A large fraction of trisomy 12, 17p⁻, and 11q⁻ CLL cases carry unidentified microdeletions of *miR-15a/16-1*

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Chronic lymphocytic leukemia (CLL) is the most common adult leukemia and is characterized by chromosomal aberrations including 13q, 11q, and 17p deletions and a trisomy of chromosome 12 (T12). 13q deletions are often associated with 11q and 17p deletions in aggressive cases. Conversely, T12 CLLs show a variable prognosis, and association with 13q deletions is uncommon. The *miR-15a/16-1* cluster is the functional target of 13q deletions, leading to *BCL2* overexpression. Chromosomal aberrations in CLL are associated with prognosis, and their identification is carried out by fluorescence in situ hybridization (FISH). Since standard FISH only detects large deletions, we investigated the presence of undetected microdeletions targeting *miR-15a/16-1* in CLL cases. We found that ∼34% of CLL samples show an unreported loss of the *miR-15a/16-1* locus regardless of their cytogenetic profile. Interestingly, 15 out of 39 (∼39%) of all CLLs with T12, carry microdeletions of *miR-15a/16-1*, indicating that, in patients with T12, *miR-15a/16-1* are mostly inactivated by microdeletions. In addition, ∼40% of CLL cases bearing T12, 17p⁻, and 11q⁻ showed unidentified microdeletions of *miR-15a/16-1*, suggesting that *miR-15a/16-1* loss cooperates with such chromosomal alterations in CLL. These data may have clinical relevance for the successful stratification of patients for treatment.

CLL | trisomy 12 | 13q deletion | *miR-15a/16-1*

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia, arising from CD5⁺ B lymphocytes (1). CLL can occur as an indolent or aggressive form (2), and is characterized by specific chromosomal abnormalities: 13q14.3 deletions (13q⁻, ∼55%), 11q22.3 deletions (11q⁻, ∼15%), 17p13.1 deletions (17p⁻, ∼5–8%), and a trisomy of chromosome 12 (T12, ∼15%) (3). In 2002, the minimal deleted region on chromosome 13 was described (4), including the microRNA (miRNA) cluster genes encoding for *miR-15a* and *miR-16-1*. Such a discovery indicated a role for miRNAs in the development of CLL (4). miRNAs are 22- to 25-nt-long noncoding RNAs that bind the 3′ untranslated region of target messenger RNAs in a sequence-specific fashion, stalling their translation (5). In 2002 to 2005, we showed that *miR-15a/16-1* is the functional target of 13q deletions in CLL and that *miR-15a/16-1* are negative regulators of the antiapoptotic gene B cell leukemia 2 (*BCL2*), an inhibitor of the intrinsic apoptotic pathway (6, 7). More recently, it was shown that *miR-15a/16-1* also targets ROR1, an embryonic oncoembryogenic antigen expressed on the surface of the CLL cell, but not in normal postpartum tissues, that enhances CLL viability, migration, and proliferation by activating the *Wnt5* pathway (8, 9). Thus, in CLL, the deletion of chromosome 13 and the consequent loss of *miR-15a/16-1* lead to an increased expression of *BCL2* (7), which in turn supports the expansion of cancer cells in the bloodstream (6, 7, 10). The 13q deletion is considered a driver event in CLL onset and, as a sole abnormality, is associated with indolent disease (3). Conversely, CLL patients with deletions of chromosomes 11 or 17 typically show an aggressive disease, with high risk of relapse, drug resistance development, and a median overall survival of 79 and 32 mo, respectively (3). Finally, T12 CLLs show atypical morphological/immunophenotypic features and the most variable prognosis (11), with an overall survival spanning between 9.5 and 15.6 y (3, 12). It was previously shown that *NOTCH1* mutations are prevalent in T12 CLL and are associated with unfavorable prognosis (13, 14). However, *NOTCH1* mutation frequency alone does not account for the extraordinary clinical heterogeneity observed among patients carrying CLL cells with a trisomy of chromosome 12 (15). Interestingly, while coexistence of 13q deletions with 11q or 17p deletions in patients showing an aggressive disease is frequent (3, 16), coexistence with 13q deletions in T12 patients is thought to be rare (17). Indeed, T12 is also considered a driver event in CLL pathogenesis. Nevertheless, 13q deletions can be identified in ∼25% of T12 cases, and are associated with shorter survival (15). Given the role of *miR-15a/16-1* loss in CLL onset/progression, we evaluated the presence of microdeletions involving the loss of *miR-15a/16-1*, which may be undetected with standard fluorescence in situ hybridization (FISH), and assessed their prevalence in all cytogenetic cohorts.

Results

FISH and *miR-15a/16-1* Copy-Number Variation Approaches. To investigate the prevalence of microdeletions involving *miR-15a/16-1*, we performed a *miR-15a/16-1* copy-number variation (CNV) analysis of all CLL samples accrued (Fig. 1). Since T12 association with the 13q deletion is uncommon (17), we initially

Significance

13q14.3 deletion is the most common genetic lesion identified in CLLs. This study shows that microdeletions affecting the *miR-15a/16-1* cluster are more frequent than expected in all CLL cohorts and are prevalent in patients carrying a trisomy 12. Copy-number variation analysis and an experimental FISH analysis revealed that ∼34% of samples carry previously unidentified microdeletions of *miR-15a/16-1*. These data may have clinical relevance for the successful stratification of patients for treatment.

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investigated whether microdeletion of *miR-15a/16-1* may be prevalent in T12 CLL. To compare *miR-15a/16-1* CNV with standard FISH data, we defined "allelic loss" (AL) as the percentage of lost *miR-15a/16-1* alleles detected by *miR-15a/16-1* CNV (microdeletions of *miR-15a/16-1*), and "chromosome loss" (ChrL) as the percentage of lost chromosome 13 detected by standard FISH (large deletions identified by FISH). Standard FISH data obtained from clinical settings showed that in our set of 90 samples (*SI Appendix*, Table S1), 44 (49%) carry a deletion of chromosome 13. Interestingly, a *miR-15a/16-1* CNV assay showed that in the same set of 90 samples, 77 (85.5%) carry a microdeletion of *miR-15a/16-1* (Fig. 1). This result indicates that in 33 samples (~37%), microdeletions involving *miR-15a/16-1* were not previously identified by FISH. To provide a parameter suitable for comparison for these two sets of data, we calculated ChrL and AL as described in *Materials and Methods*. We determined the percentage of chromosome 13 loss based on the standard FISH analysis data. The percentage of cells showing a monoallelic deletion of chromosome 13 was divided by 2 and added to the percentage of cells showing a biallelic deletion of chromosome 13, to obtain the percentage of chromosome 13 loss. As such, ChrL accounts for monoallelic and biallelic loss in one value and thus allows us to establish a parameter suitable for such comparison. For our set of samples, ChrL was between 4.5 and 95.5% and AL was between 4.5 and 98.3%. To compare ChrL and AL data for each case, we established a threshold of 12%, as described in *Materials and Methods*. Indeed, even though both FISH and CNV are able to detect a 4.5% ChrL or AL, respectively, their sensitivity is associated with different variables according to the nature of the assays. For cases where FISH analysis did not detect a deletion of chromosome 13, such a threshold excludes samples with a low AL identified by *miR-15a/16-1* CNV that may represent a background signal. For cases where FISH analysis detected a deletion of chromosome 13, this threshold provides a parameter to identify patients where the discrepancy between ChrL and AL is significant. Such a discrepancy indicates the presence of a subpopulation of CLL cells with a microdeletion of *miR-15a/16-1* not detected by FISH.

Cases without 13q Deletions Identified by FISH. Using the threshold described above, we found 16 cases showing an unexpected *miR-15a/16-1* AL (informative cases) out of 46 CLL samples (34%) in which 13q deletions were not detected by FISH. These samples include 4 out of 7 from cohort 11q$^-$, 3 out of 8 from cohort 17p$^-$, 8 out of 23 from cohort T12, and 1 out of 9 from cohort Normal Karyotype (according to *miR-15a/16-1* CNV) (Fig. 1, Table 1, and *SI Appendix*, Table S1). These samples may carry unreported microdeletions in one or both alleles.

Cases with 13q Deletions Identified by FISH. Similar to the cases without 13q deletions, we considered the difference between AL and ChrL (determined by FISH) significant if higher than 12%. All samples harboring 13q deletions alone or in combination with other abnormalities (n = 44 total) showed consistent loss of *miR-15a/16-1* as expected (determined by *miR-15a/16-1* CNV). Among these, we found 15 informative cases (34.9%) with a higher AL than expected when compared with the ChrL (2 out of 12 in cohort 13q$^-$, 4 out of 8 in cohort 13q$^-$11q$^-$, 2 out of 7 in cohort 13q$^-$17p$^-$, and 7 out of 16 in cohort 13q$^-$T12). These samples may carry a *miR-15a/16-1* microdeletion on the remaining homologous chromosome 13 in cells bearing a 13q monoallelic deletion, or in a subpopulation of CLL cells that do not harbor such chromosomal abnormality.

Thus, we found unreported microdeletions affecting *miR-15a/16-1* in a total of 31 out of 90 samples (34.4%) (Fig. 1, Table 1, and *SI Appendix*, Table S1). These data suggest that microdeletions involving *miR-15a/16-1* are at least as frequent in T12 CLL as in other CLL cohorts.

13q Microdeletions in T12 Cases without Loss of 13q by FISH. Since association of 13q deletions with T12 is less common than with other chromosomal abnormalities, microdeletions targeting *miR-15a/16-1* may have a significant role in the pathogenesis of T12 CLL. To evaluate the incidence of *miR-15a/16-1* AL in T12 CLL cases, we clustered the 23 samples from the T12 cohort into two groups, 10 indolent and 13 aggressive, according to the prognostic markers provided (Dataset S1). Eight out of 23 samples showed a significant AL of *miR-15a/16-1* (34.8%). In addition, we observed that 2 of these 8 T12 samples, showing the highest AL, were found among the 13 aggressive cases: sample 2227, AL of ~35%, and sample 2230, AL of ~20%. The remaining 6 samples with an unexpected significant AL were found among the 10 indolent cases, showing an AL between 12.2 and 17.5% (Table 1 and *SI Appendix*, Table S1). These results suggest that identification of *miR-15a/16-1* AL may be a valuable tool for risk stratification of T12 patients.

***miR-15a/16-1* Microdeletions in CLL with Other Karyotypes.** We investigated whether the findings described above may be applicable to other cohorts of CLLs. We found that while in 13q$^-$ and NK cohorts (21 patients) only 3 samples (~14%) showed unidentified *miR-15a/16-1* AL, in cohorts with T12, 11q$^-$, and 17p$^-$ (69 patients), 28 samples showed unexpected *miR-15a/16-1* AL (~40%). Based on these results, we designed

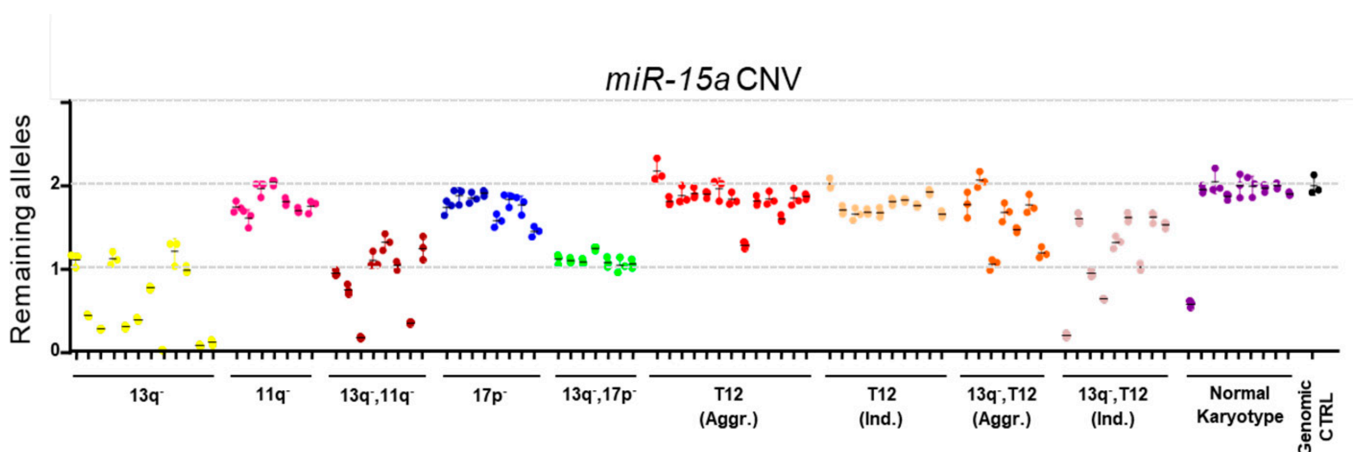

Fig. 1. *miR-15a/16-1* CNV analysis of eight CLL cohorts.

Table 1. FISH and *miR-15a/16-1* CNV analysis

Sample ID	Cohort	Class	% CLL cell with a 13q monoallelic deletion (by FISH)	% CLL cell with a 13q biallelic deletion (by FISH)	% 13q ChrL (FISH)	% *miR-15a/16-1* AL (CNV)
2152	$13q^-$	Indolent	66	14.5	47.5	85.8
2157	$13q^-$	Indolent	97.1	0	48.55	98.3
2168	$11q^-$	Aggressive	0	0	0	13.1
2169	$11q^-$	Aggressive	10	0	5	19.8
2174	$11q^-$	Aggressive	0	0	0	15.2
2176	$11q^-$	Aggressive	0	0	0	12.6
2179	$17p^-$	Aggressive	0	0	0	13.2
2183	$17p^-$	Aggressive	0	0	0	21.3
2191	$17p^-$	Aggressive	0	0	0	27.8
2210	T12	Indolent	0	0	0	15
2211	T12	Indolent	0	0	0	17.5
2213	T12	Indolent	0	0	0	16.3
2214	T12	Indolent	0	0	0	16.5
2217	T12	Indolent	0	0	0	12.2
2219	T12	Indolent	0	0	0	17.4
2227	T12	Aggressive	0	0	0	35.6
2230	T12	Aggressive	0	0	0	20
2163	$13q^-11q^-$	Aggressive	66.5	0	33.25	62.3
2164	$13q^-11q^-$	Aggressive	8	63.5	67.5	90.8
2166	$13q^-11q^-$	Aggressive	44	0	22	34
2172	$13q^-11q^-$	Aggressive	56	0	28	81.9
2178	$13q^-17p^-$	Aggressive	66.5	0	33.25	44.9
2188	$13q^-17p^-$	Aggressive	36.5	0	18.25	46
2196	$13q^-$T12	Aggressive	13	0	6.5	26.6
2198	$13q^-$T12	Aggressive	51	0	25.5	40.6
2199	$13q^-$T12	Indolent	78.5	0	39.25	89.6
2201	$13q^-$T12	Indolent	50	0	25	52.5
2205	$13q^-$T12	Indolent	10.5	0	5.25	19.3
2207	$13q^-$T12	Indolent	8.5	0	4.25	19.3
2208	$13q^-$T12	Indolent	12	0	6	23.8
2233	NK	Indolent	0	0	0	70.7

Thirty-one informative cases. Calculation of % 13q ChrL (FISH) and % *miR-15a/16-1* AL (CNV) is described in the first paragraph of *Results*.

a custom FISH probe to specifically identify microdeletions of the *miR-15a/16-1* locus (Fig. 2*A*), and performed a FISH experiment on eight selected samples (2211, 2219, and 2227 from cohort T12; 2233 from cohort NK; and 2196, 2199, 2201, and 2208 from cohort $13q^-$T12). The results of our experimental FISH support the *miR-15a/16-1* CNV data (Table 2). Indeed, samples 2211, 2219, and 2227, belonging to cohort T12, showed a loss of *miR-15a/16-1* in trisomic cells (Fig. 2 *B–D*). Sample 2233, belonging to cohort NK, showed biallelic loss of *miR-15a/16-1* (Fig. 2*E*). Interestingly, belonging to cohort $13q^-$T12, and expected to carry a monoallelic deletion of chromosome 13, samples 2199 and 2201 showed biallelic deletion of *miR-15a/16-1* both in trisomic cells and in nontrisomic cells (Fig. 2 *F* and *G*).

In conclusion, our results showed that 56 out of 90 CLL samples (~62%) had lost *miR-15a/16-1* (Table 1 and *SI Appendix*, Table S1). While standard FISH analysis indicated that ~49% of samples in our cohorts carry a gross deletion of chromosome 13, we found that ~34% of cases with no chromosome 13 deletion carry a microdeletion of *miR-15a/16-1*. In addition, we found that 28 out of 69 samples carrying a trisomy of chromosome 12, a deletion of 17p, or a deletion of 11q (40%) show unreported loss of *miR-15a/16-1*.

Discussion

To investigate the prevalence of microdeletions affecting the *miR-15a/16-1* locus on chromosome 13q14 in CLL patients with different karyotypes, we accrued eight cohorts of samples clustered according to their cytogenetic profiles. We carried out a *miR-15a/16-1* CNV assay to detect specifically the loss of the *miR-15a/16-1* locus, regardless of larger deletions of the chromosome region in which it is included. In addition, we carried out a customized FISH analysis with a *miR-15a/16-1*–specific probe, to confirm our data on selected samples. For most of the patients carrying a deletion of the 13q14 region, we found that the CNV data indicated a loss of *miR-15a/16-1*, consistent with the loss of chromosome 13q indicated by the clinical cytogenetic profile provided. However, we found 15 cases where the *miR-15a/16-1* CNV data indicated that the number of lost alleles encoding *miR-15a/16-1* was higher than expected, suggesting that subclones harboring a microdeletion of *miR-15a/16-1* may have been missed by FISH analysis. Importantly, we found 16 cases where *miR-15a/16-1* was lost in the absence of a detectable 13q deletion by FISH. For instance, CLL 2233, reported as a normal karyotype, carried a biallelic deletion of *miR-15a/16-1* according to our CNV and custom FISH data. Patients who have lost the *miR-15a/16-1* locus may be sensitive to venetoclax due to *BCL2* overexpression regardless of the 13q deletion detected by FISH. Thus, our results suggest that the FISH detection of 13q deletions in the clinical setting may not be sufficient to efficiently stratify patients for treatment, as undetected microdeletions targeting *miR-15a/16-1* may be present. In addition, loss of the *miR-15a/16-1* locus seems to be prevalent in T12 patients. This is an interesting finding, as T12 patients show the most variable clinical heterogeneity and are specifically susceptible to Richter's transformation, a serious condition characterized by

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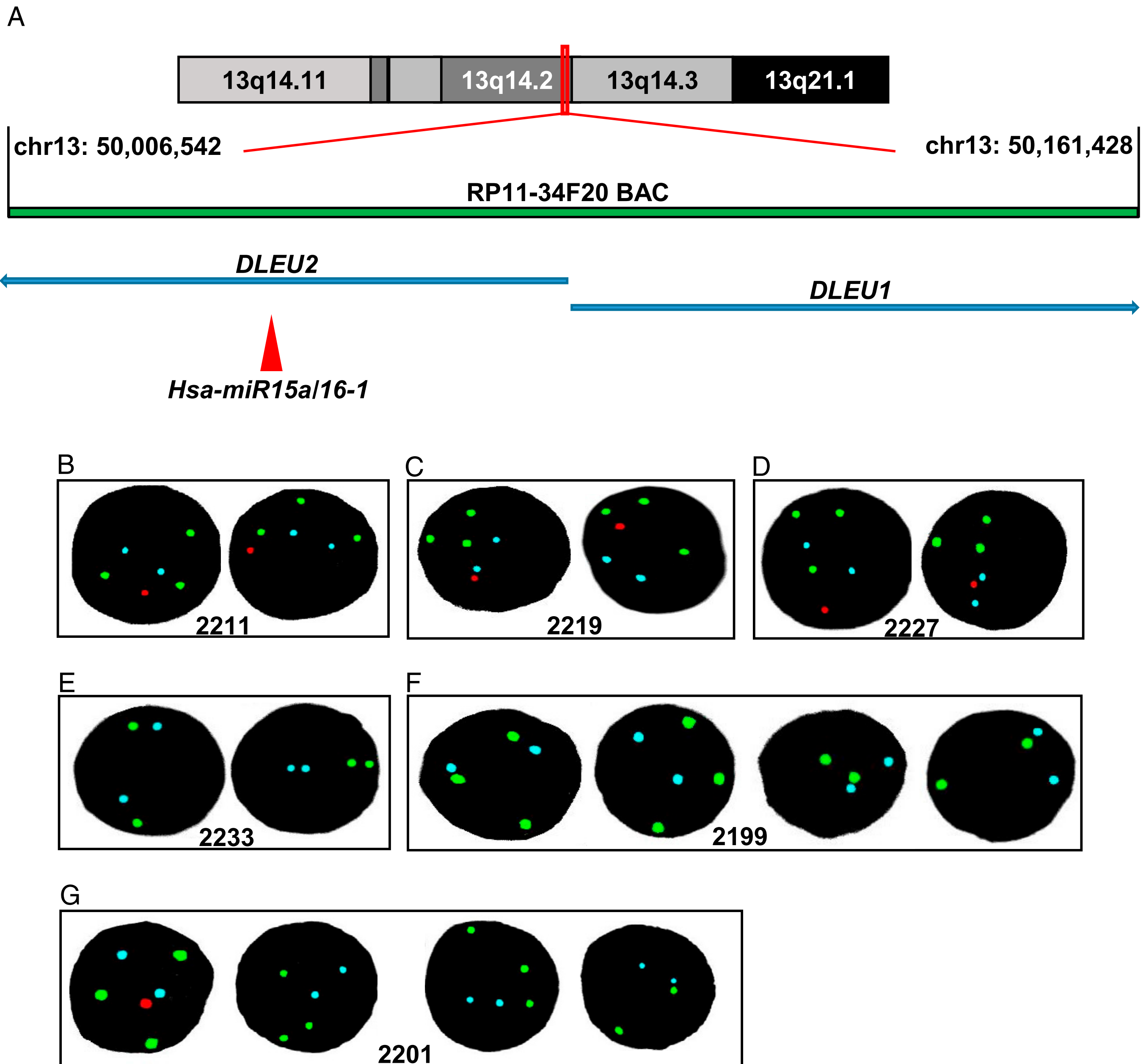

Fig. 2. FISH analysis of selected samples. (*A*) Genomic location of a custom *miR-15a/16-1* FISH probe. The *miR-15a/16-1* probe position is indicated by the red arrowhead on BAC RP11-34F20 (in green). (*B–G*) Representative images of CLL nuclei of the indicated samples. Probes used were the custom probe for the *miR-15a/16-1* locus (red) and custom probe for the *miR-195/497* locus (azure). (*B–D*, *F*, and *G*) CEP12 spectrum commercial probe for chromosome 12 (green). (*E*) Custom probe for *miR-15b/16-2* (green).

the development of a diffuse large B cell lymphoma often resistant to treatment (18, 19). Unexpectedly, when clustering the T12 samples in indolent and aggressive cases, we found that only 2 out of 13 aggressive samples showed a loss of *miR-15a/16-1*, while 6 out of 10 indolent samples showed a loss of *miR-15a/16-1*. However, the percentage of AL between aggressive and indolent cases is quite different: ∼35 and ∼20% in the aggressive cases, versus 12.2 to 17.5% in the indolent cases. The presence of subclones carrying a deletion of *miR-15a/16-1* in the T12 cohorts is also important in the evaluation for treatment. Indeed, if not taken into account when evaluating treatment strategy, such subclones in patients with a T12 mild or aggressive disease could have a selective advantage, leading to disease progression or relapse. Higher percentages of AL in aggressive cases also suggest that loss of *miR-15a/16-1* in T12 CLL can be used as a diagnostic tool to identify patients who may benefit from earlier treatment. Custom FISH analysis confirms the *miR-15a/16-1* CNV observations on the selected samples analyzed. Thus, our observations indicate that microdeletion of the *miR-15a/miR-16-1* locus is more common than expected in all CLL types, regardless of cytogenetic profile, and that the presence of undetected subclones can lead to relapse if not taken into account when the treatment plan is designed. Therefore, it is important to identify loss of the *miR-15a/16-1* locus, as this may have a significant impact on the design of personalized and efficient therapeutic plans.

Table 2. Custom FISH results for selected samples

Sample ID	Cohort	$13q^-$ category	Custom FISH analysis (%)
2196	$13q^-$T12	Monoallelic	2R2G2A (22)
			2R3G2A (57)
			1R3G2A (21)
2199	$13q^-$T12	Monoallelic	2R2G2A (4)
			0R2G2A (13)
			0R3G2A (83)
2201	$13q^-$T12	Monoallelic	0R2G2A (2)
			1R2G2A (6)
			2R2G2A (43)
			0R3G2A (24)
			1R3G2A (25)
2208	$13q^-$T12	Monoallelic	2R2G2A (41)
			1R2G2A (16)
			2R3G2A (43)
2211	T12	Normal	2R2G2A (25)
			1R3G2A (7)
			2R3G2A (68)
2219	T12	Normal	2R2G2A (30)
			1R3G2A (10)
			2R3G2A (60)
2227	T12	Normal	2R2G2A (20)
			2R3G2A (74)
			1R3G2A (6)
2233	NK	Normal	2R2G2A (42)
			1R2G2A (1)
			0R2G2A (57)

Probes used were a custom probe for the *miR-15a/16-1* locus (R) and a custom probe for the *miR-195/497* locus (A). A CEP12 spectrum commercial probe for chromosome 12 (G) was used in all samples except 2233. A custom probe for *miR-15b/16-2* (G) was used for sample 2233. For each sample, the last column reports the percentage of cells showing a specific allelic combination. For example, in sample 2196, 22% of CLL cells have two alleles for each probe target and 57% of CLL cells have three copies of chromosome 12 and two alleles for *miR-15a/16-1* and *miR-195/497*.

Materials and Methods

Study Design. Ninety samples from primary untreated CLL patients were collected. Each patient provided written informed consent in compliance with the Declaration of Helsinki. This study was approved by the institutional review boards of the University of California San Diego and The Ohio State University. Peripheral blood mononuclear cells (PBMCs) were isolated from patients enrolled in the CLL Research Consortium who satisfied diagnostic and immunophenotypic criteria for CLL. The cytogenetic profile of each patient was provided to classify patients into cohorts: Standard FISH analysis was carried out with commercial probes and the percentage of cells with any chromosomal abnormality was recorded. DNA was isolated from PBMCs to carry out CNV analysis. An additional experimental FISH analysis was carried out with a custom-made *miR-15a/16-1*–specific probe to confirm *miR-15a/16-1* CNV data (Table 2).

Patient Cohorts. Patients were grouped into eight cohorts according to standard FISH data. Four cohorts showed a single chromosomal abnormality: cohort $13q^-$ ($n = 12$), cohort $11q^-$ ($n = 7$), cohort $17p^-$ ($n = 8$), and cohort T12 ($n = 23$). Three cohorts showed a combination of the 13q deletion with another abnormality: cohort $13q^-11q^-$ ($n = 8$), cohort $13q^-17p^-$ ($n = 7$), and cohort $13q^-$T12 ($n = 16$). One cohort showed a normal karyotype: cohort NK (n = 9). A detailed list of patient samples is reported in Table 1 and *SI Appendix*, Table S1.

DNA Isolation and CNV. DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen; 69504) following the manufacturer's instructions. DNA concentration and quality were examined using the NanoDrop 2000 spectrophotometer. A CNV assay was performed using a custom probe to detect *miR-15a* in 10 ng of genomic DNA. Quantitative real-time PCR TaqMan copy-number assays were performed using the miR-15a–specific FAM dye–labeled custom probe, designed on genomic coordinates chr13: 50,049,119 to 50,049,201 (Applied Biosystems). TaqMan CNV reactions were performed in triplicate and normalized using a VIC dye–labeled telomerase reverse transcriptase (TERT) control output (Thermo Fisher Scientific; 4403316). Commercial human genomic DNA was used as a biallelic reference (Promega; G147A). The analysis for the *miR-15a/16-1* CNV assay was performed by setting a value of 2 for the biallelic controls, to indicate the presence of two alleles (Fig. 1). All data were then normalized against the biallelic control and converted to provide a percentage of AL (Table 1 and *SI Appendix*, Table S1).

Custom FISH Probe Design and FISH Analysis. Standard FISH analysis for chromosome 13 deletions in a clinical setting was performed with a Vysis D13S319 (13q14.3) SpectrumOrange probe (Abbott; 05J86-011; GTIN 00884999012776). Experimental FISH analysis was performed with custom probes. Three human bacterial artificial chromosomes (BACs), RP11-34F20, RP11-227J5, and RP11-61B20, which span the *miR-15a/16-1* locus on chromosome 13, *miR-15b/16-2* locus on chromosome 3q, and *miR-195/497* locus on chromosome 17p, respectively, were purchased from the BACPAC Resources Center. Locations of RP11-34F20 (chr13: 50,006,542 to 50,161,428), used in Fig. 2 B–G and spanning *miR-15a/16-1*, is shown in Fig. 2A. BAC DNAs, purified with the NucleoBond Xtra BAC Kit (Takara Bio USA), were subjected to fluorescent labeling by the nick translation method in the presence of either SEEBRIGHT Red 598 dUTP (RP11-34F20), SEEBRIGHT Green 496 dUTP (RP11-227J5), or SEEBRIGHT Aqua 431 dUTP (RP11-61B20), purchased from ENZO Life Sciences. The RP11-34F20 BAC of 154,887 bp spanning the *miR-15a/16-1* locus on chromosome 13 was chopped up to obtain a final product of homogeneous 200-bp pieces by nick translation. A nick translation reaction was prepared on ice in a thin-wall PCR tube containing 1.8 μg BAC DNA, 50 mM Tris·HCl (pH 7.4), 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 30 μM each dATP, dCTP, and dGTP, 15 μM each dTTP and fluorophore-dUTP, 90 U *Escherichia coli* DNA polymerase I (New England Biolabs), 22.5 ng DNase I (Enzo Life Sciences), and 9 μL of 80-fold diluted in dilution buffer. The reaction was started by incubating at 15 °C for 3 h and quenched by adding 5 μL of 0.5 M ethylenediaminetetraacetic acid. Free dNTPs and fluorophore–dUTP were removed by applying on Bio-Spin P-6 gel columns (Bio-Rad). In this condition, a probe with an average size of 200 bp could be produced. Cryopreserved samples were thawed and then cultured in RPMI media supplemented with 20% fetal bovine serum, L-glutamine, lectin pokeweed mitogen, phorbol 12-myristate 13-acetate (Life Technologies), and CpG ODN 685 (Sigma-Aldrich) for 72 to 96 h. Colcemid (Life Technologies) was added 30 min prior to harvesting. Samples were fixed in Carnoy's fixative and slides were made following standard laboratory procedures. FISH was performed with a mixture of custom-made BAC probes and CEP 12 Spectrum Green (Abbott Molecular). The BAC probes were combined with human Cot-1 DNA (Life Technologies), ethanol-precipitated, and resuspended in Vysis IntelliFISH hybridization buffer (Abbott Molecular). The CEP 12 probe was then added to the mixture. Prior to hybridization, slides were pretreated with 0.005% pepsin in 0.01 N HCl followed by a postfix solution (0.9% formalin, 12.5 mM $MgCl_2$ in phosphate-buffered saline). FISH was performed according to the manufacturer's recommendations. Probes and samples were codenatured for 5 min at 73 °C and then hybridized overnight at 37 °C. Slides were washed in 2× saline sodium citrate, 0.1% Nonidet P-40 for 5 min at 42 °C, dried, and counterstained with DAPI. Signals were enumerated using a fluorescence microscope (Zeiss Axioscope 40) and images were captured with Applied Spectral Imaging software. One hundred cells were analyzed per patient.

Statistical Analysis. To compare the *miR-15a/16-1* CNV results with the data offered by standard FISH, we determined the percentage of chromosome 13 loss based on the standard FISH analysis data. The percentage of cells showing a monoallelic deletion of chromosome 13 was divided by 2 and added to the percentage of cells showing a biallelic deletion of chromosome 13 to obtain the percentage of chromosome 13 lost. Based on our results for sample 2207, which has the lowest percentage of ChrL identified, we established a threshold of 4.25% ChrL as the lowest limit of detection for standard FISH (σ). To compare standard FISH with *miR-15a/16-1* CNV, we set a threshold of 2σ (~8.5%) that identifies a significant difference between the two groups. However, five samples (2173, 2187, 2215, 2221, and 2228) show an AL, identified via *miR-15a/16-1* CNV assay, very close to this threshold. Thus, to minimize the detection of samples that may not be representative of a significant difference, we adjusted the threshold to 12%.

Data Availability. All study data are included in the article and/or supporting information.

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