Journal of Molecular Diagnostics, Vol. 9, No. 1, February 2007

DOI: 10.2353/jmoldx.2007.060088

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 linage. 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