Telomeric *IGH* Losses Detectable by Fluorescence *in Situ* Hybridization in Chronic Lymphocytic Leukemia Reflect Somatic V_H Recombination Events

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Routine interphase fluorescence *in situ* hybridization (FISH) analysis of chronic lymphocytic leukemia (CLL) with LSI *IGH*/CCND1 assay, applied to differentiate CLL from leukemic mantle cell lymphoma, identified a subset of cases (42/174) with translocation-like *IGH* signal pattern. To unravel the underlying 14q32/*IGH* aberrations, 14 of these cases were subjected to cytogenetic, detailed FISH, and V_H mutation analyses. FISH identified cryptic losses of various portions of the *IGHV* region in all 14 cases. Fine mapping of these V_H deletions revealed a strict correlation between their distal border and localization of the used V_H gene, suggesting that they are not oncogenic but reflect physiological events accompanying somatic *V-D-J* assembly. This hypothesis was further supported by FISH analysis of 20 CLL and hairy cell leukemia cases with the known V_H usage showing a constant loss of sequences proximal to the used gene, identification of V_H deletions in normal B cells, and their exclusive demonstration in B cell malignancies, but not of T cell and myeloid lineage. Given that these cryptic physiological V_H losses in B cells may seriously complicate analysis of B cell leukemia/lymphoma and lead to false conclusions, FISH users should take them into consideration when interpreting *IGH* aberrations in these malignancies. (J Mol Diagn 2007, 9:47–54; DOI: 10.2353/jmoldx.2007.060088)

Chromosomal translocations affecting the immunoglobulin loci, particularly immunoglobulin heavy chain (*IGH*) genes complex at 14q32, are a hallmark of B cell malignancies.^{1,2}

They usually result in deregulated expression of involved oncogenes (eg, *BCL1*, -2, -3, -6, *CMYC*, and *PAX5*) juxtaposed to the regulatory elements of *IGH*. As some of these translocations are associated with specific subtypes of mature B cell lymphoma and have prognostic significance, their detection is of clinical importance. In daily practice, *IGH* translocations have been routinely analyzed by fluorescence *in situ* hybridization (FISH) using either a common LSI *IGH* dual-color, break-apart rearrangement assay or dual-color, dual-fusion oncogene-specific probe, such as LSI *IGH*/CCND1, *IGH*/BCL2, and *IGH*/CMYC.

The *IGH*-mediated translocations are relatively rare in B cell chronic lymphocytic leukemia (CLL),^{3–5} which is the most common form of leukemia in adults and shows a highly variable clinical course. The CLL cells display a phenotype of mature activated B lymphocytes expressing CD19, CD5, and CD23 and having reduced levels of membrane IgM, IgD, and CD79b. The co-expression of CD19 and CD5, however, is also characteristic for mantle cell lymphoma (MCL), which, in contrast to CLL, is usually an aggressive disease hallmarked by the t(11;14)(q13;q32)/*IGH*-CCND1 rearrangement. Differential diagnosis between CLL and leukemic MCL is sometimes challenging.⁵ Given that up to 30% of MCL cases have immunophenotypic features characteristic of B-CLL, albeit usually with atypical, pleomorphic morphology,⁶ immunophenotyping alone is insufficient to exclude a diagnosis of MCL. Therefore, in the Belfast City Hospital (Belfast, Northern Ireland) all suspected CLL cases have been routinely examined by rapid interphase FISH with the LSI *IGH*/CCND1 assay to identify t(11;14)-positive MCL cases, in addition to examination for CLL typical cytogenetic aberrations. During this analysis, a subset of CLL

Supported in part by the Fund for Scientific Research of Flanders (FWO-Vlaanderen), Grant G.0338.01, K.U. Leuven Research Foundation (BIL03/12), and by the Belfast City Hospital Trust Haematology Research Funds. C.M. was funded by the Northern Ireland Department of Education and Learning. V.V. is a fellow of the Belgian Foundation against Cancer, and P.V. is a Clinical Investigator of FWO-Vlaanderen. This work presents research results of the Belgian Programme of Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming.

Accepted for publication August 18, 2006.

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cases with *IGH* signal pattern suggestive of *IGH* translocation different from t(11;14) has been identified. Fourteen of these cases were subjected to cytogenetic, further FISH, and V_H mutation analysis. Results are discussed below.

Materials and Methods

Patients

Peripheral blood samples from 14 of 42 CLL patients with the previously identified *IGH* abnormalities attending the Hematology Outpatients Clinic, Department of Hematology, Belfast City Hospital, between September 2004 and April 2005 were collected for this study. The study had local Ethics Committee approval and the patients gave informed consent to participate in the investigation. The diagnosis in all CLL patients was confirmed by immunophenotyping and all had the typical CD19+CD20WPCD23+CD43+CD79bWPCD5+CD10− phenotype and demonstrated light chain restriction. Additional cases of B cell, T cell, and myeloid malignancies were collected from the files of Department of Human Genetics, K.U. Leuven (Leuven, Belgium). The HDML2, L-1236, and KM-H2 cell lines came from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Cytogenetic and Fluorescence in Situ Hybridization (FISH) Analysis

Cytogenetic analysis was performed on peripheral blood cells cultured for 72 hours after stimulation with 12-O-tetradecanoyl phorbol-13-acetate. In each case, 3 to 26 metaphase cells were analyzed. Karyotypes were described according to ISCN.⁷

Probes used for FISH analysis included the LSI *IGH*/CCND1, *IGH*/CCND1 XT, *IGH*, and *IGH*/BCL2 assays (Vysis, Downers Grove, IL), 14q32 subtelomeric probe (P1 60H4),⁸ and a set of nine bacterial artificial chromosome (BAC) clones covering *IGH* (11771, 417P24, 676G2, 965B13, 112H5, 101G24, 12F16, 47P23, and 2548B8).⁹ Noncommercial probes were directly labeled with Spectrum Orange- and Spectrum Green-dUTP (Vysis) using nick translation. Two *IGH* BAC clones, 676G2 and 965B13, co-hybridized with 15q11, and 2548B8 co-hybridized with the 16p11.2 region.⁹ Co-hybridization signals, however, were weaker and did not significantly perturb interphase FISH analysis.

FISH experiments were evaluated using an Axioplan 2 fluorescence microscope equipped with a charge-coupled device Axiophot 2 camera (Carl Zeiss Microscopy, Jena, Germany) and a MetaSystems Isis imaging system (MetaSystems, Altlußheim, Germany). One to 18 abnormal metaphases and/or 200 interphase cells were evaluated in each FISH experiment.

Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasm (FICTION) analysis with CD20 antibody (DakoCytomation, Glostrup, Denmark) and two pairs of *IGH* BAC

clones performed on smears from normal peripheral blood and reactive lymph node followed previously published protocol.¹⁰

V_H Mutation Analysis

Mutational status of V_H was determined using the BIOMED-2 method as previously described.^{11,12} Mutations and V_H segment usage were identified by comparison with the germline sequence using Ig Blast database (<http://www.ncbi.nlm.nih.gov/igblast/>) and IMGT (<http://imgt.cines.fr/>). Sequences containing more than 2% deviation from the germline were considered as somatically mutated rather than genetic polymorphisms.

Results

Routine interphase FISH analysis with LSI *IGH*/CCND1 applied to 174 CLL cases identified 42 cases (24%) with aberrant three green (G) signals from the *IGH* probe included in this assay and two regular red (R) (11q13/CCND1) signals (Figure 1A). Given that the accompanying normal cells in these samples showed either two separated green signals (Figure 1B) or two double green signals (together four green signals) (Figure 1C), molecular events underlying this aberrant signal constellation (2R3G) were not clear. We presumed that three *IGH*/green signals arose by *IGH* breakage due to chromosome translocation (split of one of two green signals in Figure 1, B cells), but a partial deletion of *IGH* (loss of one of four green signals in Figure 1, C cells) or a fragmental excision of the *IGH* locus followed by its insertion into another gene locus or a trisomy 14q32 could not be excluded. To clarify these intriguing FISH findings, 14 of these CLL cases (further referred to as index cases) with available peripheral blood cells at time of the study were subjected to cytogenetic and further detailed FISH analysis.

Chromosome studies led to identification of clonal or nonclonal karyotypic changes in eight and four cases, respectively (Table 1). The involvement of 14q32 harboring the *IGH* locus was detected in case 1 with a del(14)(q24q32) and in case 13 with a t(14;18)(q32;q21). The typical CLL abnormalities, −13/del(13q), trisomy (or tetrasomy) 12, del(6q), and loss of chromosome 17/17p, were found in six, three, two, and two cases, respectively.

All 14 cases have been simultaneously re-analyzed by FISH with LSI *IGH*, a dual-color, break-apart rearrangement assay. In contrast to the previously used LSI *IGH*/CCND1 comprising a mix of two Spectrum Green-labeled probe segments, one covering the *IGHC* and 3' *IGH* region (450 kb) and the second hybridizing to the terminal part of *IGHV* region (450 kb), the latter assay comprises two differently labeled probes, the Spectrum Orange-labeled 3' *IGH* flanking probe (250 kb) (further referred as 3' *IGH* FP) and the Spectrum Green-labeled *IGHV* probe (900 kb) covering the entire *IGHV* region (Vysis).

FISH analysis with LSI *IGH* was performed on interphase and, when applicable, on metaphase cells. This

Figure 1. Examples of FISH results. Analyzed cases: case 5 (**A–C**, **F**), case 1 (**D**), case 3 (**E**), diffuse large B cell lymphoma with $t(3;14)(q27;q32)$ and $+der(3)t(3;14)(q27;q32)$ (**G**) and normal peripheral B cells analyzed by FICION with CD20 (**H**). Applied probes: LSI IGH/CCND1 (**A–C**), LSI IGH (**D–F**), 965B13-SO/47P23-SG (**G**), and 47P23-SG/2548B8-SO (**H**). **Arrow** and **arrowheads** in **G** indicate normal chromosome 14 and two $der(3)t(3;14)$, respectively. Extra red signals seen in **G** correspond to 15q11, a region of co-hybridization of 965B13.⁹

analysis showed aberrant signal constellations in 10 to 88% of analyzed cells from all 14 cases (Table 1). The patterns included loss of the 3' IGHFP red signal (1F1G) detected in case 1 with $del(14)(q24q32)$ (Figure 1D), complete or partial loss (diminished) of the IGHV/green

signal (1F1R or 1F1RG_d, respectively) found in 11 cases (nos. 2 to 12) (Figure 1, E and F), and the split 1F1R1G pattern observed in case 13 with a $t(14;18)$ and in case 14 without cytogenetically detectable $t(14q32)$ (LSI IGH/BCL2 negative). As a control, we examined nine new CLL

Table 1. Results of Cytogenetic, FISH, and V_H Analysis

Case	Cytogenetics	FISH with LSI IGH		V_H analysis	
		Signal pattern	No. of metaphase cells/% of interphase cells	V_H gene usage	Mutation status
1	48,XY,+12,del(14)(q24q32),+del(18)(q21) [8]	2F	2/40	4-39	UM
2	46,XY,del(13)(q13q14)[2]/46,XY[14]	1F1G	3/60		
		2F	4/66	1-69	UM
		1F1R	8/34		
3	43-48,XX,del(3)(q27)[2],+4[2],del(13)(q13q14),+1-2mar[cp4]/46,XX[4]	2F	3/60	3-48	M
4	46,XY,del(13)(q)[9]/46,XY [10]	1F1RG _d	2/40		
		2F	1/70	4-31	UM
		1F1RG _d	4/30		
5	46,XY,-10,+11,del(13)(q12q14)[1]/46,XY [6]	2F	7/42	3-48	M
		1F1RG _d	7/58		
6	46,XX,der(7)t(1;7)(q25;q32),del(13)(q13q14)[3][cp7]/46,XX[1]	2F	7/79	4-34	UM
7	46,XY,del(6)(q23)[1]/46,XY [2]	1F1RG _d	8/21		
		2F	0/27	1-69	UM
		1F1R	0/73		
8	45-47,X,-Y[5],del(6)(q13q15),+7[2],-17[2],+mar[3][cp7]/46,XY[1]	2F	1/13	1-69	UM
9	45-47,XY,add(12)(p13)[1],der(14)t(14;17)(p12;q11)[2],add(14)(p12)[3],-17[4],add(22)(p12)[1],+mar[3][cp5]	1F1R	17/87		
		2F:	1/12	1-69	UM
10	47,XY,-2,+12,+12[1]/46,XY[17]	1F1R	5/88		
		2F	1/17	1-69	M
		1F1R	5/83		
11	46,XX[16]	2F	0/43	3-53	M
		1F1RG _d	0/57		
12	46,XY[5]	2F	3/45	1-69	M
		1F1R	6/55		
13	47,XY,+12,t(14;18)(q32;q21)[6]/46,XY[20]	2F	1/61	3-30	UM
		1F1R1G	3/39		
14	46,XX,-13,+mar[1]/46,XX[8]	2F	0/90	1-2	M
		1F1R1G	0/10		

F, fused; R, red; G, green; d, diminished signal; NS, not successful; M, mutated; UM, unmutated.

cases recently registered in our laboratory. In three of them complete or partial loss of the *IGHV*/green signal was also found (data not shown). In control experiments performed on four blood samples from normal individuals (200 interphase evaluated in each experiment), the cut-off level of diminished/lost *IGH* signals was 1%. Further evidence of the predominant *IGHV* deletions underlying the aberrant "three green" *IGH* signal pattern in the present cases was provided by metaphase FISH analysis with LSI *IGH*/CCND1, LSI *IGH*/CCND1 XT, and LSI *IGH*/BCL2. In contrast to LSI *IGH*/CCND1, the *IGH* probe included in the latter assays is made up of a single 1.5-Mb probe covering essentially the entire locus. FISH analysis with these three assays performed on four cases (nos. 3, 8, 9, 10) showed the same results on metaphase cell level: normal green signal on one chromosome 14 and diminished green signal on a second chromosome 14. The difference was noticed at the interphase cell level because only LSI *IGH*/CCND1 produced a two double green signals pattern in a significant number of cells. Normal interphase cells hybridized with LSI *IGH*/CCND1 XT and LSI *IGH*/BCL2 displayed two regular green signals, and abnormal cells were hallmarked by the 1F1RG_d pattern, supporting our previous conclusion.

The *IGHV* deletions detected in the index cases were further mapped by FISH using nine BAC clones covering the entire *IGH* locus labeled with either Spectrum Orange- (SO) or Spectrum Green (SG)-dUTP (Figure 2).⁹ This analysis performed on 11 cases (nos. 2 to 12), with loss of the *IGH*- "green signal," showed presence of the *IGHC* sequences covered by 417P24 and 11771 and loss of signal from various *IGHV* BAC clones (Figure 2). Loss of signals from three, four, and five *IGHV* clones was detected in three, three, and six cases, respectively. All of them showed normal presence of two 47P23 (*IGHV* terminal) signals. Cases 8 and 9 were additionally analyzed with the 14q subtelomeric probe (P1 60H4), which in both cases gave two regular signals. Unexpectedly, loss of signals from two or three *IGHV* BAC clones were also found in two control cases without LSI *IGH*- "green signal" losses, including case 1 with del(14)(q24q32) (1F1G- "red signal" deletion) and case 13 with a t(14;18) (1F1R1G-split signals) (Figure 2, bottom panel).

The V_H mutational analysis performed on these index CLL cases showed that eight cases had unmutated (um) V_H genes and six cases had mutated (m) V_H genes that represented eight different V_H segments (Table 1). The most frequently used was V_H1-69 , identified in six cases

Figure 2. FISH mapping of *IGH* deletions in 11 CLL cases. Schematic presentation of the *IGH* region (top panel), applied probes (center panel), and FISH results (bottom panel) (not to scale). Checkered and dotted bars indicate Spectrum Green- and Spectrum Orange-labeled probes, respectively. Open bars mark regions found to be present on both chromosomes 14; filled bars point sequences lost on one homolog 14.

(2 m/4 μ m). The *V_H3-48* was used in two cases (m), and single cases used the *V_H1-2* (m), *V_H3-30* (um), *V_H3-53* (m), *V_H4-31* (um), *V_H4-34* (m), and *V_H4-39* (um) genes.

Comparison of fine *V_H* FISH mapping data available in 13 cases with results of the *V_H* analysis showed that in all studied cases a distal breakpoint of *V_H* correlated with the genomic localization of the used *V_H* gene (Figure 2). This finding suggested that the detected deletions represent physiological loss of genomic sequences associated with a somatic *V-D-J* assembly in precursor B cells.

To confirm this supposition, we further examined by FISH the status of the *V_H* region in a cohort of B cell, T cell, and myeloid malignancies. Given a common deletion of 965B13 (the most proximal *V_H* BAC clone) and constant presence of 47P23 (the most terminal *V_H* BAC clone) in the index CLL cases (Figure 2), we used these two clones labeled with SO- and SG-dUTP, respectively, for FISH analysis of a further 17 B cell leukemias/lymphomas, six T cell leukemias/lymphomas, and nine cases of myeloid malignancies listed in Table 2. The balanced co-localized signals (2–4) were detected in two B cell lymphomas and in all analyzed myeloid and T cell malignancies, including HDML2, the Hodgkin's lymphoma cell line of T cell origin. The remaining 13 B cell leukemia/lymphoma cases revealed aberrant FISH patterns manifested by either the diminished 965B13 signal (2/17) or by loss of one (10/17) or two (1/17) 965B13 signals. The two additional Hodgkin's lymphoma cell lines of B cell origin included in this study, L-1236 and KM-H2, displayed either a total loss of 965B13 signals (L-1236) or complex unbalanced (1R4–8G) FISH pattern (KM-H2).

Fifteen of these B cell leukemia/lymphoma cases were documented by various known 14q32/*IGH* translocations including t(3;14)(q27;q32) (3x), t(8;14)(q24;q32) (3x), t(11;14)(q13;q32) (3x), t(14;18)(q32;q21) (3x), and t(14;19)(q32;q13) (3x) (Table 2). Metaphase FISH analysis of these cases showed a common loss of the *V_H* proximal signal from chromosome 14 not involved in translocation and presence of normal co-localized *V_H* signal on the derivative partner chromosome (eg, der(3)t(3;14); Figure 1G). In one case with t(8;14), however, loss of both proximal (965B13) signals from normal chromosome 14 and derivative chromosome was found.

To check whether in other B cell leukemia cases size of *V_H* deletions also correlate with a genomic localization of used *V_H* gene segments, we analyzed by FISH 15 CLL and 5 hairy cell leukemia (HCL) cases with the known expression of *V_H1-2* (2x), *V_H3-7* (2x), *V_H3-11* (2x), *V_H3-15* (1x), *V_H3-21* (1x), *V_H3-23* (3x), *V_H3-30* (1x), *V_H3-33* (1x), *V_H3-48* (2x), *V_H4-34* (3x), *V_H4-39* (1x), and *V_H4-59* (1x). Each of these cases was analyzed with an individual pair of differentially labeled BAC clones, one covering the particular used *V_H* gene segment and the second covering the adjacent proximal *V_H* region (eg, in the case expressing *V_H3-48*, the 12F16 clone harboring *V_H3-48* and the proximal 101G24 clone were applied). Sixteen of 20 cases showed the presence of a hybridization signal from the *V_H* carrying clone and loss of signal from a proximal BAC. Two cases displayed loss of both proximal signals (biallelic deletion), and in the two remaining cases expressing the very proximal *V_H1-2*, two co-localized signals were detected (undetectable deletion).

Finally, we examined the occurrence of *V_H* deletions in normal B cells. For this purpose, FICTION analysis using CD20 antibody and the 47P23-SG/254B88-SO FISH assay were performed on two samples of normal peripheral blood and reactive lymph node. In these experiments a subset of CD20-positive cells with a 1F1G/1F1GRd signal pattern, similar to that seen in B cell leukemia/lymphoma, was identified (Figure 1H). In contrast, CD20-negative cells showed normal 2F signal configuration.

Discussion

We have shown that aberrant *IGH* FISH signal patterns, incidentally detected in a subset of CLL cases analyzed with LSI *IGH*/CCND1 assay, only rarely designate leukemia-associated *IGH* abnormalities (translocation, deletion). In the majority of cases, these FISH aberrations reflected somatic *V_H* deletions caused by loss of intervening DNA sequences between the targeted *V_H* segment and the *DJ* region that accompanies normal, physiological *IGH* rearrangement in B cells. Given the clonal nature of leukemia, all neoplastic cells manifested the same *V_H* deletion characteristic for the precursor CLL cell. Of particular interest, these physiological deletions were heralded by a peculiar "three green signal" pattern in interphase CLL cells. The *IGH* probe included in the applied LSI *IGH*/CCND1 assay comprises *de facto* two Spectrum Green-labeled probe segments covering the

Table 2. Results of FISH with the 965B13-SO and 47P23-SG *IGHV* Probes

Case (cell line)	Diagnosis ^a /14q aberration	Interphase FISH pattern	Metaphase FISH pattern
B cell malignancies			
1	DLBCL/t(3;14), +der(3)	2F1G	14G, 2xder(3)F
2	DLBCL/t(3;14)	1F1G	14G, der(3)F
3	DLBCL/t(3;14)	1F1G	14F, der(3)G
4	B-ALL/t(8;14)	1F1G	14G, der(8)F
5	BL/t(8;14)	2F	14F, der(8)F
6	BL/t(8;14)	2G	14G, der(8)G
7	MCL/t(11;14)	1F1G	14G, der(11)F
8	MCL/der(1)t(1;14), t(11;14)	1F1G	der(1)G, der(11)F
9	MCL/t(11;14)	2F	14F, der(11)F
10	FL/t(14;18)	1F1GR _d	14GR _d , der(18)F
11	FL/t(14;18)	1F1GR _d	14GR _d , der(18)F
12	FL/t(14;18), +der(18)	2F1G	14G, 2xder(18)F
13	CLL/t(14;19)	1F1G	14G, der19F
14	CLL/t(14;19)	1F1G	14G, der19F
15	CLL/t(14;19)	1F1G	14G, der19F
16 (L-1236)	HL/t(1;14),der(8)t(1;8;14), inv(14)	4G	der(1)G, der(8)G, inv(14)G/G
17 (KM-H2)	HL/-14, add(14)(p12)	1R4-8G	marR, 2-4x(?)i(14)G/G
T cell malignancies			
18	ALK+ALCL	4F	3x14F, marF
19	ALK+ALCL	2F	2x14F
20	gdHSTCL	2F	2x14F
21	T-ALL/t(10;14)	2F	14F, der(10)F
22	T-PLL/t(X;14)	2F	14F, der(X)F
23 (HDLM2)	T-HL/der(14)	2-4F	der(14)F, marF/2xder(14)F, 2xmarF
Myeloid malignancies			
24	Ph+ CML	2F	2x14F
25	Ph+ CML	2F	2x14F
26	Ph+ CML	2F	2x14F
27	Ph+ CML	2F	2x14F
28	Ph+ CML	2F	2x14F
29	MDS	2F	2x14F
30	AML M4	2F	2x14F
31	AML M4	2F	2x14F
32	AML M3	2F	2x14F

^aDiagnosis of the selected cases was reviewed prior to FISH studies; DLBCL, diffuse large B cell lymphoma; ALL, acute lymphoblastic leukemia; BL, Burkitt lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; HL, Hodgkin lymphoma; ALCL, anaplastic large cell lymphoma; gdHSTCL, γ/δ hepatosplenic T cell lymphoma; PLL, polymorphocytic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; F, fused; R, red; G, green; d, diminished signal.

proximal and distal *IGH* regions separated by approximately 450 kb (Figure 2, center panel). The discontinuity of this probe and different levels of chromatin condensation in interphase cells possibly account for occurrence of either two or four (two double) green *IGH* signals in normal cells (Figure 1, B and C). Our further studies clearly showed that these three green signals found in interphase CLL cells mostly represented loss of one of four normal (two double) signals due to the partial *IGH* deletions (V_H in 11/14 and C_H in 1/14 cases) and only rarely was split of one of two green signals due to the initially expected t(14q32/*IGH*) (2/14 cases). In no case was the aberrant *IGH* FISH pattern caused by a cryptic excision/insertion of *IGH* or trisomy of 14q32/*IGH*. Our supposition was supported by metaphase FISH analysis of these CLL cases with LSI CCND1 XT and LSI *IGH*/BCL2, which constantly generated one regular and one diminished (or lost) green signal on chromosomes 14. Further evidences supporting this model were provided by cytogenetic, detailed FISH and molecular V_H analysis of 14 cases representing this subset of CLL and by additional FISH studies of the V_H region performed on approximately 40 B cell leukemia/lymphoma cases with known and unknown

V_H usage. All V_H deletions detected in these cases were submicroscopic, interstitial, varying in size but constantly involving the most proximal V_H sequences adjacent to the *D-J* region, and in all of them the distal breakpoint strictly correlated with a genomic localization of the used V_H gene. They were detected exclusively in B cell neoplasms and not found in malignant proliferations of T cell and myeloid origin. Interestingly, these cryptic V_H deletions were also demonstrated in both Hodgkin's lymphoma cell lines of B cell origin (but not in HDML2 of T cell origin), providing additional evidence of somatic *IGH* rearrangements in typical Reed-Sternberg cells. Moreover, using FICTION we documented V_H deletions in a subset of normal CD20+ B cells from the peripheral blood and reactive tonsil but not in CD20-negative cells.

It is also worth noting that our metaphase FISH studies, performed on several t(14;var)(q32;var), demonstrated that V_H deletions constantly affected the normal chromosome 14 expected to carry a functionally rearranged *IGH* locus (Figure 1G), and only rarely chromosome 14 involved in translocation. These findings are fully concordant with the known mechanisms of somatic *V-D-J* recombination and allelic exclusion resulting in expression of only single productive immunoglobulin heavy and light

chain rearrangements in a given lymphocyte.^{13–16} According to this concept, B cells with failures in a primary *V-D-J* assembly (*VDJ*[−]/*VDJ*⁰), achieve productive rearrangement by ongoing independent recombination of *IGH* segments on the other chromosome 14 (*VDJ*[−]/*VDJ*⁺). We believe that biallelic *V_H* deletions found by FISH in a few analyzed B cell leukemia/lymphoma cases reflect either this latter mechanism or a defective immunoglobulin allelic exclusion leading to expression of two functional *IGH* alleles (*VDJ*⁺/*VDJ*⁺), as detected in approximately 6% of CLL cases.^{17,18} Moreover, we presume that analogous deletions in the *C_H* region might be detected by FISH in malignancies originating from mature B cells subjected to immunoglobulin class switch recombination, as shown using fiber-FISH approach¹⁹ and array CGH.²⁰ This mechanism, however, was not operating in the index CLL cases expressing IgM (data not shown).

Genomic losses from the 14q32/*IGH* region have been occasionally detected in CLL and other B cell lymphomas,^{4,21–23} generating difficulties in evaluation of FISH results.²⁴ Recently, Fink et al²² postulated that loss of the *IGHV* signal in CLL represents usage of a telomeric *IGHV* gene segment, as 9 of 11 of their cases expressed distally located *V_H* 1–69, *V_H* 3–72, and *V_H* 3–74. In contrast to our study, however, they noted a low incidence of *IGHV* deletions in other B cell malignant disorders (1/6). This discordance (17% versus 87% in the present series) is caused by a low resolution of applied LSI *IGH* assay when compared with our BAC FISH strategy.

Finally, we want to stress that the present *IGH* alterations were identified by chance during routine interphase FISH analysis of CLL cases with LSI *IGH*/CCND1, and in this particular condition *V_H* deletions were manifested by the unusual “three green signal” pattern in interphase CLL cells. In our daily practice, however, diminished or lost *V_H* signal has been recurrently observed in a wide spectrum of B cell malignancies routinely analyzed with LSI *IGH* and other FISH assays containing *IGH* probe. Although the phenomenon of physiological *V_H* deletions in B cells is well known, practical implications of these cryptic genomic losses for routine FISH analysis of B cell malignancies are often overlooked. As shown here, these somatic events may seriously complicate molecular cytogenetic analysis of B cell leukemia and lymphoma and lead to false conclusions. Therefore, to avoid misinterpretation of FISH data, FISH users should be aware not only of different molecular characteristics of commercial *IGH* probes routinely used for diagnostic purposes but also of cryptic *V_H* deletions that in B cell disorders do not manifest oncogenic events but normal physiological features of precursor B cells. To unravel *IGH* abnormalities generating aberrant FISH patterns in B cell malignancies, we propose two slightly different decision trees, one for users of LSI *IGH*/CCND1 (Figure 3A) and one for users of LSI *IGH*/CCND1 XT, -/BCL2, and -/CMYC (Figure 3B). Given the different characteristics of *IGH* probes included in these assays (discontinuous in LSI *IGH*/CCND1 and continuous in the remaining assays), these probes generate different aberrant interphase FISH patterns (Figure 3, A and B). In any case, however, when an

Figure 3. Decision trees to identify *IGH* aberrations not specific for the applied FISH assay or observed in B cell malignancies analyzed with either LSI *IGH*/CCND1 (**A**) or LSI *IGH*/CCND1 XT, -/BCL2, and -/CMYC (**B**). The latter assay is combined with the control Spectrum Aqua CEP 8, which is not included in this scheme. Cells harboring biallelic *V_H* deletions are marked by an asterisk.

aberrant (not specific for the applied assay) *IGH* signal pattern is seen, we recommend use of the LSI *IGH* dual-color break-apart rearrangement probe to distinguish between true 14q32/*IGH*-associated translocation and other acquired *IGH* aberrations (eg, nonreciprocal translocation, interstitial 14q deletion) or somatic *V_H* deletions. The latter events may affect one or both chromosomes 14

(biallelic V_H deletions are marked by asterisk in Figure 3) hallmarked by either a diminished or lack of *IGHV* green signal. Note that the 1F1R pattern is produced by cells with either a nonreciprocal t(14;var)(q32;var) (loss of a partner derivative chromosome) or an extended somatic V_H deletion that can be distinguished by further FISH analysis with, eg, 14q subtelomeric probe (loss of one signal in the former case). Likewise, the 1F1G signal pattern is produced by cells with either a nonreciprocal t(14;var)(q32;var) [loss of der(14)] or interstitial 14q deletion involving IGH that can be further distinguished by analysis with, eg, a near-centromeric 14q11 probe (loss of one signal in the former case). Of course, all *IGH*-associated problems could be avoided by direct use of gene-specific dual-color break-apart rearrangement probes (LSI CCND1, LSI BCL2, LSI CMYC). However, their thresholds are much higher than those of *IGH*-related dual-fusion assays and these characteristics limit their application in cases with a low incidence of abnormal cells. Finally, we recommend replacement of LSI IGH/CCND1 by LSI IGH/CCND1 XT, which, like LSI IGH/BCL2 and /CMYC, comprises a single large *IGH* probe. The evaluation of FISH experiments performed with these latter assays is much easier, and results are less misleading because their *IGH* probes are not producing a two double green signal pattern in normal cells.

Acknowledgments

We thank A. Hagemeijer and Lucienne Michaux for critical reading of the manuscript and for stimulating discussion. We thank U. Pluys for technical assistance in FISH analysis and R. Logist for clerical help.

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