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Association of *Ig/BCL6* translocations with germinal center B lymphocytes in human lymphoid tissues: implications for malignant transformation

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Chromosomal translocations (CTs) between immunoglobulin (*Ig*) genes and the *BCL6* proto-oncogene are frequently associated with diffuse large B-cell lymphomas (DLBCLs) and follicular lymphomas (FLs) and are implicated in the development of these lymphomas. However, whether *Ig/BCL6* translocation per se is sufficient to drive malignant transformation is not clear. To understand the biology of *Ig/BCL6*-translocated cells prior to their malignant transformation, we developed a system capable of detecting 1 to 3 *Igμ/BCL6* CT cells in 1 million mixed cells through the detection of chimeric Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts that reflect reciprocal *Igμ/BCL6* translocations. The chimeric transcripts that existed in the vast majority of normal lymphoid tissues are due to *Igμ/BCL6* CT and were not generated from trans-splicing. Both Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts were coexpressed in the same cell populations. The *Ig/BCL6* recombination junctions themselves were isolated from B-cell subpopulations expressing the Iμ-BCL6 transcripts. The appearance of *Igμ/BCL6* CT was associated with cells expressing germinal center but not naive B-cell markers. This study shows that *Ig/BCL6* translocations occur in germinal center–stage B cells in healthy humans, and that *Ig/BCL6* CTs per se are not likely sufficient to cause the malignant transformation in the context of human B cells. (Blood. 2006;108:2006-2012)

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Introduction

The proto-oncogene B-cell lymphoma 6 (BCL6), a critical transcriptional repressor for germinal center (GC) reaction,[1] is a frequently targeted partner for chromosomal translocations (CTs) associated with various lymphomas such as diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL).[1-4] Reciprocal CTs between the immunoglobulin (Ig) gene locus on 14q32 and the BCL6 gene on 3q27 (t(3;14)(q27;q32)) are found in a significant portion of DLBCL and FL,[1] and they strongly implicate *Ig/BCL6* CT in the development of these lymphomas. *Ig/BCL6* CT primarily occurs between the Ig switch regions and the noncoding exon 1–intron 1 region of the *BCL6* gene,[2-6] presumably mediated by the similar mechanism for Ig class switch recombination (Ig CSR).[7] As a result, expression of the BCL6 from the translocated locus comes under the transcriptional control of Ig region promoter/enhancer, a genetic alteration known as promoter substitution.[6] The replacement of the natural BCL6 promoter, which is subjected to tight control during GC reaction,[8] by the Ig promoters/enhancers results in dysregulation of BCL6 expression.[6] As BCL6 expression is critical for B-cell proliferation, differentiation and survival in GC, the deregulated BCL6 expression is believed to contribute to B-cell malignant transformation for a subgroup of DLBCL and FL.[6-9] The long-held speculation that deregulated BCL6 expression driven by Ig promoters/enhancers plays an important role in B-cell malignant transformation during lymphomagenesis gains support from recent data showing in mice models that Ig promoter/enhancer-controlled BCL6 expression promotes lymphomagenesis.[10,11]

Although *Ig/BCL6* CT has been implicated in the lymphomagenesis as related to DLBCL and FL, it is not clear what the exact role of the CT is during the malignant transformation, and whether the *Ig/BCL6* CT per se is sufficient to lead to the malignant transformation in the context of human B cells. These important questions regarding the biology of the *Ig/BCL6*-translocated cells prior to their malignant transformation have not been previously addressed because of the lack of an assay capable of detecting and measuring such rare *Ig/BCL6*-translocated cells in their precancerous stages. We reasoned that one way to gain insight into the role of *Ig/BCL6* translocation in B-cell malignant transformation and lymphomagenesis is to examine the *Ig/BCL6* translocations prior to malignant transformation. In this paper, we report the results of using a novel system to detect and measure rare *Igμ/BCL6* CT events in mixed cell populations and to characterize the CT cells and quantify their frequencies in lymphoid tissues of otherwise healthy humans.

Materials and methods

Cells and cell lines

Human tonsils and spleens were collected following tonsillectomy or splenectomy performed at the UCLA Center for Health Sciences and Kaiser Permanente Medical Center at Panorama City, CA, after obtaining appropriate institutional review board approval. Human peripheral mononuclear

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X.Y. and K.L. contributed equally to this study.

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cells (PBMCs) from healthy donors were obtained from the Core Virology Laboratory of the Jonsson Cancer Center at UCLA. Diffuse large B-cell lymphoma tissues were previously collected from patients with this diagnosis and stored. Informed consent was obtained in accordance with the Declaration of Helsinki. The human B-cell line Ramos 2G6 was purchased from ATCC (Manassas, VA).[12,13] BL-2 and DG75 cells were provided by Dr Q. Pan-Hammarstrom (Karolinska Institute, Sweden),[14] and CL-01 was provided by Drs P. Casali (UC Irvine, CA) and A. Cerutti (Cornell University, NY).[15] All the cells and cell lines were cultured or maintained in complete RPMI 1640, as described previously.[13]

Cell-isolation procedures

Tonsillar B cells were purified from tonsil mononuclear cells as described previously.[16] Specific cell populations (B cells and subpopulations, T cells, dendritic cells) were isolated by positive magnetic bead selection (Dynal beads; Invitrogen, Carlsbad, CA) with an appropriate antibody as follows. Antibodies to human CD19 (HIB-19), IgD (1A6-2), CD38 (HIT2), CD77 (5B5), CD3 (HIT3a), and CD209 (DCN46) (BD Pharmingen, San Diego, CA) were incubated with B cells (for B-cell subpopulations) or total tonsillar cells (for T cells and dendritic cells) for 30 minutes at 4°C with agitation, followed by incubation with goat anti–mouse IgG– or anti-IgM (for CD77)–coated Dynal beads at a ratio of 1 projected target cell per 3 beads for 1 hour at 4°C with agitation. The magnetic bead attached cells were selected 3 times with a magnet stand and subsequently subjected to RNA or DNA preparation. In some experiments, the $CD38^+$, IgD^+IgM^+, $CD10^+$, or $CD77^+$ B cells were isolated by fluorescence-activated cell sorting (FACS) that was labeled with anti-CD38–APC, anti-IgM–PE, anti-IgD–FITC, anti-CD10–FITC (HI10a), or anti-CD77–FITC.

Reverse-transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen) as described.[13] Total RNA (1 μg) or RNA from a given number of cells as indicated was reverse transcribed to cDNA by murine Moloney leukemia virus (MMLV) transcriptase priming with oligo-dT_{15}, as previously described.[13] Detection of the chimeric Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts was performed with a nested PCR protocol with regular Taq polymerase in the presence of 2.5 mM $MgCl_2$ (Promega, Madison, WI).[13] The first-round PCR was conducted at 95°C for 2 minutes to inactivate the MMLV, followed by 95°C for 15 seconds, 65°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. For the second-round PCR, 2 μL of the first-round PCR products was served as DNA templates, and the PCR was conducted at 95°C for 15 seconds, 68°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. The 5′ primer Iμ3.1 (5′-CGACTGGCTGCTCAGGCCCCAGC-3′) and 3′ primer BCL6-3C4.1 (5′-ACTGGCATGGCGGGTGAACT-3′) were used for first-round, and the 5′ primer Iμ4 (5′-TAATGGACTTGGAGGAATGATT-3′) and 3′ primer BCL6-3C1 (5′-GATACAGCTGTCAGCCGGCGAGGCCA-3′) for second-round PCR for the amplification of Iμ-BCL6E2 transcripts. For BCL6E1-Cμ1 transcripts, the 5′ primer BCL6E1a (5′-AATTGAGCTCT-GTTGATTCTTAG-3′) and 3′ primer CHμ1.2 (5′-CCTCTCAGGACTGAT-GGGAAGC-3′) were used for the first-round, and 5′ primer BCL6E1 (5′-GTTCTTAGAAGTGGTGATGCAAG-3′) and 3′ primer CHμ1.0 (5′-TGGAGGAGAAAGTGATGGAGTCG-3′) for the second-round PCR. To monitor the cDNA loading, GAPDH was simultaneously amplified with the first-round PCR with a forward primer (5′-TGAAGGTCGGAGTCAACG-GATTTGGT-3′) and a reverse primer (5′CCATGTAGTTGAGGTCAAT-GAA-3′) that amplify a 120-bp product. For detection of Igμ germ-line transcripts (Iμ-Cμ transcripts), the primers Iμ3.1 and Cμ1.1 (5′-TGCTCTGATGTCAGAGTTGTTC-3′) were used to amplify a 255-bp product. For BCL6 transcripts, the primers BCL6E1 and BCL6-3C4.1 were used to amplify a 147-bp product. The PCR product was electrophoresed with 2% TBE agarose gel. To prevent possible cross contamination, cell manipulation, RNA preparation, and cDNA synthesis were performed in a different location from that of PCR product analysis. An aliquot of PCR master mix without template DNA was always included as negative control for both first- and second-round PCR.

For quantification of chimeric Iμ-BCL6E2 transcript–expressing cells, total tonsillar cells were 2-fold serially diluted in duplication starting at 4×10^6 cells. The RNA prepared from each cell dilution was subjected to RT reaction and PCR with the method described in the preceding paragraph. The end dilution with at least one positive signal from the duplicates was scored as positive and arbitrarily defined as one translocation event. The translocation frequency was normalized to event(s) in every 1×10^6 total tonsillar cells.

Amplification, cloning, and sequencing of the Ig/BCL6 translocation fragments

Genomic DNA was prepared from DG75 cell line, DLBCL cells, tonsil and spleen B cells, and isolated cell subpopulations using DNA extraction kits (Promega), as previously described.[13] DNA (400 ng/reaction) was amplified using an expanded PCR kit (Roche Applied Science, Indianapolis, IN). The primer pair Sμ3B (5′-ACTCAGATGGCTAAACTGAGCCTAAGCT-3′) and BCL6-5B (5′-CTGCACCGCCTCCTCTATATCTC-3′) was used for first-round PCR under the following conditions: 95°C for 5 minutes, 60°C for 5 minutes, and 72°C for 10 minutes for one cycle followed by 95°C for 30 seconds, 65°C for 1 minute, and 72°C for 5 minutes for 40 cycles. For the nested PCR, 2 μL of the first-round PCR product was the source of the DNA templates, and the primer pair Sμ3C (5′-AGCCTGAGCTAACAG-GCTGAACT-3′) and BCL6-4.9B (5′-GTGGGTTGTGAGTGTCATTAC-CCTGT-3′) was used under the following conditions: 95°C for 30 seconds, 68°C for 1 minute, and 72°C for 5 minutes for 40 cycles. PCR bands were purified from 0.8% agarose gels and cloned into the Topo-zero blunt cloning vector (Invitrogen). The plasmids were sequenced with M13 forward and M13 reverse sequencing primers.

Results

Chimeric Iμ-BCL6 transcripts are found in normal human tonsil and spleen cells

The CT between the *Igμ* gene on chromosome 14 and the *BCL6* gene on chromosome 3 in cells from a subset of DLBCL patients generates a fusion gene that expresses chimeric Iμ-BCL6 (Figure 1)[6] or VDJ-BCL6 transcripts.[17] Since any *Igμ/BCL6* translocation occurring within the germ line or switched Igμ switch (Sμ) region

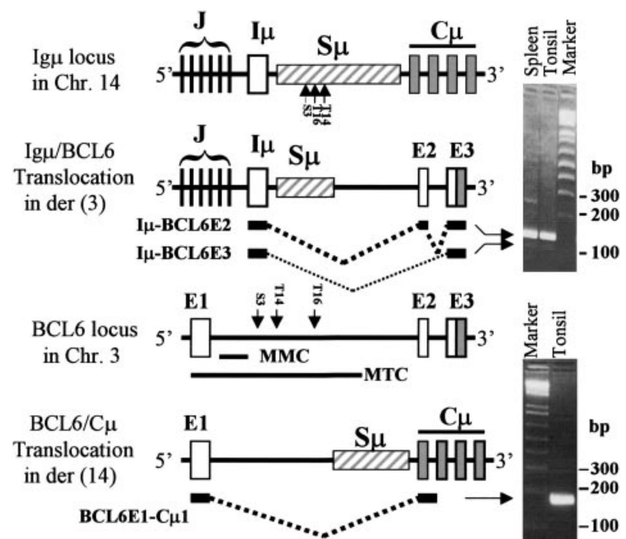

Figure 1. Chimeric transcripts produced from *Igμ/BCL6*-translocated B cells. Shown on the left is a diagram of the germ-line BCL6 locus in chromosome 3 (Chr. 3) and Ig locus in Chr. 14, their reciprocal Igμ/BCL6-translocated loci, and the generation of the chimeric Iμ-BCL6 and BCL6E1-Cμ transcripts from the fusion genes. The chimeric transcripts amplified from tonsillar and/or splenic B cells are shown on the right. For Iμ-BCL6 transcripts, the major product of the chimeric transcripts is Igμ-BCL6E2, whereas Igμ-BCL6E3 is the minor product. The arrows present the Igμ/BCL6 recombination junctions isolated from human $CD38^+$ B cells shown in Figure 4. MMC indicates major mutation cluster; MTC, major translocation cluster.

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(~ 5 kb for germ-line Igμ, and various sizes for Sμ-Sx switched locus, where Sx is a switch region of the downstream Ig isotype)[18] and the exon 1–intron 1 region of the *BCL6* gene (~ 10 kb)[5,6] should uniformly produce the chimeric Iμ-BCL6 transcripts derived from the splicing of the Iμ exon to the exon 2 of the *BCL6* gene,[6] we reasoned that such chimeric transcripts could serve as a marker for the rare *Igμ/BCL6* translocation events that we predict occur in heterogeneous cell populations prior to their malignant transformation.

To test this possibility, we established a nested RT-PCR assay to detect the expression of the chimeric Iμ-BCL6 transcripts. As shown in Figure 1 (top right panel), the assay was able to detect such Iμ-BCL6 transcripts from both human tonsillar and splenic B cells. Cloning and sequence analysis demonstrated that the major RT-PCR products (145 bp) from the human tonsil and spleen cells were derived from the accurate splicing of the Iμ exon of the Igμ gene to the exon 2 of the BCL6 gene as has been reported with Igμ/BCL6-translocated DLBCL[6] (hereafter referred as to Iμ-BCL6E2 transcripts) (Supplemental Figure S1A, available on the *Blood* website; click on the Supplemental Figures link at the top of the online article). A minor RT-PCR product (106 bp) that represents alternative splicing of the Iμ to the exon 3 of the BCL6 gene (hereafter referred as to Iμ-BCL6E3 transcripts) was also observed from most tonsil- and spleen-cell preparations (data not shown; see Figure 2 for examples). These data show that the Iμ-BCL6 transcripts, the product of one side of reciprocal *Ig/BCL6* translocations in chromosome der(3) that are expected to lead to the deregulated expression of BCL6, can be detected in normal human tonsillar and splenic B cells.

Chimeric BCL6E1-Cμ transcripts are detected from human tonsils

Igμ/BCL6 CT, as defined in lymphomas, is a balanced translocation between the *Igμ* gene locus on 14q32 and the *BCL6* gene on 3q27. Such a reciprocal CT also creates a fusion gene comprised of the 5′ region of the *BCL6* gene, including the BCL6 promoter and BCL6 exon 1 region, juxtaposed to the 3′ region of the *Igμ* gene in d(14) chromosome, including the 3′ Sμ region and the Igμ heavy chain constant region (*Cμ*) gene (Figure 1). As a result, the Cμ gene comes under the transcriptional control of the BCL6 promoter, and this fusion gene would theoretically produce chimeric RNA BCL6E1-Cμ transcripts representing splicing from the exon 1 of the *BCL6* gene (BCL6E1) to *Cμ* gene. Such transcripts have been detected from DLBCL with *Ig/BCL6* CT.[19] As existence of the reciprocal BCL6E1-Cμ transcripts in our cells would provide independent evidence to corroborate the genomic origin of the *Igμ/BCL6* translocation–derived chimeric Iμ-BCL6 transcripts, we established an RT-PCR assay to detect BCL6E1-Cμ transcripts in human tonsillar B cells. Indeed, BCL6E1-Cμ transcripts (190 bp) were detected from the same human tonsil B-cell preparations that were positive for the Iμ-BCL6E2 transcripts (Figure 1 lower right panel). Sequencing revealed that in the BCL6E1-Cμ transcripts, the BCL6 exon 1 sequence is accurately spliced to the splicing acceptor site of the exon 1 of the *Cμ* gene (Cμ1) (Figure S1B). This result demonstrates that the chimeric BCL6E1-Cμ1 transcripts, the products of the other side of reciprocal *Ig/BCL6* translocations in chromosome d(14), are present in human tonsillar B cells.

Coexpression of chimeric Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts

The reciprocal nature of *Ig/BCL6* translocations theoretically should lead the CT cells to express both chimeric Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts in the same cells. To determine whether these 2 types of chimeric transcripts are simultaneously expressed, we examined their expression in the same cell populations. As shown in Figure 2A, the BCL6E1-Cμ1 transcripts were detected in the same CD38+ B-cell subpopulations that also were expressing Iμ-BCL6E2 from different tonsils (n = 5). In contrast, those cell populations that did not express Iμ-BCL6E2 transcripts, including IgD+ B cells, and CD77+ and CD38+ B cells from tonsil no. 32 (T32), also did not express BCL6E1-Cμ1 transcripts (Figure 2A). These results indicate that both types of chimeric transcripts are correlatively expressed in the same cell subpopulations. Comparison of the assay sensitivities between the Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts revealed that the assay sensitivity for the Iμ-BCL6E2 transcripts was about 9-fold higher than that for the BCL6E1-Cμ1 transcripts (Figure 2B). Therefore, we chose the assay for Iμ-BCL6E2 transcripts as the primary detection method in further studies.

Human B-cell lines expressing both germ-line Igμ and BCL6 transcripts do not produce chimeric Iμ-BCL6E2 or BCL6E1-Cμ1 transcripts

Chimeric Iμ-BCL6E2 or BCL6E1-Cμ1 transcripts can serve as a specific marker for *Igμ/BCL6* translocations only if they are transcribed from the translocated chromosomes and do not result from other mechanisms potentially capable of generating such transcripts (for example, trans-splicing between the pre-mRNAs of the germ-line Igμ transcripts [Iμ-Cμ] and BCL6 transcripts). To test whether the chimeric transcripts can be generated from the trans-splicing between the Iμ-Cμ and BCL6 pre-mRNA, we searched for the Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts from several human B-cell lines that spontaneously produce both Iμ-Cμ and BCL6 transcripts. As shown in Figure 3, human Burkitt lymphoma cell lines Ramos, DG75, CL-01, and BL-2 all produce both Iμ-Cμ and BCL6 transcripts, but no Iμ-BCL6 or BCL6E1-Cμ transcripts were detected in nonstimulated or IL-4 plus anti-CD40–stimulated cultures even when using an assay capable of detecting Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts from human tonsil cells that served as a positive control (Figure 3). These results indicate that both Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts are unlikely to be derived by the mechanism of trans-splicing between the Iμ-Cμ and BCL6 transcripts.

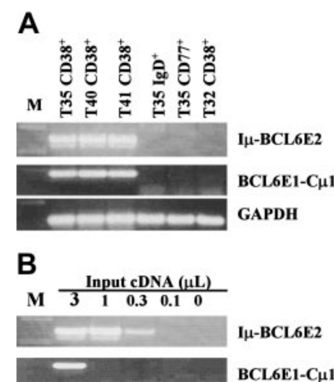

Figure 2. Coexpression of the Iμ-BCL6E2 with BCL6E1-Cμ1 transcripts. (A) The magnetic bead–purified B-cell subpopulations as indicated were simultaneously subjected to detection for the expression of both types of chimeric transcripts. Equal amounts of cDNA (3 μL) from each sample were input. (B) Sensitivity comparison of the assays for both types of chimeric transcripts. Serially diluted cDNA from the same sample as indicated was compared for sensitivity, where sensitivity for the Iμ-BCL6E2 was about 9-fold higher than that of the BCL6E1-Cμ1 transcripts.

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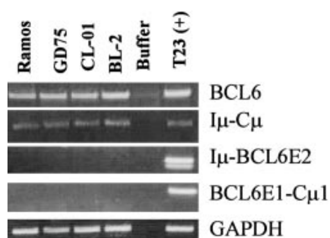

Figure 3. Trans-splicing is not likely the responsible mechanism for the generation of chimeric transcripts. Burkitt lymphoma cell lines (Ramos, DG75, CL-01, and BL2) cells, while expressing both BCL6 and germ-line IgM transcripts, do not express the chimeric Iμ-BCL6E2 or BCL6E1-Cμ1 transcripts. Tonsil no. 23 (T23) cells were included as a positive control. The results represent 3 independent experiments.

Isolation of *Igμ/BCL6*-translocated DNA products from primary B cells

To clearly demonstrate that *Ig/BCL6* translocations occur in human primary lymphoid tissues, we sought to isolate and identify the recombination junctions of the *Igμ/BCL6* translocations with a genomic DNA PCR approach. To optimize this PCR assay for amplification of *Igμ/BCL6* translocations from primary human tonsillar B cells, we used previously diagnosed DLBCL cells with a known *Igμ/BCL6* translocation as a DNA template for the amplification (Figure 4A). A series of these DLBCL cells diluted in the background of 1×10^5 DG75 cells (equivalent of 400 ng DNA) were subjected to nested PCR amplification. This PCR strategy was reliably able to amplify *Igμ/BCL6*-translocated products (approximate size of 2.5 kb) from 10 copies of DLBCL cells but not reliably from fewer than 3 copies (Figure 4A). Fifty-eight attempts to amplify *Igμ/BCL6*-translocated products from the unfractionated tonsillar cells failed. However, from a total of 48 amplifications from CD38$^+$-enriched B cells of 3 different donors (tonsil 14, tonsil 16, and spleen 3), we successfully amplified and isolated 3 clones with *Igμ/BCL6* translocations (Figure 4C). Sequence analysis revealed that all 3 clones joined their Sμ sequences directly to the intron 1 region sequence of the BCL6 gene (Figure 4D; Figure S2). The minimum nucleotide overlaps in the recombination junctions (1 nt in T14, 3 nt in T16, and 2 nt in S3) suggest that the translocations were mediated through the nonhomologous end joint process responsible for Ig CSR. There was a 213-bp internal deletion of the Sμ region between 587 and 818 of the GenBank accession number X56795 in T14. Two mutations in the vicinity of the recombination breakpoints were found in 1 of the 3 clones (T16). These results, which show the presence of the recombination breakpoints, further demonstrate with an additional approach that Igμ/BCL6 translocation occurs in normal human primary B cells.

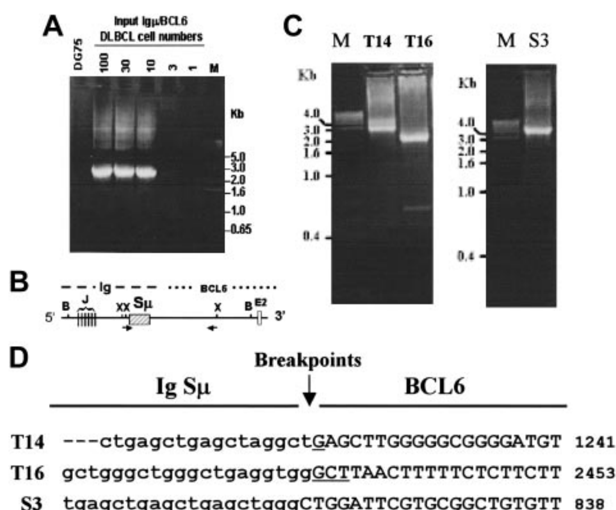

Figure 4. *Igμ/BCL6* translocation products from human primary B cells. (A) Sensitivity of the genomic DNA PCR assay for *Igμ/BCL6* translocation. Various DLBCL cells known to have *Igμ/BCL6* translocation were diluted into the background of DG75 cells as DNA template for PCR, and the sensitivity of the assay was determined to be between 3 and 10 copies/reaction. (B) Diagram of the primer position for the PCR. (C) PCR amplification of the *Igμ/BCL6* translocation products from CD38$^+$ B cells from spleen (S3) and tonsils (T14 and T16). (D) Recombination breakpoints of the 3 isolated translocation products are shown. The lowercase letters present the sequences from *Ig* Sμ; and the capital letters, for the intron 1 sequence of the *BCL6* gene. The overlapped nucleotides in the breakpoints are underlined. The internal deleted portion of the Sμ in the clone T14 is indicated with the dashed line. The sequence numbers correspond to the *BCL6* gene of GenBank accession no. AY189709. The breakpoint positions for these clones are diagrammed in Figure 1.

Igμ/BCL6-translocated cells are associated with germinal center markers in normal lymphoid tissues

To further analyze the *Igμ/BCL6* translocation events in normal human lymphoid tissues and in circulation, we investigated the distribution of the Iμ-BCL6E2 transcripts in human lymphoid tissues and PBMCs. We detected Iμ-BCL6E2 transcripts in 39 (96%) of 41 randomly collected human tonsils (Figure 5A; Table 1) and 3 (100%) of 3 human spleens (Figure 5B) by using a total of 1×10^6 tonsillar or splenic cells. T32 tonsil was the only sample that has been confirmed to be negative for both types of chimeric transcripts from both total tonsil cells and CD38$^+$ B cells through multiple tests (Figure 2A; Table 1). We could not confirm the negative Iμ-BCL6 expression results for T9 (Figure 5A) with CD38$^+$ B cells, as it was a previously frozen sample. These results show that Igμ/BCL6-translocated cells are present in lymphoid tissues in the vast majority, if not all, of otherwise healthy individuals. However, peripheral-blood mononuclear cells (PBMCs) from 14 donors were negative when 1 to 2×10^6 cells were analyzed for Iμ-BCL6 transcripts (Figure 5C) ("Discussion").

Analysis of magnetic bead selected cells was undertaken to define the distribution of Iμ-BCL6E2 transcripts with B-cell subpopulations. Iμ-BCL6E2 transcripts were consistently detected in CD19$^+$, CD38$^+$, and IgD$^-$ B-cell populations, as well as in total tonsillar cells, but not in CD3$^+$ or CD209$^+$ tonsillar-cell populations (Figure 5D; Table 1; n = 7). The association of Iμ-BCL6E2

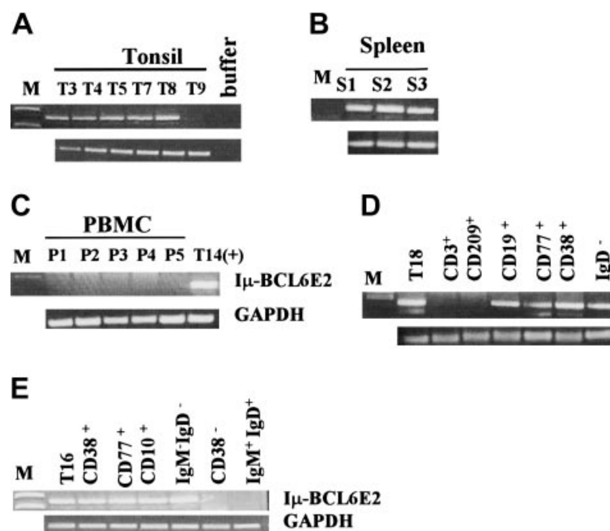

Figure 5. Characterization of the Iμ-BCL6 transcript producing cells from normal lymphoid tissues and PBMCs. Chimeric Iμ-BCL6 transcripts detected from the representative tonsil (A), spleen (B), and PBMC (C) donors are shown. Associations of the chimeric Iμ-BCL6 transcripts with the tonsillar B-cell subpopulations positively selected by magnetic beads (D) and sorted by FACS (E) are presented.

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Table 1. Summary of the detection of Iμ-BCL6 transcripts from human lymphoid tissue

	Age, y	Iμ-BCL6E2 transcripts					Frequency, events per 10^{-6}
		MCs*	$CD38^+$ B cells	$CD77^+$ B cells	$CD3^+$ T cells	$CD209^+$ DCs	
Tonsil no.							
1†	na	+	nd	nd	nd	nd	nd
2†	na	+	nd	nd	nd	nd	nd
3†	na	+	nd	nd	nd	nd	nd
4	11	+	nd	nd	nd	nd	nd
5	8	+	nd	nd	nd	nd	nd
6	10	+	nd	nd	nd	nd	nd
7	11	+	nd	nd	nd	nd	nd
8†	na	+	nd	nd	nd	nd	nd
9†	na	–	nd	nd	nd	nd	nd
10	11	+	+	nd	nd	nd	nd
11	19	+	+	nd	nd	nd	nd
12	6	nd	+	+	–	–	8
13	5	+	+	+	–	–	4
14	8	+	+	nd	nd	nd	nd
15	9	+	+	nd	nd	nd	nd
16	3	+	+	+	–	–	16
17	10	+	+	+	–	–	0.5
18	9	+	+	+	–	–	8
19	10	+	+	nd	nd	nd	2
20	17	+	+	nd	nd	nd	8
21	6	+	+	nd	nd	nd	1
22	5	+	+	–	–	–	16
23	11	+	+	nd	–	–	0.5
24	27	+	+	nd	nd	nd	2
25	na	+	+	nd	nd	nd	0.5
26	na	+	+	nd	nd	nd	nd
27	3	+	+	nd	nd	nd	2
28	7	+	+	nd	nd	nd	nd
29	4	+	+	nd	nd	nd	nd
30	21	+	+	nd	nd	nd	nd
31	14	+	+	nd	nd	nd	nd
32	7	–	–	nd	nd	nd	nd
33	12	nd	+	nd	nd	nd	nd
34	8	nd	+	nd	nd	nd	nd
35	21	+	+	–	nd	nd	nd
36	19	nd	+	nd	nd	nd	nd
37	4	nd	+	nd	nd	nd	nd
38	11	nd	+	nd	nd	nd	nd
39	9	nd	+	nd	nd	nd	nd
40	7	nd	+	nd	nd	nd	nd
41	12	nd	+	nd	nd	nd	nd
Spleen no.							
1	na	+	+	nd	nd	nd	nd
2	27	+	+	nd	nd	nd	nd
3	40	+	+	nd	nd	nd	nd

na indicates not available; nd, not done.
*Mononuclear tonsil cells.
†Frozen samples.

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with $CD77^+$ marker was less consistent, as 5 of 7 enriched $CD77^+$ B cells were positive (Figure 2A; and data not shown). Using FACS sorting to extend these studies, Iμ-BCL6E2 transcripts were detected in B cells, $CD38^+$, $CD77^+$, $CD10^+$, and IgM^-IgD^- cells, but not in IgM^+IgD^+ or $CD38^-$ B-cell populations (Figure 5E, n = 3). Overall, these results show that the Iμ-BCL6E2 transcripts primarily exist in $CD38^+$ and/or $CD10^+$ B-cell subpopulations, and indicate that Iμ-BCL6 transcripts are associated with GC-stage B cells, but not naive B cells, T cells, or dendritic cells.

Frequency estimation of *Igμ/BCL6* translocation cells in human tonsils

We next sought to define the sensitivity of our assay for the quantitative measurement of chimeric transcripts as a biomarker for *Ig/BCL6* translocation in mixed cell populations. As shown in Figure 6A, the assay could reliably detect chimeric Iμ-BCL6E2 transcript in the RNA equivalent of 1 to 3 translocated DLBCL cells (0.25-0.75 pg RNA). This was determined by serially diluting Iμ-BCL6E2–positive DLBCL-cell RNA into the RNA equivalent of 1×10^6 DG75 cells (4 μg RNA), a Burkitt lymphoma cell line that harbors an Ig/c-myc but not an *Ig/BCL6* translocation.[14] To estimate the frequency of *Igμ/BCL6*-translocated cells, we performed a semiquantitative analysis of limiting dilution to determine the frequencies of the Iμ-BCL6E2 transcript–expressed cells in human tonsillar cells. The frequency of the *Igμ/BCL6*-translocated cells was estimated by using the highest dilution of the input cells able to give a positive Iμ-BCL6E2 signal as "one Ig/BCL6 CT event," as shown in Figure 6B, where the frequency was 1 event for

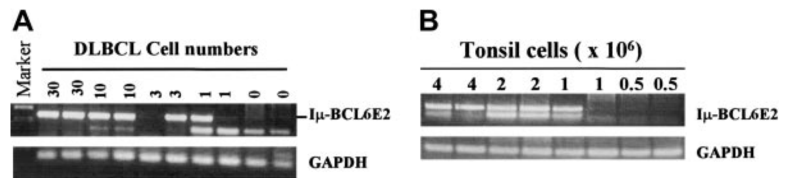

Figure 6. Sensitivity of the RT-PCR assay for the chimeric transcripts. (A) The sensitivity for Iμ-BCL6E2 with DLBCL cells. The RNA equivalent for the indicated DLBCL-cell number was diluted into RNA from 1×10^6 DG75 cells for the assay done in duplicate. (B) The assay sensitivity for tonsillar cells. The tonsillar cells were serially diluted as indicated; the highest dilution (eg, lowest cell number) with a positive Iμ-BCL6E2 signal from at least one sample of the duplicates is scored as the end dilution.

every 10^6 tonsillar cells. Hence, one CT "event" is at least one (or more than one) CT cell in the given cell population. Since the transcriptional rate for Iμ-BCL6 transcripts may be different between DLBCL and primary B cells carrying an *Igμ/BCL6* CT, the assay sensitivity obtained from the reference DLBCL cells may not exactly apply to the primary B cells. The frequency was further normalized to the number of Iμ-BCL6E2 expression events in every 1×10^6 cells. Thirteen tonsillar cells analyzed with this approach showed that the frequencies of the *Igμ/BCL6*-translocated cells were in the range of 0.5 to 16 CT events in every 1×10^6 tonsil cells (Table 1).

Discussion

Analysis of *Ig/BCL6* CT cells in humans prior to their full malignant transformation has not been possible previously due to the lack of an appropriate system capable of detecting and/or measuring *Ig/BCL6*-translocated cells in mixed cell populations. Previously developed *Ig/BCL6* CT detection systems used for tumor tissue analysis are simply not sensitive enough to effectively detect and/or quantify the rare precancerous cells with this CT.[20] To better understand the biologic nature of the *Ig/BCL6* CT cells prior to their malignant transformation, we established a sensitive RT-PCR assay for detection of the chimeric Iμ-BCL6 transcript as a marker reflecting *Igμ/BCL6* CT. Because trans-splicing mechanism is not likely responsible for the production of the observed chimeric transcripts, the chimeric transcripts are therefore the reliable markers for the *Igμ/BCL6* CT. Indeed, several lines of evidence—including the coexpression of both chimeric Iμ-BCL6E2 and BCL6E1-Cμ1 transcripts reflecting the reciprocal *Igμ/BCL6* CTs from the same B-cell subpopulation, the special association of the chimeric transcripts with GC markers, and the isolation of the *Ig/BCL6* DNA recombinational products from GC B cells—further support the notion that the chimeric transcripts can serve as the markers for *Ig/BCL6* CT in mixed cell populations.

It is generally believed that a CT is a critical minimum factor in the development of many types of leukemias/lymphomas, as many such CTs are strongly associated with specific leukemias/lymphomas.[21] However, several types of CTs per se are known to be insufficient to result in full lymphomagenesis (for example, Ig/BCL2 [t(14;18)(q32;q21)],[22] Ig/c-myc [t(8;14)(q24;q32)],[23] and NMP/ALK [t(2;5)(p23;q35)])[24] in humans and various types of CT in mice.[21] These latter data support a "2-hit" hypothesis,[25] in which another factor(s), including genetic or epigenetic alterations in addition to the CT per se, is required for malignant transformation in these specific CT-associated lymphomas. However, one cannot simply extrapolate this outcome to other lymphoma-associated CTs and, especially, in those involved in *Ig/BCL6* CT that are frequently associated with DLBCL and FL. With an assay capable of detecting as few as 1 to 3 *Igμ/BCL6* CT cells in 10^6 cells, we were able to identify and to quantify the frequencies of these CT cells in human tonsils, spleen, and blood. Essentially, all tonsil and spleen samples demonstrated the presence of the chimeric transcripts indicative of the *Igμ/BCL6* CT, while such cells were not found in PBMCs. Therefore, we showed that *Ig/BCL6* CT cells exist in human lymphoid tissues of otherwise healthy individuals. Our data also show that the frequent occurrence of an *Ig/BCL6* CT in human lymphoid tissues is not sufficient to result in malignant transformation. This is of particular interest in that the deregulated BCL6 expression driven by an Iμ-promoter is not sufficient to lead to lymphomagenesis in the context of human B cells, while it is able to promote DLBCL-like pathology in a transgenic mouse model.[11]

The association of *Ig/BCL6* CT cells with GC phenotypes suggests that the CT occurs at the GC stage of B-cell development (indicating that these CT cells are the products of the GC reaction). This is consistent with the GC origin of DLBCL.[7] Indeed, the machinery for Ig SHM and CSR, 2 major genetic alteration events for antibody diversification that occur during GC reactions,[26,27] has been implicated in the generation of *BCL6* mutations[28] and in *Ig/BCL6* translocations (this study), as they are characteristics of the activation-induced cytidine deaminase (AID)–dependent Ig SHM and Ig CSR.[6,28,29] Therefore, both *BCL6* SHM and *Ig/BCL6* CT are likely the byproducts reminiscent of the GC reactions mediated by the machinery for Ig SHM and CSR.

The fate of the cells that carry the observed *Ig/BCL6* CT remains to be fully explored and will be of great interest in terms of the potency of the precancerous cells for the subsequent malignant transformation. Those CT cells that do not express B-cell receptors (BCRs) are not expected to survive the GC selection, since CT-associated lymphomas with GC origin usually express BCR and the CT cells are predominantly involved in the nonproductively rearranged *Ig* alleles.[7,30] Even though the number of *Ig/BCL6* CT cells that will survive through GC selection is not clear at this time, a portion of these CT cells is expected to express BCR, and therefore is likely able to survive the GC selection, as there is no typical reason for why the *Ig/BCL6* CT should selectively occur in the nonproductive Ig alleles. Potentially *Ig/BCL6* CT cells surviving the GC selection and entering the peripheral pool would become memory B cells and, therefore, they probably would have a higher chance of undergoing malignant transformation due to their exposure to antigen stimulation, or genetic or epigenetic alterations. Alternatively, the *Ig/BCL6* CT cells could also gain an additional hit for malignant transformation inside the GC, without undergoing GC selection and/or exiting GC reaction, because AID-mediated mutational/recombinational activities for further genetic alterations theoretically could also occur in the GC stages of CT cells. Malignant transformation also could occur when the *Ig/BCL6* CT event hit the cells already harboring other genetic alterations, such as mutations in other proto-oncogenes, as such mutations are frequently associated with DLBCL.[31]

Once the *Ig/BCL6* CT cells survive the GC selection, they should exit GC and become memory B cells; therefore it is possible that such CT-bearing memory B cells would appear in the circulation. We could not detect *Ig/BCL6* CT cells in PBMCs, but whether this is because they are truly absent or simply too few in percentage of total PBMCs remains to be addressed. The total amount of PBMCs used (1-2 $\times 10^6$ cells) likely contains too few post–GC-stage B cells for detection as only about 4% of PBMCs are memory B cells. To identify whether *Ig/BCL6* CT cells are

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present in PBMCs, a specific B-cell population–enriched fraction (such as CD27$^+$ memory B cells) needs to be tested.

The correlated expression of both types of chimeric transcripts in the same cell subpopulations not only provides support for the notion that the chimeric transcripts are the sensitive markers for this type of CT, but also suggests that the *Igμ/BCL6* CT in primary B cells is reciprocal, as it is present in lymphomas, and indicates that these CT cells are very likely the precursors of those lymphomas harboring *Ig/BCL6* CT. However, the conclusion that the *Igμ/BCL6* CT in primary B cells is reciprocal can be confirmed at the single-cell level only with appropriate technology such as fluorescent in situ hybridization (FISH), which can formally demonstrate reciprocal CT. Unfortunately, due to the sensitivity limitation of the FISH assay,[32] an attempt to use FISH to demonstrate the reciprocal nature of the CT in primary B cells failed (data not shown).

With this sensitive assay, we will be able not only to detect the rare *Igμ/BCL6* CT in lymphoid tissue but also to measure the frequency of this CT as the potential precursor for DLBCL and FL in different samples. This can be done while examining the effects of variables such as age, gender, infection (HIV), chemical exposure, or other potential risk factors. It is also possible that a quantitative assessment of *Igμ/BCL6* CT via this chimeric transcript approach will serve as a broad indicator for general genomic instability associated with GC reactions in subjects. Detection and measurement in blood B cells will be a big step forward in this regard. In addition, enrichment of the CT cells will allow study of their growth characteristics in vitro and their further molecular characterization. Finally, our assay will allow the detection of residual CT tumor cells after therapy in appropriate lymphoma patients.

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