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Abstract

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In summary, we provide evidence that *IGH*-mediated chromosomal aberrations affecting the 14q32.13/*TCL1A*-*TCL6* region are recurrent in mature B-cell neoplasms with villous lymphocytes. Despite extensive qRT-PCR studies, molecular consequences of these novel aberrations remain elusive.

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Abstract

Genetic background of mature B cell neoplasms with villous lymphocytes is poorly understood. We identified a novel breakpoint region at 14q32.13 that was rearranged together with *IGH*/14q32.33 in 4 *BRAF*V600E-negative leukemia/lymphoma cases with villous lymphocytes carrying either t(14;14)(q32.13;q32.33) (3 patients) or del(14)(q32.13q32.33) (1 patient). The 14q32.13 breakpoints were mapped by FISH in the region harboring the *TCL1A/TCL1B/TCL6* genes, known to be affected by *TCRA/D*-mediated t(14;14)(q11;q32)/inv(14)(q11q32) occurring in T-cell leukemia/lymphoma. To identify the target of t(14;14)(q32.13;q32.33) and del(14)(q32.13q32.33), qRT-PCR analysis of 25 candidate genes located centromerically and telomerically to the 14q32.13 breakpoint was performed. Any of the analyzed genes was commonly overexpressed in the presented cases. Of note, upregulated transcription of *TCL1A* was observed in 2 cases.

In summary, we provide evidence that *IGH*-mediated chromosomal aberrations affecting the 14q32.13/*TCL1A-TCL6* region are recurrent in mature B-cell neoplasms with villous lymphocytes. Despite extensive qRT-PCR studies, molecular consequences of these novel aberrations remain elusive.

Introduction

Lymphoproliferative disorders with villous lymphocytes are rare and include 3 indolent B-cell malignancies sharing several pathological features: hairy cell leukemia (HCL), HCL-variant (HCL-v) and splenic marginal zone lymphoma (SMZL) [1]. The neoplastic cells of HCL and HCL-v have a characteristic villous morphology [2], that also typifies circulating cells of SMZL [3]. These cells display a mature B-cell phenotype and primarily infiltrate the spleen, bone marrow and peripheral blood, but only rarely lymph nodes [4]. Despite these similarities, it has been recently hypothesized that HCL, HCL-v and SMZL represent distinct lymphoid neoplasms, and showed that HCL-v display more features in common with SMZL than with HCL [5,6]. The biological differences between HCL and HCL-like disorders mainly concern the status of *IGHV* genes (commonly mutated in HCL, and unmutated in 22% of HCL-v and 15-41% of SMZL), the usage of *IGHV* genes (restricted in HCL-v and SMZL, but not restricted in HCL), clinical response to purine analogues (good in HCL, and poor in HCL-v/SMZL) and median survival (>20 years in HCL, 9 years in HCL-v and 13 years in SMZL) [2,7-16]. This hypothesis has been very recently supported by identification of the somatically acquired V600E mutation of the *BRAF* gene in all analyzed HCL cases but not in other indolent B-cell neoplasms, including HCL-v and SMZL [17-19].

HCL-v was first identified by Cawley et al. [20]. The WHO classification of lymphoid tumors recognized HCL-v as a provisional entity and temporarily placed it

in a category of splenic lymphoma/leukemia, unclassifiable [4]. Given the rarity of HCL-v and difficulties underlying a clear-cut diagnosis of this leukemia, very little is known about the genetic events that may contribute to pathogenesis of HCL-v [21].

We report here results of cytogenetic and fluorescence *in situ* hybridization (FISH) studies that led to the identification of recurrent 14q32.13 aberrations in 4 cases of *BRAF*V600E-negative mature B-cell neoplasms with circulating villous lymphocytes, including 1 case of HCL-v. Molecular cytogenetic analysis was complemented by qRT-PCR studies of more than 20 candidate genes potentially targeted by these rearrangements.

Materials and methods

Patients

The reported cases were selected from the lymphoma database of the Center for Human Genetics, KUL, Leuven, Belgium (cases 1, 2 and 4) and the Centre de Génétique Humaine, Gosselies, Belgium (case 3). The selection criteria included presence of cytogenetically proven t(14;14)(q32;q32) or related abnormalities potentially involving 14q32.13 as well as 14q32.33, like del(14q31/32) and add(14)(q32), or del(14)(q31q32). Control cases used for FISH and qRT-PCR were selected from the database of the Center for Human Genetics, KUL, Leuven, Belgium and the Palacky University, Olomouc, The Czech Republic. The pathological features and immunophenotype of splenectomy specimen of case 1 and bone marrow trephine of case 4 were reviewed. Given presence of circulating villous lymphocytes, differential diagnosis of the reported cases included HCL, SMZL and splenic lymphoma/leukemia, unclassifiable, which include splenic diffuse red pulp small B-cell lymphoma and HCL-v [4]. To establish diagnosis of the selected cases, we followed diagnostic criteria of these tumors described in the WHO classification of lymphoid tumors [4] and mutation status of *BRAF* [17-19].

Conventional Cytogenetics and FISH

Cytogenetic analysis of spleen (case 1), peripheral blood (cases 2, 3 and 4), and bone marrow (case 4) cells was performed after a short term culture. G-/R-banded chromosome analysis and FISH followed routine methods. The applied FISH probes are listed in Supplemental Table 1. Noncommercial probes were directly labeled with SpectrumOrange- or SpectrumGreen-dUTP (Abbott Molecular, Ottignies, Belgium) using random priming. FISH images were acquired with a fluorescence microscope equipped with an Axiophot 2 camera (Carl Zeiss Microscopy, Jena, Germany) and a MetaSystems ISIS imaging system (MetaSystems, Altlussheim, Germany). One to 10 abnormal metaphase cells and/or 200 interphase nuclei were evaluated in each FISH experiment.

VH Mutation Analysis

Monoclonality was detected using fragment analysis [5]. Rearranged *IGH* genes were amplified using *IGH* leader primers and the antisense primers J_H1245, J_H3 and J_H6. Direct sequencing was performed on both DNA strands using the same primers as in the amplification. The sequencing procedure was performed using an ABI PRISM 3130xl (Applied Biosystems, Halle, Belgium), following the manufacturer's procedure. Mutations were identified by comparison of the sequence (adjusted with SeqMan) with the germline *IGH* sequence by IMGT/V-Quest (<http://imgt.cines.fr>). The cut-off of 97% homology to the germline sequence was chosen to identify mutated cases.

BRAF Mutation Analysis

BRAF mutations were sequenced after a mutant enrichment PCR. In this PCR the wildtype sequence is blocked by a LNA probe. The *BRAF*-F primers comprised of 5'-CCTAAACTCTTCATAATGCTTGCTC-3' and *BRAF*-R contained 5'-GACCCACTCCATCGAGATTT-3'. The obtained fragments were sequenced with BigDye terminators (Applied Biosystems). The analysis was performed

Double Immunolabeling

Diagnosis-certified primary antibodies targeting the nuclear antigen BSAP/PAX5 (NCL-L-PAX-5, Novocastra, Newcastle, UK) and Annexin 1 (Transduction Laboratories, Lexington, KY) were applied overnight. Enzymatic detection was performed using amplification kits containing alkaline phosphatase (AP) and horseradish peroxidase (HRP)-polymeric conjugates (EnVision, Dako, Glostrup, Denmark). Finally, we used 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich, St Louis, Missouri) and Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche, Basel, Switzerland) for enzymatic HRP- and AP-visualization of the signals, respectively.

Genomic Profiling

Genomic profiling of 2 cases was performed using the Affymetrix Cytogenetics Whole-Genome 2.7M arrays (Affymetrix, Santa Clara, CA, USA), following the manufacturer's protocol. After hybridization, arrays were washed and scanned using a GeneChip Fluidics Station 450 and a GeneChip Scanner 3000 7G. The obtained images were automatically processed and results were analyzed using the software "Chromosome Analysis Suite" (CHAS) (Affymetrix) and "Array Studio" (OmicSoft Corporation).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Genomic DNA was removed by DNaseI (Roche, Basel, Switzerland) treatment and RNA samples were further purified on RNeasy mini columns (Qiagen, Hilden, Germany). A total of 1 µg RNA was reverse transcribed into cDNA using random hexamers and Superscript III (Invitrogen).

Primers (Supplemental Table 2) were designed using Lightcycler probe design software (Roche). RT-PCR was performed on Lightcycler LC480 (Roche) using qPCR MasterMix Plus for SYBR Green I (Eurogentec, Seraing, Belgium). Specific PCR amplification was assessed by dissociation curve analysis. Quantification was determined using the delta Ct method comparing region of interest with the reference gene *HPRT1* (normal spleen).

Results

We report here 4 cases of mature B-cell neoplasms with villous lymphocytes harboring 14q32.13/14q32.33 aberrations proven by cytogenetics and FISH. Relevant clinical and genetic data of these cases with are summarized in Table I. There were 3 male patients and 1 female patient ranging in age from 54 to 75 years (median 65). Patient 1 was diagnosed with HCL-v. He presented with hepatosplenomegaly, thrombocytopenia and sporadic circulating hairy-like cells with a typical immunophenotype. Splenectomy was performed and no further therapy was required. The patient died after 69 months from unrelated reason. Patient 2 was originally diagnosed with HCL (1995), however, molecular analysis did not show the V600E mutation of *BRAF*. Unfortunately, further classification of this leukemia was not possible because laboratory and clinical data of this patient are missing. Patients 3 and 4 also presented with circulating lymphocytes with villous projections and immunophenotype resembling HCL-v. Both patients were asymptomatic and only displayed moderate lymphocytosis. Their disease was indolent, and did not require therapy. One of these patients is lost to follow-up and the second is alive and survived more than 22 years. Cases 1, 3 and 4, like case 2, showed lack of the V600E mutation of *BRAF* (data not shown) and did not express Annexin A1 by IHC (example in Supplemental Figure 1), two features characteristic for HCL [18,22]. Mutation analysis of *IGVH* performed in cases 2-4 displayed mutated *VH* genes and usage of *VH4-39*, *VH3-7* and *VH3-30*, respectively. Given incomplete immunophenotypic and clinical data, a clear-cut diagnosis of the reported cases could not be established. Therefore, they will be further referred as "mature B-cell neoplasms with villous lymphocytes, not further classifiable".

Cytogenetic analysis of case 1 (further referred as index case) identified t(14;14)(q32.13;q32.33) as the sole aberration in all 6 karyotyped metaphases (Table I). Both breakpoints of this translocation were characterized by FISH with probes shown in Figure 1A. LSI *IGH* breakapart assay showed an abnormal hybridization pattern: green/*IGVH* probe hybridized with a shorter der(14) and a red/3' *IGH* signal colocalized with a normal fused *IGH* signal on a longer der(14) (Figure 2A). The partner breakpoint was mapped by FISH walking strategy using a set of 13 14q32.13 BAC clones: 7 hybridized to both der(14) and 6 gave 2 signals on the longer der(14) (Figure 1B). These findings pointed the breakpoint in the region of RP11-1070N10/RP11-164H13, harboring 3 tightly clustered T-cell lymphoma/leukemia (*TCL*) genes: *TCL1A*, *TCL1B* and *TCL6* (www.ensembl.org).

Further analysis with 3 fosmid clones covering *TCL1A/TCL1B/TCL6* (G248P83752B7, G248P82004A4 and G248P81893E11) mapped the 14q32.13 breakpoint within the region flanked by G248P82004A4 and G248P81893E11 (Figure 1B-C, Figure 2C).

To identify additional cases with t(14;14), we search for B-cell leukemia/lymphoma cases with 14q32 aberrations affecting both chromosomes 14. One case with del(14q) and add(14q), further identified as t(14;14)(q32.13;q32.33), was found in our database (case 2). Of interest, this case showed features of HCL. Another case with t(14;14)(q32.13;q32.33) (case 3), originally diagnosed with HCL-v, was selected from the database of our collaborators. Both cases analyzed by FISH with LSI *IGH* and BACs flanking the *TCL1A/TCL1B/TCL6* genes cluster displayed the same hybridization pattern as the index case. FISH analysis with 3 fosmid clones covering *TCL1A/TCL1B/TCL6* mapped the 14q32.13 breakpoint in regions flanked either by G248P83752B7 and G248P82004A4 or by G248P82004A4 and G248P81893E11 in case 2 and case 3, respectively (Figure 1C). Further FISH analysis of cases 1-3 with 14q32.33 BAC clones narrowed down the *IGH* breakpoint of case 1 to the *CH* region flanked by RP11-11771/*IGCHEP1* and RP11-417P24/*J1->IGHG1* (Figure 1B). This finding suggests a mechanism other than somatic *V-D-J* recombination or immunoglobulin class switch recombination in a generation of this translocation. Cases 2 and 3 were not informative due to somatic deletions of *IGH*.

The fourth case (no 4) which also displayed featured of HCL-v harbored a variant aberration, del(14)(q32.13q32.33), which was identified by us during the previous molecular cytogenetic studies of B-cell leukemia/lymphoma with *IGH*-involving interstitial del(14) [23]. The distal 14q32.33 breakpoint was hallmarked by loss of the red/3' *IGH* (Figure 2D) and RP11-11771/*IGCH* signals, and the proximal breakpoint was marked by a diminished signal of RP11-164H13 (Figure 2E) and loss of the G248P81893E11 signal.

Cases 1 and 4 were further subjected to genomic array analysis using the Affymetrix Cytogenetics Whole-Genome 2.7M platform. The first case displayed balanced genomic profile (data not shown). Results of aCGH analysis of case 4 were suboptimal (low bone marrow infiltration by neoplastic cells); however, loss of sequences telomeric to *TCL1A* was evident (Figure 2F).

The 14q32.13/14q32.33 regions were further examined by interphase FISH in 15 additional cases of lymphoproliferative disorders with villous lymphocytes documented by splenectomy specimens, of which 11 harbored the *BRAFV600E* mutation and 3 showed a wild type configuration of the gene. All these cases revealed a normal FISH status of the *TCL1A-6* and *IGH* regions.

To identify the gene(s) affected by these 2 variant 14q32.13 aberrations, we analyzed expression of the *TCL1A*, *TCL1B* and *TCL6* mRNA by qRT-PCR in all 4 cases. The applied primers are shown in Supplemental Table 2. In addition, we examined 3 available HCL-v cases with a normal FISH status of *IGH* and *TCL1A/TCL1B/TCL6*, and mRNAs from 3 T-PLLs with the

inv(14)(q11.2q32.13)/*TCRA/D-TCL1A* rearrangement. As controls, non-malignant splenic cells and mature (CD19+) B- and T-cells sorted from peripheral blood of healthy individuals were analyzed. Results are shown in Supplemental Table 3. *TCL1A* mRNA was detected in cases 1 and 2 with expression levels slightly higher than in normal CD19+ B-cells (2.9-3.5 vs 2.2), whereas cases 3 and 4 showed no *TCL1A* mRNA expression. In control HCL-v cases expression of *TCL1A* ranged from 0.3 to 0.9, thus was lower than in normal CD19+ B-cells. Control T-PLLs showed a similar level of *TCL1A* mRNA expression as cases 1 and 2 (0.4-3.8). Expression of this gene, however, was not detected in normal mature T-cells. None of the analyzed cases expressed mRNA of *TCL1B* or *TCL6*, except for one T-PLL case showing very high *TCL1B* expression. Further qRT-PCR experiments were performed to determine whether the 14q32 aberrations might alter the expression of genes in the vicinity of *TCL1A*. None of the five candidate genes located centromeric to *TCL1A-6* (*DICER1*, *SERPINA9*, *DDX24*, *ITPK1*, *GOLGA5*) were commonly upregulated. In addition, expression analysis of 12 candidate genes located telomeric to *TCL1A* (*c14orf132*, *BDKRB2*, *ATG2B*, *C14orf129*, *AK7*, *PAPOLA*, *VRK1*, *AL163872.1*, *LOC100129345*, *C14orf177*, *BCL11B* and *SETD3*) and of 3 non-coding genes (*ucr379*, *ucr380*, *ucr381*), known as T-UCRs mapped upstream of *VRK1* (www.ensembl.org) did not identify a common target gene. Either a weak or absent expression of these genes was observed in the analyzed cases (Supplemental Table 3). Furthermore, global expression profiling of case 1 revealed no significantly up-regulated gene on chromosome 14, when compared to normal spleen (data not shown).

Discussion

Our study identified novel *IGH*-mediated chromosomal aberrations affecting the 14q32.13/*TCL1A-TCL6* region in 4 cases of mature B-cell neoplasm with circulating villous lymphocytes, not further classifiable, including the index case originally diagnosed as HCL-v. Despite of diagnostic difficulties of these „archival“ cases, it is intriguing that all of them revealed rearrangements of the same chromosomal regions, 14q32.13 and 14q32.33. Given that both affected loci are separated by an approximately 10 Mb region (Figure1C), t(14;14)(q32.13;q32.33) and del(14)(q32.13;q32.33) can be easily overlooked during routine cytogenetic analysis.

The t(14;14)(q32.13;q32.33) represents a classical chromosomal aberration in B-cell leukemia/lymphoma which usually activates proto-oncogene by its juxtaposition to strong *IGH* regulatory elements. The known example, among others, includes the *CEBP*-targeted t(14;14)(q11;q32) in B-cell precursor acute lymphoblastic leukemia [24]. The analogous aberrations identified in T-cell leukemia/lymphoma, t(14;14)(q11;q32), and a variant inv(14)(q11q32), are mediated by *TCRA/D* (14q11) and activate the *TCL1A/TCL1B/TCL6* genes [25,26]. The *TCL1A* and *TCL1B* genes, together with *MTCP1* at Xq28, are members of the

TCL1 gene family which encodes small, intracellular, β -barrel-shaped proteins which bind and augment AKT kinase activity, thereby regulating signal transduction and enhancing cell proliferation and survival. *TCL6* mapped 7 kb upstream of the *TCL1B* locus, expresses at least 11 isoforms through complex alternative splicing, including splicing with the *TCL1B* gene. These isoforms encode at least five open reading frames and are expressed mainly in the B-cell lineage but not in T-cells. The oncogenic potential of *TCL1A* was demonstrated *in vivo* in *TCL1A* transgenic mice that developed a spectrum of T- and B-cell malignancies, including tumors resembling human chronic lymphocytic leukemia (CLL) [27]. Further studies showed an increased expression of *TCL1A* in CLL and several other B-cell lymphomas [28], including SMZL in which upregulation of *TCL1A* correlated with loss of 7q32 microRNAs [29]. Collectively, these findings suggest a role for *TCL1A* in B-cell tumorigenesis, although 14q32.13/*TCL1A* breakpoints have not been identified in B-cell malignancies so far.

In light of this data, our finding of recurrent and frequently sole 14q32.13/*TCL1A*-6 aberrations in mature B-cell neoplasms with villous lymphocytes, not further classifiable, is particularly interesting. Involvement of *TCL1A*, the best candidate gene, however, was not proven by qRT-PCR. Transcription level of *TCL1A* was only slightly higher than in normal CD19+ B-cells in 2/4 index cases and the gene was not expressed in 2 cases. In addition, expression analysis of more than 20 additional genes located up- and downstream of the breakpoint did not identify any commonly overexpressed gene. Either the targeted gene escaped our analysis or it was only slightly upregulated.

In conclusion, t(14;14)(q32.13;q32.33) and del(14)(q32.13q32.33), are novel chromosomal aberrations associated with mature B-cell neoplasms with circulating villous lymphocytes including one case of HCL-v. The best candidate gene targeted by these aberrations is *TCL1A*, the well known and powerful oncogene implicated in pathogenesis of T-cell leukemia/lymphoma and aberrantly expressed in a wide spectrum of B-cell neoplasms. Its upregulation, however, was not common in the presented cases.

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JUST ACCEPTED

Figure Legends

Figure 1

FISH mapping of the 14q32.13 and 14q32.33 breakpoints. (A) Scheme of a normal chromosome 14 and localization of the applied FISH probes. (B) Scheme of $t(14;14)(q32.13;q32.33)$ and hybridization pattern of FISH probes in case 1. The 14q32 breakpoints on both derivative chromosomes are marked by dashed lines and LSI IGH probes are in grey boxes. (C) Graphic representation of the 14q32.13-32.33 region (not in scale) harboring *IGH* and the *TCL1-6* genes cluster (upper part), the applied FISH probes (middle part) and the assigned breakpoints (bottom part). Commercial LSI IGH probes, BACs and fosmids (not in scale) are marked by light, darker and dark gray bars, respectively. Arrows representing particular genes indicate their transcriptional orientation.

Figure 2

Examples of FISH and aCGH analysis. FISH analysis was performed on case 3 with t(14;14) (A-C), and case 4 with del(14) (D-E). The applied FISH probes included LSI IGH (A, D), 14q32.13 BACs (RP11-1070N10-SO and RP11-164H13-SG) (B, E), and 14q32.13 fosmids (G248P82004A4-SG and G248P81893E11-SO) (C). Arrow and arrowhead in (A-C) indicate a longer and shorter der(14), respectively. Arrow and arrowhead in (D-E) indicate a normal chromosome 14 and del(14q), respectively. Note: (A) split of LSI IGH signals and co-localization of the red and fused signals on a longer der(14). Interphase cell with the corresponding 1F-R1G pattern is shown in the inset; (B and C) split of *TCL1A*-6 BACs and fosmids signals, respectively; colocalization of red and fused signals is clearly visible on interphase cells (inset); (D) loss of LSI IGH-SO signal from del(14) and (E) loss of the RP11-164H13 (green) but not the 1070N10 (red) signal on del(14). (F) Genomic profile of chromosome 14 in case 4 generated with the Affymetrix Cytogenetics Whole-genome 2.7M array (upper panel), zoomed on the 14q32.13 region (lower panel). Note the loss of sequences distal to *TCL1A*.

Table Legend

Table I

Relevant clinical and genetic features of the reported cases.

Case	Sex/ Age	Peripheral blood	Immunophenotype	Clinical presentation	Sample/ Status	Cytogenetic findings* (corrected after FISH)	VH status / used genes	Therapy	Outcome	Survival (months)
1	M/63	slight lymphocytosis, thrombocytopenia	CD20+/CD5-/CD23-/CD25-/CD103-/CD11c+/TRAP - /Tbet+/PAX5+/Annexin A1-	hepatosplenomegaly	S/D	46,XY,t(14;14)(q32.13;q32.33) [6]	ND	splenectomy (for diagnostic purposes)	died in CR due to unrelated cause	69
2	M/75	NA	NA	NA	PBL/D	46,XY,t(14;14)(q32.13;q32.33) [10]	m / V4-39	NA	lost to follow-up	NA
3	F/68	slight lymphocytosis	CD19+/CD20+/CD103+/CD11c+/CD25-/Annexin A1-	asymptomatic	PBL/P	45,X,-X,add(11)(q22)[2],del(13)(q22q32)[2],t(14;14)(q32.13;q32.33) [cp10]	m/V3-7	none	alive, asymptomatic, but concomitant colon carcinoma	264+
4	M/54	slight lymphocytosis	CD20+/CD5-/CD23-/CD25-/CD11c+/TRAP+/Tbet+/PAX5+/Annexin A1-	asymptomatic	PBL/P BM/P	46,XY,del(14)(q32.13q32.33) [16] 46,XY,del(14)(q32.13q32.33) [8]	m/V3-30	none	lost to follow-up	142+

M, male; F, female; NA, not available; ND, not done; PBL, peripheral blood; S, spleen; BM, bone marrow; D, at diagnosis; P, at progression; VH, variable region of immunoglobulin heavy chain locus; m, mutated; CR, complete remission;

*Chromosomal aberrations are presented in accordance with the International System of Human Cytogenetic Nomenclature, 2009.