of tumor DNA and approximately 700 kb of normal DNA. The positions of the mutations are reported with respect to the precursor microRNA molecule. The list of 42 microRNAs analyzed includes 15 members of the specific signature or members of the same clusters, *miR-15a*, *miR-16-1*, *miR-23a*, *miR-23b*, *miR-24-1*, *miR-24-2*, *miR-27a*, *miR-27b*, *miR-29b-2*, *miR-29c*, *miR-146*, *miR-155*, *miR-221*, *miR-222*, and *miR-223* and 27 other microRNAs (randomly selected): *let-7a2*, *let-7b*, *miR-17-3p*, *miR-17-5p*, *miR-18*, *miR-19a*, *miR-19b-1*, *miR-20*, *miR-21*, *miR-30b*, *miR-30c-1*, *miR-30d*, *miR-30e*, *miR-32*, *miR-100*, *miR-105-1*, *miR-108*, *miR-122*, *miR-125b-1*, *miR-142-5p*, *miR-142-3p*, *miR-181a*, *miR-187*, *miR-193*, *miR-206*, *miR-224*, and *miR-346*. FISH denotes fluorescence in situ hybridization, and LOH loss of heterozygosity.

† When normal correspondent DNA from buccal mucosa was available, the alteration was identified as germ-line when present or somatic when absent, respectively.

‡ Data were only for one patient.

microchip analysis and Northern blotting showed that CLL cells from both patients had a substantial reduction in the expression of *miR-16-1* as compared with that of normal CD5+ cells (Table 3). In most CLL cells from these patients, we also detected a monoallelic deletion at 13q14.3 by fluorescence in situ hybridization and loss-of-heterozygosity analysis (data not shown). This substitution was

not found in any cells from 160 control subjects (P<0.05 by the chi-square test). In both patients, normal cells from the buccal mucosa were heterozygous for this abnormality. Therefore, the C→T change is a germ-line mutation or a very rare polymorphism; the finding that the mother and sister of one of the patients have CLL and breast cancer, respectively, supports the existence of a germ-line

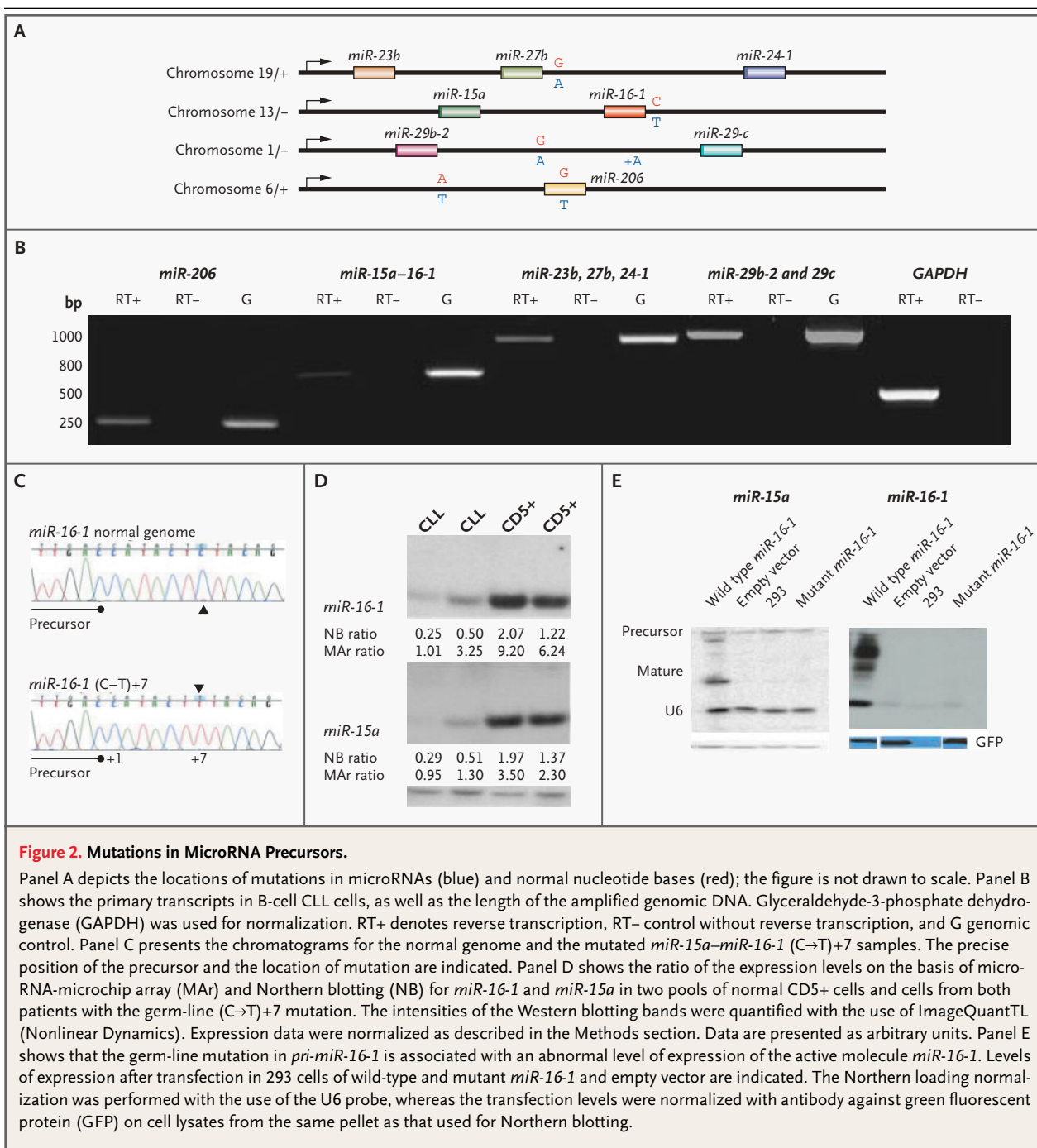


Figure 2. Mutations in MicroRNA Precursors.

Panel A depicts the locations of mutations in microRNAs (blue) and normal nucleotide bases (red); the figure is not drawn to scale. Panel B shows the primary transcripts in B-cell CLL cells, as well as the length of the amplified genomic DNA. Glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. RT+ denotes reverse transcription, RT- control without reverse transcription, and G genomic control. Panel C presents the chromatograms for the normal genome and the mutated *miR-15a-miR-16-1* (C→T)+7 samples. The precise position of the precursor and the location of mutation are indicated. Panel D shows the ratio of the expression levels on the basis of microRNA-microchip array (MAR) and Northern blotting (NB) for *miR-16-1* and *miR-15a* in two pools of normal CD5+ cells and cells from both patients with the germ-line (C→T)+7 mutation. The intensities of the Western blotting bands were quantified with the use of ImageQuantTL (Nonlinear Dynamics). Expression data were normalized as described in the Methods section. Data are presented as arbitrary units. Panel E shows that the germ-line mutation in *pri-miR-16-1* is associated with an abnormal level of expression of the active molecule *miR-16-1*. Levels of expression after transfection in 293 cells of wild-type and mutant *miR-16-1* and empty vector are indicated. The Northern loading normalization was performed with the use of the U6 probe, whereas the transfection levels were normalized with antibody against green fluorescent protein (GFP) on cell lysates from the same pellet as that used for Northern blotting.

mutation. This family fulfills the minimal criteria for familial CLL: two or more cases of CLL in first-degree relatives.²⁶

To identify a possible molecular effect of the C→T substitution, we prepared vectors containing either the wild-type allele of the *miR-15a-miR-16-1* cluster or the mutated allele. The 293 cells, which

have low endogenous expression of this cluster, were transfected with the vectors. As a control we used the empty vector. The mutant transfectants expressed *miR-16-1* and *miR-15a* at levels that were significantly lower than those of the wild-type transfectants and similar to that of non-transfected cells (Fig. 2). These results indicate that the C→T

substitution affects the level of expression of mature microRNAs.

DISCUSSION

In this study of CLL, we found a significant association between the expression of certain microRNAs and the expression of ZAP-70, the mutational status of *IgV_H*, and the time between diagnosis and initial treatment. The time from diagnosis to initial treatment is an important factor associated with disease activity, since therapy for CLL is usually withheld until symptoms, advanced disease, or both develop.²⁷ Using a microRNA microarray composed of 190 human genes, we found a unique 13-gene molecular signature associated with each prognostic factor. Therefore, we believe that microRNA expression can be included in the markers with prognostic significance in CLL.

Besides its relevance as a prognostic marker, the microRNA signature we found may be relevant to the pathogenesis of CLL. Several facts provide support for its functional importance. First, all the microRNAs of this signature represent the active parts of the transcript that interact with messenger RNA, even though about 20 percent of the oligomers in the microarray are specific for pre-microRNA, a functionally inactive microRNA. Second, the signature consists of microRNAs that are abnormally expressed in CLL (*miR-15a* and *miR-16-1*) or other leukemias (*miR-155*) or are located in regions involved in cancers (*miR-23b*, *miR-24-1*, *miR-29b-2*, and *miR-195*).¹⁰⁻¹³ Third, 7 of 13 of these microRNAs are members of microRNA clusters, and their level of expression is similar, suggesting a common mechanism of gene regulation that marks the differences in these two prognostic categories of CLL samples.

The finding of mutations in two microRNA genes, *miR-16-1* and *miR-15a*, in CLL is important. Our previous data indicated that *miR-16-1* and *miR-15a* behave as tumor suppressors in CLL. The combination of loss of heterozygosity plus a germline mutation that we found in two patients is characteristic of the Knudson model of inactivation of a tumor-suppressor gene. The presence of pathogenic mutations in the *miR-15a-miR-16-1* cluster, as well as the identification of various mutations in other microRNAs, indicates that this new class of genes is involved in CLL²¹ and that at least some microRNAs can function as tumor-suppressor genes.^{10,13,28} Because the 40 bases before and after

the pre-microRNA can influence the transcription of the microRNA,²⁷ it is possible that the single-base mutation C→T may affect the expression of microRNA.

Most of the sequence abnormalities we identified in microRNA genes were not found in 160 subjects without cancer, and in several instances, they were also found in DNA from normal cells in the same patient. In a recent study of 96 healthy subjects, 10 polymorphisms of microRNA genes were found, but none were in any of the five mutated microRNAs we found in CLL.²⁹ Since CLL is a disease with a frequent association in families (10 to 20 percent of patients have at least one first-degree relative with CLL) as well as other cancers,³⁰ microRNA mutations may be a predisposing factor for the cancers associated with CLL. Since we identified mutations in both signature-specific and signature-independent microRNAs and we screened less than one fifth of the known microRNAs,³¹ the frequency of the mutations that we reported here (15 percent) may be an underestimate.

MicroRNAs are a recently identified class of regulatory RNAs that function primarily by targeting specific messenger RNAs (mRNAs) for degradation or inhibition of translation and thus decreasing the expression of the resulting protein. Our finding that all but one of the microRNAs that predict the time to initial therapy are overexpressed suggests that the down-regulation of target mRNAs plays a role in disease progression. Several genes are targeted by two different microRNAs, such as *WTAP*, the Wilms' tumor-1-associated protein isoform 1, which is targeted by both *miR-221* and *miR-223* (Table 2). Moreover, the anti-apoptotic *BCL2* gene is reported to be overexpressed in 65 to 70 percent of B-cell CLLs,³² whereas deletions or down-regulations of *miR-16-1* were reported in the same proportion of CLL samples.¹⁰ We have shown that *BCL2* is a target of microRNAs *miR-15* and *miR-16* and that down-regulation of *BCL2* protein by these microRNAs triggers apoptosis.³³

In conclusion, our study shows that a unique microRNA signature is associated with prognostic factors and disease progression in CLL and that mutations in microRNA genes are frequent and may have functional importance.

Supported by Program Project grants (P01CA76259, P01CA81534, and P30CA56036, to Drs. Kipps and Croce) from the National Cancer Institute, by a Kimmel Scholar award (to Dr. Calin), and by grants from the Italian Ministry of Public Health, the Italian Ministry of University Research, and the Italian Association for Cancer Research (to Drs. Negrini and Volinia).

REFERENCES

1. Bullrich F, Croce CM. Molecular biology of chronic lymphocytic leukemia. In: Cheson BD, ed. *Chronic lymphoid leukemia*. New York: Dekker, 2001:9-32.
2. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004; 351:893-901.
3. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003;348:1764-75.
4. Orchard JA, Ibbotson RE, Davis ZA, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. *Lancet* 2004;363:105-11.
5. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999;94: 1840-7.
6. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;94:1848-54.
7. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med* 2005;352:804-15.
8. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000;343:1910-6.
9. Calin GA, Trapasso F, Shimizu M, et al. Familial cancer associated with a polymorphism in ARLTS1. *N Engl J Med* 2005;352: 1667-76.
10. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 2002;99:15524-9.
11. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-97.
12. Lim LP, Lau NC, Garrett-Engle P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;433:769-73.
13. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* 2004;101:2999-3004.
14. Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* 2003;1: 882-91.
15. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004;64:3753-6.
16. Metzler M, Wilda M, Busch K, Viehmann S, Borkhardt A. High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* 2004;39:167-9.
17. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* 2005;102:3627-32.
18. Ota A, Tagawa H, Karnan S, et al. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 2004;64:3087-95.
19. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834-8.
20. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A* 2004;101:9740-4.
21. Calin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci U S A* 2004;101:11755-60.
22. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120: 15-20.
23. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303:83-6.
24. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood* 1996;87:4990-7.
25. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* 2005;120:21-4.
26. Ishibe N, Sgambati MT, Fontaine L, et al. Clinical characteristics of familial B-CLL in the National Cancer Institute Familial Registry. *Leuk Lymphoma* 2001;42:99-108.
27. Kipps TJ. Chronic lymphocytic leukemia and related diseases. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligson U, eds. *Williams hematology*. New York: McGraw-Hill, 2001:1163-94.
28. McManus MT. MicroRNAs and cancer. *Semin Cancer Biol* 2003;13:253-8.
29. Iwai N, Naraba H. Polymorphisms in human pre-miRNAs. *Biochem Biophys Res Commun* 2005;331:1439-44.
30. Catovsky D. Definition and diagnosis of sporadic and familial chronic lymphocytic leukemia. *Hematol Oncol Clin North Am* 2004;18:783-94.
31. Griffiths-Jones S. The microRNA Registry. *Nucleic Acids Res* 2004;32(Database Issue):D109-D111.
32. Korz C, Pscherer A, Benner A, et al. Evidence for distinct pathomechanisms in B-cell chronic lymphocytic leukemia and mantle cell lymphoma by quantitative expression analysis of cell cycle and apoptosis-associated genes. *Blood* 2002;99:4554-61.
33. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 2005; 102:13944-9.

Copyright © 2005 Massachusetts Medical Society.

JOURNAL EDITORIAL FELLOW

The *Journal's* editorial office invites applications for a one-year research fellowship beginning in July 2006 from individuals at any stage of training. The editorial fellow will work on *Journal* projects and will participate in the day-to-day editorial activities of the *Journal* but is expected in addition to have his or her own independent projects. Please send curriculum vitae and research interests to the Editor-in-Chief, 10 Shattuck St., Boston, MA 02115 (fax, 617-739-9864), by November 1, 2005.

CORRECTION

A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia

A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia . On page 1796, in Table 2, the column head "Level of Expression in Group 4" and the corresponding footnote should have referred to "Group 1." The article has been corrected on the *Journal's* Web site at www.nejm.org.