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ORIGINAL ARTICLE: RESEARCH

Molecular characterization of neoplastic and normal ‘sister’ lymphoblastoid B-cell lines from chronic lymphocytic leukemia

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Shortened running title: Authentic CLL lymphoblastoid B-cell lines

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Key words: Chronic lymphocytic leukemia (CLL), Lymphoblastoid cell line (LCL), *IGHV*-sequence, Cell-of-origin, Natural antibodies, Antigen structure, CD5+ innate B cells

Abstract

Chronic lymphocytic leukemia (CLL) B-cells resemble self-renewing CD5⁺ B-cells carrying auto/xeno-antigen-reactive B-cell receptors (BCRs), and multiple innate pattern-recognition receptors, such as Toll-like receptors and scavenger-receptors. Integration of signals from BCRs with multiple surface membrane-receptors determines whether the cells will be proliferating, anergic, or apoptotic. To better understand the role of antigen in leukemogenesis, CLL cell lines producing monoclonal antibodies (mAbs) will facilitate structural analysis of antigens and supply DNA for genetic studies. We present here a comprehensive genotypic and phenotypic characterization of available CLL and normal B-cell-derived lymphoblastoid cell lines (LCLs) from the same individuals (n=17). Authenticity and verification studies of CLL-patient origin were done by *IGHV*-sequencing, FISH, DNA/STR-fingerprinting. Innate B-cell features, ie. natural Ab-production and CD5-receptors, were present in most CLL cell lines, but in none of the normal LCLs. This panel of immortalized CLL-derived cell lines is a valuable reference representing a renewable source of authentic Abs and DNA.

Introduction

Chronic lymphocytic leukemia (CLL) cells develop in the bone marrow (BM) and lymph node (LN) ‘proliferative’ compartments in crucial support by cellular and humoral milieu components [1]. The characteristic CD5⁺/CD19⁺/CD23⁺/CD27⁺/CD43⁺ phenotype and drastically restricted antibody (Ab) repertoire expressed by the tumor clones resemble those of a putative human innate B cell population, similar to mouse B1 cells [2,3], albeit the cell-of-origin is still debated [4,5]. For a better understanding of the role of antigens and growth factors in the microenvironment, multiple experimental models have been reported. However, none of these animal or co-culture systems completely mirrors the *in vivo* scenario [6]. Nevertheless, the importance of pro-survival influences from stromal-cell derived thioredoxin 1 (Trx1) [7-10], stem-cell factors produced by nurse-like cells [11], CD40L-transfected feeder cells [3], and macrophage feeder [12] have been highlighted. Without such support, CLL cells do not proliferate *in vitro* and quickly die by growth factor deprivation. The analytical options of these co-cultures are limited even though the CLL cells can be activated and stimulated for several divisions [8].

Cultures of CLL-derived lymphoblastoid cell lines (CLL-LCLs) continue to produce mAbs and express genomic aberrations such as del(13q14), trisomy 12, del(11q), del(17p) identical to the *in vivo* patient leukemic clone [3]. One functionally crucial exception would be that LCLs are EBV-transformed in contrast to the majority of primary CLL cells. Resting peripheral blood (PB) B cells from healthy donors are readily transformed by EBV into LCLs with retained traits, including Ab production [13-15], whereas CLL B-cells are generally resistant to EBV-induced transformation. While the CLL cells carry the virus receptor CD21 and can be infected, these cells usually do not transform to cell lines, due to a very restricted expression of the EBV-encoded genes lacking LMP1 (latency IIb program) [16]. Consequently, upon exposure of a CLL sample to EBV in culture, it is the normal B cell fraction that is more easily transformed [17,18].

A few rare CLL-LCLs have been established from such *in vivo* infected clones, however, most cell lines that do exist were derived from *in vitro* EBV-infections, and both types of cell lines exhibit full latency III program. Considering the higher accessibility to EBV transformation of normal B cells over CLL B cells in such a mixed culture, the few long-term CLL-LCLs that do exist have been questioned as to their correct neoplastic origin.

In this study, we present a detailed genomic and phenotypic analysis of a panel of such CLL cell lines [17,19-23], including those claimed to be normal B cell derived LCLs from the same donors [17]. The main purpose of this study was to collect evidence of unique clonal

identity and authenticity of the cell lines. We have analyzed the most relevant molecular features such as Ab-reactivities, genetic aberrations, and certain activation markers (i.e. CD38, ZAP0, TCL1, CLLU1), with particular emphasis on neoplastic-to-normal B cell compartment. The LCLs represent models for activated CLL cells useful for analyses of DNA aberrations in leukemic cells and they are a renewable source of natural antibodies in studies of antigen structures.

Materials and methods

CLL cell lines

CLL cell lines (Table 1) were cultured in RPMI 140 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Immunoglobulin gene sequencing

DNA was extracted according to standard procedures using proteinase K. *IGHV*, *IGKV* and *IGLV* subgroup-specific PCR amplification and nucleotide sequencing was performed as published [24]. The DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Piscataway, NJ) was used for sequence reactions to be analysed by an automated DNA sequencer (MegaBACE 1000 DNA Analysis System, Amersham Bioscience, Sunnyvale, CA). Sequences were submitted to the IMGT/V-QUEST database. Germline identity of <98% to the corresponding *IGHV*, *IGKV* and *IGLV* germline genes was defined as mutated. All HCDR3 sequences were compared with published sequences for stereotyped subsets [25].

Real-time quantitative PCR (RQ-PCR)

RQ-PCR for *FMOD* and *CLLU1* was performed according to previously published procedures [26,27]. For *TCL1* we used RT-PCR (forward primer: GGAGAAGTTCGTGTATTTGG, reverse: CGCCGTCAATCTTGATG). RT-PCR data represent β-actin normalized levels. DoHH2 B cells and their stably *TCL1*-transfected sublines DoHH2-TCL1 served as controls [28].

FISH analysis

Cell lines were analyzed for genomic aberrations using a CLL FISH probe panel including probes for 11q22.3 (ATM), 17p13.1 (p53), 12cen (CEP12), 13q14.3 (D13S319) and 13q34 (VYSIS, Downers Grove, IL, USA). Fluorescence signals were enumerated in at least 200 interphase cells. The cut-off for positive values was set at >10%.

High resolution short tandem repeat (STR) analysis

DNA quantitative fluorescence PCR (QF-PCR) was performed in order to determine whether the CLL clone and its normal LCL counterpart originated from the same patient. Seventeen polymorphic microsatellite markers on chromosome 13, 18, 21, X and Y were amplified by PCR, using a ChromaQuantTM kit (CyberGene, Stockholm, Sweden) including fluorochrome-

labelled primers, according to the manufacturer's instructions. The PCR fragments were analysed by capillary electrophoresis on a MegaBACE 500.

Protein expression/secretion analysis

CD5, CD19, CD20, CD23, as well as IgM, Ig- κ and λ -light chains were analyzed by flow cytometry as previously described [3]. IgG-production was analyzed by ELISA in IgM-negative cell lines. CD38 (HB-7, Becton Dickinson, Heidelberg, Germany) and TCL1 expression (clone 1-21 as PE- and APC-conjugates) were assessed by flow cytometry in 3 independent measurements for most cultures with isotype-controlled mean fluorescence intensity (MFI) reported to determine relative expression levels and with indicated percentage of positive cells (cut-off's 30%). ZAP70 expression was assessed using a customized flow cytometry protocol [29]. TCL1 and ZAP70 levels (Ab #D1C10E, Cell Signalling Technology Inc., Beverly, MA) were confirmed in all cases by Western blot (WB) with categorizations for TCL1 as published) [30]. Jurkat E6-1 cells and normal PB T cells represented positive controls for the WB and flow-cytometric analysis of ZAP70.

ELISA for autoantigens

Chemoluminescent-based ELISAs were used for analysis of Ab reactivities previously identified in tissue-arrays and protein-arrays [24]. Vimentin, oxidized LDL (oxLDL), proline-rich acidic protein-1, or cofilin-1 were coated onto MicroFluor2-plates, then cell line-derived Abs were incubated overnight. Alkaline-phosphatase-anti-IgM was used as indicator Ab and plates were developed with LumiPhos530 substrate. Luminescence-intensity was recorded in a Wallac Victor2 luminometer. Competition-ELISA was used for validation. Abs were then preincubated with increasing concentrations of antigen for 18 h.

Results

Table I summarizes the panel of 17 CLL patient-derived cell lines, of which 10 were claimed to be of neoplastic (CLL-LCLs) and 7 of normal B cell origin (LCLs). The clinical information available for the CLL patients is presented together with the genetic and immunophenotypic characteristics of their corresponding established cell lines (Supplemental Table A-I). If *IGHV/IGKV/IGLV* gene analysis, FISH cytogenetics, DNA genotyping and immunophenotype analysis were not included in the primary reports, we performed these tests on the cell lines, and also on patient blood samples received from biobanks. Here, we have followed the nomenclature suggested by Drexler [31] as follows: “Authentication” of derivation refers to conclusive proof of derivation from a particular patient; “verification” of neoplasticity refers to unequivocal proof of neoplastic origin of the cell line. The term ‘verified’ was used for molecular evidence supporting CLL neoplastic origin, but in contrast to the term ‘authentic’, the verified-only cell lines could not be assigned to a particular patient due to unavailability of blood sample.

CLL and normal B cell lines from the same individuals

Initially six CLL/normal LCL pairs were available: I83-E95/I83-LCL, 232B4/232A4, WaC3CD5⁺/Wa-osel, PGA1/PG-B95-8, CI/CII, AI-60/AII, AIII. However, on the basis of *IGHV* gene sequence data, we discovered that in the two pairs -WaC3CD5⁺/Wa-osel and CI/CII, the normal ‘sister’ counterpart had identical *IGHV*-gene rearrangement with the leukemic clone. Furthermore, the AI-60 partner in the AI-60/AII, AIII pair ceased to proliferate.

I83-E95/I83-LCL pair: The prime value of this CLL/normal LCL pair [17] is the authentic origin of both cell lines, with the leukemic partner showing maintained CD5-expression (Figure 2), +12, 13q-, 17p- markers, and release of natural IgM, λ , *IGHV3-30*-mutated monoclonal Abs (Figure 1A) with anti-oxLDL and vimentin specificity reacting with apoptotic Jurkat cells, whereas the normal LCL was IgM, λ of unknown specificity, CD5-negative, normal karyotype, and identical DNA-fingerprint to patient (Table I, Supplemental Table A, Supplemental Figure 1A). The cell lines were previously established from 7-d cultures of EBV-infected CLL cells grown in the presence of Trx1, IL2 and SAC (heat inactivated *S. aureus*, Cowan I) followed by plating on irradiated human embryonal fibroblasts.[17] We conclude that I83-E95 represents the authentic malignant CLL clone and that I83-LCL was derived from the patient’s normal B cells.

WaC3CD5⁺/Wa-osel pair [17]: This pair is noteworthy because it exemplifies biclonality in CLL. WaC3CD5⁺ is an IgM⁺/κ⁺/CD5⁺/CD19⁺ (Figure 1B and Figure 2) /13q-/17p- line expressing two different functional *IGHV3-30*-unmutated clones (Figure 1B), the first belonging to stereotyped subset-32 was dominant with a 360 bp HCDR3 sequence CARGPGDYYDSSGYYWWNYYYY GMDVW, while the second minor clone had a shorter 310 bp HCDR3 sequence CAKGRPLTGYW with no subset membership (Table I, Supplemental Table B). The latter rearrangement was identical to that in patient's blood cells and in the Wa-osel line. At the time of establishment, the WaC3CD5⁺ line had the dominant 360 bp-rearrangement only, but after initial culturing, the minor 310 bp-rearrangement could also be detected. WaC3CD5⁺ produced natural Abs with polyspecific binding including apoptotic cell membranes and *S. pneumoniae* polysaccharides. Noteworthy, the Wa-osel sister cell line is an IgM⁺/κ⁺/CD5⁻ (Figure 2) monoclonal line expressing the second shorter HCDR3-sequence only. Based on DNA-fingerprint (supplemental Figure 1B) and FISH data, we conclude that the Wa-osel line is an authentic neoplastic clone, and that WaC3CD5⁺ is a biclonal authentic CLL line. The two clones continued to coexist in long-term cultures. The method of establishment was similar to that described above for I83-E95, except that anti-CD5 magnetic beads were used for pre-selection during WaC3CD5⁺-establishment.

232B4/232A4 is another example of oligoclonality in CLL. Based on *IGHV*-gene rearrangement analysis (Figure 1C), this CLL/normal LCL pair [17] represents outgrowth of one (with a 325 bp *IGHV3-48/IGHD3-10/IGHJ3* rearrangement, named 232B4-CLL line) of the two leukemic clones (310 bp and 325 bp long) present in patient's blood and a normal B cell (232A4) of the same patient that had an unrelated and mutated *IGHV1-46/IGHD5-5/IGHJ6* gene rearrangement. 232B4 secreted IgG, κ Abs with affinity for cofilin-1, an actin binding and depolarizing protein that co-localizes with Arp2/3 and the Wiskott-Aldrich syndrome protein WASp complex in apoptotic blebs [24]. CD5-expression was absent in both cell lines (Figure 2, Table I, Supplemental Table C). The method of establishment was similar to that described above for I83-E95. We conclude that the two cell lines are authentic based on DNA/STR fingerprint analysis (Figure 3), and *IGHV* sequence analysis: 232B4 of malignant CLL patient origin and 232A4 of normal B cell origin from the same person.

HG3 was established by in vitro EBV-infection from an *IGHV1-2* unmutated IgM, λ CLL patient clone in the presence of CD40L-expressing human L-cell fibroblasts [3]. Transformed cells expressed EBNA2 and LMP and had identical IG gene rearrangements and biallelic 13q14 deletion with genomic loss of *DLEU7*, miR15a/miR16-1 as the patient clone. The surface CD5⁺/CD19⁺/CD20⁺/CD27⁺/CD43⁺ expression, and release of IgM natural Abs

reacting with oxLDL-like and filamin-B epitopes on apoptotic cells (*cf.* stereotyped subset-1) is reminiscent of human B1 cells (Table I and Supplemental Table D). We conclude that HG3 line is of authentic origin.

MEC1/MEC2 represents spontaneous outgrowth of CLL clones from a CLL patient in prolymphocytic leukemia (PLL) transformation [32]. CD5 expression was present in early cultures, but it was eventually lost. Patient's leukemic B cells were EBNA-negative, but cell lines were EBNA-positive, which may be explained by an infection of a small number of the patient's neoplastic cells *in vivo* with patient's circulating EBV, or an *in vitro* infection by normal EBV-carrying B cells releasing the virus. Both cell lines showed *IGHV4-59*-mutated sequence (Table I and Supplemental Table E), identical to the sequence of the patient clone as reported in the primary reference. It is not known whether the patient had concurrent CLL and PLL clones [33] and in case of concurrency, which one gave rise to MEC1 and MEC2 [32]. Both cell lines are of certified leukemic origin without conclusive resolution of their CLL or PLL origin.

CI/CII pair: Based on maintained CD5-expression (Figure 2) and stereotyped subset-5 (unmutated *IGHV1-69/IGHD3-10/IGHJ6* and *IGLV1-4/IGLJ3*) CDR3 sequence (Figure 1F), a true CLL origin in both CI and CII could be verified. In the primary reference, CI was described as a normal B cell [21]. Originally, the CII line contained two subpopulations, one with a 47,XX,+12 karyotype and a with a 47,XX, inv(11),+del(12) t(12;15) karyotype. The patient's normal tissue expressed both type-A and type-B of the isozyme glucose-6-phosphate dehydrogenase (G6PD), whereas the CII cell line expressed exclusively type-A G6PD, i.e. identical to the patient's blood CLL clone. CII and the patient's clone were IgM, λ . In contrast, the CI cell line was karyotypically normal, expressed IgM, κ , and type-B G6PD, thus Karande et al. [21] assumed that CI was derived from non-leukemic normal B cells (Table 1 and Supplemental Table F). We analyzed CI and CII from three different laboratories and found that all CI cells showed *IGHV1-69*/subset-5 rearrangements, identical with the CII cell line. These data together with the CD5⁺/CD19⁺ -expression and trisomy 12 strongly indicate that CI was cross-contaminated early in the establishment history with the neoplastic CII clone. In this scenario, the original culture contained a few neoplastic cells along with normal B cells. We conclude that CI and CII are of verified CLL origin derived from the same patient (STR-analysis in Supplemental Figure 1C), foremost based on the BCR belonging to the stereotyped subset-5.

PGA1/ PG/B95-8: PGA1 is another example of an *in vivo* EBV-transformed CLL clone growing out spontaneously *in vitro* without addition of B95-8 strain of EBV [22]. Its original

marker was a ring chromosome 15 that was also present in patient blood. This was eventually lost in cultures, whereas 13q- and +12 were maintained. CD5-expression was down-regulated (Figure 2). The PG/B95-8 represents a normal CD5-negative LCL with normal karyotype derived from the same donor (Supplemental Figure 1D). We conclude that PGA1 is authentic, carrying trisomy 12, and an IGHJ-RFLP pattern identical to the patient's clone, and that PG/B95-8 sister cell line was derived from normal B cell of the PG patient.

AI-60/AII, AIII: The AI-60 was IgM, κ with a 45X,-X karyotype and A-type G6PD identical to the blood CLL cells [34]. The AII and AIII were of leukemic origin on initial examination, but overgrown by normal cells during the first year. AI-60 showed a mutated *IGHV1-69/IGHD2-2/IGHJ4*-rearrangement. STR-analysis showed unequivocal identity between AI-60, AII and AIII (Table I, Supplemental Figure 1E). Due to the growth cessation of AI-60 in the AI-60/AII/AIII group and lack of patient's ex vivo cells, we conclude that AII and AIII are verified normal LCLs derived from a CLL patient. (Footnote: Presently, it is unknown to us if AI-60 cell line grows or is deposited in other laboratories (besides our own in Linköping, Uppsala and Stockholm) – as we have no complete information as to which labs the original cell line was distributed since the establishment in 1980. However, we feel that the CLL community deserves to be informed about the molecular data on these cells despite this drawback).

EHEB: This CLL-LCL had a normal karyotype and identical *IGH* and *IGL* rearrangements with the patient's CLL cells [23], originally CD5⁺/CD19⁺/ μ ^{low}/cytoplasmic κ ⁺. We found in this study a mutated *IGHV1-18/IGHD5-12/IGHJ1* gene rearrangement with 82.8% germline identity (Figure 1). FISH analysis showed extra copies of 11q (Table I) and FACS analysis revealed a CD5⁻CD19⁺/CD20⁺/CD23⁺/ μ ⁻/ κ ⁺ surface phenotype (Figure 2 and Supplemental Table G). We could not conclusively re-confirm/verify a neoplastic origin of the EHEB cell line in this study due to loss of CD5-expression, lack of CLL-markers, and inaccessibility to patient's blood cells.

Quantitative gene expression levels of FMOD and CLLU1

Figure 4A, B shows that *FMOD* and *CLLU1* mRNA expression levels were low in comparison with a panel of patients' CLL cells (n=175) previously analyzed [35].

CD5 expression

CD5 is an obligatory marker for classification of CLL. It is also present on most, but not all, innate human B cells, resembling murine B1 B cells, and it is considered to be associated with

an activated phenotype [2], but not always. We found differential CD5 expression on CLL cell lines (Figure 2), exemplified by maintained expression on HG3, WaC3CD5⁺, CI, and CII, lower expression on I83-E95, and lost expression on 232B4, PGA1, EHEB (see also Table I). None of the normal ‘sister’ LCLs were CD5-positive. HG3 was already shown to express a combination of B1-cell associated markers (CD5⁺/CD19⁺/CD27⁺/CD43⁺/natural Ab⁺) [3].

CD38, ZAP70 and TCL1

Protein expression values of CD38, ZAP70, and the TCL1 proto-oncogene are summarized in Table II and Figure 5. In addition, TCL1 mRNA-expression was analyzed (Figure 5B). I83-E95 was CD38⁺/TCL1⁺/ZAP70^{dim}, whereas its normal sister line I83-LCL was CD38⁺/TCL1⁺/ZAP70⁻. 232B4 and 232A4 were both CD38-negative, but showed ZAP70 and TCL1-expression, which tended to be higher in the 232B4 CLL line than in the normal 232A4 LCL (Table II, Figure 5B). PGA1 (CLL-LCL) and PG/B95-8 (normal LCL) were both low in CD38 and TCL1 expression, although slightly increased levels were observed in PGA1 compared with PG/B95-8. ZAP70 was absent in both cell lines (Table II). In these LCLs, the activation marker CD38 showed an expression gradient with a high degree of spread across one culture, while ZAP70 and TCL1 were more uniformly expressed. There was no obvious association of CD38, ZAP70, or TCL1 expression with each other or with *IGHV* mutation status.

Discussion

We here present detailed authenticity characterization of a collection of 17 CLL patient-derived cell lines, of which 8 were found to be authentic neoplastic origin, 2 of verified CLL origin, and 5 derived from the patients' normal B-cells. We comprehensively catalogue the differences between leukemic LCLs and normal B-cell LCLs derived from the same CLL patients including analyses of gene and protein expression patterns of CLL-relevant molecules.

A substantial challenge in understanding CLL biology is to understand the role of antigen in the proliferative lymphoid milieu in contrast to peripheral blood (resting) compartment, where antigen is absent. Although animal models have contributed considerably to closing many knowledge gaps in CLL, these have certain limitations, such as cell subset access and fidelity issues [6]. Therefore, and in light of the relentless apoptosis induction occurring in primary CLL cultures, the generation of immortalized CLL cell lines has been highly desirable, as these represent important supplemental tools with benefits of high reproducibility and easy-access, in particular for mAb (*IG* gene/protein).

With the recent advent of co-culture systems for prolonged cultivation of CLL cells using macrophages [12], CD40L-transfected fibroblasts [3], or patient-derived stromal cells [7], there has been an improvement in the frequency of obtained EBV-transformed CLL clones. For stable long-term Ab-production, fusion of the EBV-transformed CLL cells with the K6H6/B5 heteromyeloma (mouse NS-1-Ag4 myeloma × human nodular lymphoma cells) appears to be a very valuable tool [12]. For genetic studies, however, these hybrids suffer from the additional DNA supplied by the mouse×human fusion-partner, in comparison with CLL-LCLs, which retain the karyotype of the patient's *in vivo* clone. In our panel of 17 CLL patient-derived cell lines characterized in this study (Table1), authenticity was certified in 8 cell lines: I83-E95, MEC1 (CLL/PLL), MEC2 (CLL/PLL), WaC3CD5⁺, Wa-osel, 232B4, PGA1, and HG3, by *IGHV*-sequence matching with patients' blood cells and DNA/STR-fingerprint-data, whereas verified CLL origin was evidenced in CI and CII based on CDR3 sequence similarity to stereotyped subset #5. Five cell lines were confirmed normal diploid LCLs: I83-LCL, 232A4, AII, AIII, and PG/B95-8. Finally, AI-60 and EHEB could not conclusively be verified to be of CLL-derivation due to unavailability of patient blood cells for sequence comparisons.

A key feature of the CLL clone is the IG structure/specificity, since it provides information on the function and differentiation of the CLL B cell progenitor. CLL B cells share several properties with CD5⁺ B1 cells described in mice [2], but also with mature CD5⁺

B cells [5]. CLL cells have a highly restricted “stereotyped” BCR repertoire in ~30% of cases [25,36]. We found that 6 of the 8 CLL-LCLs produced ‘natural’ Abs against oxidation-specific self-antigens and the mAb production was maintained in the cell lines over long time. The natural Ab repertoire has previously only been described for murine CD5⁺ B1 cells [24,37]. Overall, with respect to BCR ‘anatomy’ (ie. CDR3 sequence, *IGHV* gene segment usage, surface IgM-expression) and Ab-secretion, but also to a large degree based on immunophenotype, our set of cell lines represents key features of primary human CLL.

The expression levels of CD38, ZAP70, TCL1, FMOD, CLLU1 were generally lower in the cell lines compared with levels in CLL patients’ blood cells, but interestingly ZAP70 and TCL1 tended to be expressed at slightly higher levels in the CLL cell lines than in their same-patient LCL sister cell lines. No conclusive answer can be provided as to why TCL1 levels found in these CLL cell lines tended to be lower than in fresh primary CLL samples (not shown [30,38]). It is also known that EBV-gene products regulate TCL1 in a latency-program dependent fashion [39,40]. Furthermore, we found that *FMOD* expression did not differ in CLL-LCLs vs. LCLs from normal B-cells, although the expression was low as compared with primary CLL cells (Figure 4A, B). Interestingly, EBV specifically interferes with and mimics micro-milieu fed activation pathways that are most central in CLL, such as BCR and CD40L cascades. Küppers and co-workers described EBV overriding the gene expression patterns of B-cell differentiation [41]. Overall, influences by EBV and the altered culture environment likely affect ZAP70, CD38, CLLU1, FMOD, and TCL1 expression through pathway and regulatory interferences.

Although EBV-transformed LCLs have provided a conveniently accessible and renewable resource for functional studies of gene expression and epigenetic regulation [42], their utility of LCLs as a surrogate model for primary tissues – B cells in particular, has been questioned due to the effect of EBV-transformation. Çalışkan et al. [42]. found that 6464 genes were differentially expressed between primary B cells and LCLs, but that most of the expression differences were of small magnitude with only 33 genes differentially expressed with a fold change of >1.5. Based on their detailed global expression arrays and methylation profiling, they conclude that LCLs may be faithful models for expression quantitative trait loci (eQTL), and suggest that inference of the genetic architecture that underlies regulator variation in LCLs can typically be generalized to primary B cells, whereas inference based on functional studies in LCLs may be more limited to the cell lines

In summary, we here explored the authenticity of malignant CLL cell lines based on genetic and phenotypic analyses, and we showed the individual CLL-representative nature of

these systems including innate CD5⁺ B cell features. Inferences based on factors responsible for survival and proliferation of CLL cells cannot be made due to EBV imprinting in these cell lines. We regard this collection of authentic CLL and sister LCLs as valuable tools for studies on antibody-specificities, and genetic (DNA) aberrations during the leukemogenesis. As any model, these LCLs have to be analyzed ideally in conjunction with primary tissues.

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Table I. CLL lymphoblastoid cell line characteristics

Cell line	Patient age/sex/ Binet,Rai	Surface markers	<i>IGHV/D/J</i> ¹	<i>IGKV/J</i> <i>IGVL/J</i>	CDR3	BCR- ligands	FISH/ karyotype	STR/DNA- fingerprint	Authenticity	Details/Ref.
I83-E95	75/Male/ Binet A	IgM, λ CD5 ⁺ /CD19 ⁺	3-30/D3-16/J4-M	<i>IGLV3-3/J3</i>	CAKVGWFRGQF GGALGSFDYW	vimentin oxLDL	tetraploid, +12, 13q-, 17p-, t(2;11) also in patient's blood	I83-E95 = I83- LCL	<i>IGHV</i> : line = patient blood: <u>authentic</u>	Suppl A Ref[17]
I83-LCL	75/Male/ Binet A	IgM, λ /CD5 ⁺ /CD19 ⁺					normal	I83-LCL = I83-E95	<u>normal B cells</u> from patient	Suppl A/Ref[17]
WaC3CD5 ⁺	-/Male/ Rai I	IgM, κ /CD5 ⁺ /CD19 ⁺	<i>A</i> : 3-30.3/D3-22/J6- <i>UM-subset-32</i> (360bp) <i>B</i> : 3-30/D7-27/J2UM (310bp)	<i>IGKV3-20</i>	A. CARGPG DYYDSSGYWW NYYYYGMDVW subset-32 B. CAKGRPLTGYW	vimentin oxLDL PcC polys ¹	13q-, 17p-	WaC3CD5+ = Wa-osel	<i>IGHV</i> A and B present in patient blood: <u>authentic</u>	Suppl B/Ref[17]
Wa-osel	-/Male/ Rai I	IgM, κ /CD5 ⁺ /CD19 ⁺	<i>B</i> : 3-30/D7-27/J2UM (310bp)		B. CAKGRPLTGYW		tetraploid	Wa-osel = WaC3CD5+	<i>IGHV</i> B present in patient blood: <u>authentic</u>	Suppl B/Ref[17]
232B4	-/Male/ Rai 0	IgG, κ /CD5 ⁺ /CD19 ⁺	3-48/D3-10/J3 M (325bp) ²	<i>IGKV3-11</i>	CARDVGGLRASD LW	cofilin-1	+17, 11×4, 13×4, 12×4	232B4 = 232A4 = patient blood	<i>IGHV</i> : line = patient blood: <u>authentic</u>	Suppl C/Ref[17]
232A4	-/Male/ Rai 0	IgM ^{low} , κ /CD5 ⁺ /CD19 ⁺	1-46/D5-5/J6 M				normal	232A4 = 232B4= patient blood	normal B cell from patient	Suppl C/Ref[17]
HG3	70/Male/ Rai II	IgM, λ /CD5 ⁺ /CD19 ⁺	1-2/D6-13/J6 UM	<i>IGLV2-14</i>	CARASYAADYYY YGMDVW	oxLDL filamin B	13q- ×2		<i>IGHV</i> : line = patient blood: <u>authentic</u>	Suppl D/Ref[3]
MEC1 ³	58/Male/ Rai II	IgM, κ /CD5 ⁺ /CD19 ⁺	4-59/D2-21/J4 M		SQGVLTADY		12-, 17p- ⁴		<i>IGHV</i> : line = patient blood: <u>authentic</u> ⁴	Suppl E/Ref[32]
MEC2 ³	58/Male/ Rai II	IgM, κ /CD5 ⁺ /CD19 ⁺	4-59/D2-21/J4 M		SQGVLTADY		12-, 17p- ⁴		<i>IGHV</i> : line = patient blood: <u>authentic</u> ⁴	Suppl E/Ref[32]
CII	47/Female/ Rai II	IgM, λ /CD5 ⁺ /CD19 ⁺	1-69/D3-10/J6UM <i>subset-5</i>	<i>IGLV1-4</i>	CARDLVRGVIIIM GDYYYYYMDVW	PRAP-1 ¹	Trisomy/tetrasomy 12 , tetrasomy 11q/13q/17p	CII = CI	Stereotyped subset-5	Suppl F/Ref.[21]
CI	47/Female /Rai II	IgM, λ /CD5 ⁺ /CD19 ⁺	1-69/D3-10/J6UM <i>subset-5</i>		CARDLVRGVIIIM GDYYYYYMDVW	PRAP-1 ¹	11q-, +12	CI = CII	Stereotyped subset-5	Suppl F/Ref.[21]
PGA1	-/Male/-	IgG, λ /CD5 ^{dim} /CD19 ⁺	4-39/D4-17/J5M		CARRCGAYGDYP NWFPW		13q-, +12	PGA1 = PG/B95-8	<i>IGHV</i> : line = patient blood: <u>authentic</u> ⁴	Suppl G/Ref[22]
PG/B95-8	-/Male/-	IgM ^{low} , κ /CD5 ⁺ /CD19 ⁺	1-69/D2-2/J4				normal	PG/B95-8= PGA1	<u>normal B cells</u> from patient	Suppl G/Ref[22]
EHEB	69/Female/ -	IgG, κ	1-18M/5-12/J1		CARDDGGGKGD YGRLW		normal or +11		Inconcl, patient blood unavail.	Suppl H/Ref[23]
AI-60	54/Female/ -	IgM, κ	1-69/D2-2/J4 M					AI-60 = AII = AIII		Suppl I
AII	54/Female/ -		3-33/D3-16/J4 M					AI-60 = AII = AIII		Suppl I
AIII	54/Female/ -		4-34/D3-10/J4 M		CATVPGAW	nuclear antigen		AI-60 = AII = AIII		Suppl I

¹M, mutated; UM, unmutated; PcC, pneumococcal polysaccharides; PRAP-1, proline-rich acidic protein-1. ²Patient blood had two *IGHV3-48* UM rearrangements: one minor 310 bp and one dominating 325 bp long. ³MEC1 and MEC2 were established from a CLL patient that progressed into polymphocytic leukemia. ⁴Data from primary ref.[32]

Table II. Differential expression of CD38, ZAP70, and TCL1 in CLL LCL and LCL derived from normal B-cells

Cell Line	CD38 (% pos. cells / MFI fold-increase)		ZAP70 (% pos. cells / MFI fold-increase)		TCL1 (% pos. cells / MFI fold-increase)	TCL1 (Western blot / RT-PCR)
I83-E95	92%	31×	58%	/ 3×	82% / 5×	+
I83-LCL	78%	59×	13%	/ 2×	88% / 8×	+
WaC3CD5 ⁺	15%	2×	3%	/ 2×	37% / 3×	neg.
Wa-osel	<10%	1×	18%	/ 2×	49% / 6×	-/(+) [*]
232B4	<10%	1×	49%	/ 3×	76% / 5×	+
232A4	<10%	1×	36%	/ 3×	47% / 3×	(+)
CLL-HG3	89%	58×	44%	/ 4×	67% / 4×	(+)
MEC1	40%	7×	2%	/ 1×	71% / 5×	(+)
CII	69%	12×	83%	/ 6×	74% / 4×	+
CI	-	-	-	/ -	-	-
PGA1	25%	9×	1%	/ 1×	54% / 3×	-/(+) [#]
PG/B95-8	44%	6×	16%	/ 2×	20% / 2×	neg.
EHEB	-	-	-	/ -	-	-
AI-60	-	-	-	/ -	-	-
AII	97%	53×	25%	/ 3×	61% / 4×	++
AIII	29%	4×	30%	/ 2×	62% / 4×	+

% cells: categorization (see comments in Supplemental Figures) according to a 30% cut-off.

MFI: charted as ratio to isotype control and considered positive (see comments in Supplemental Figures) when > 2.5 fold for Zap70 and > 3.5 fold for TCL1.

Jurkat E6-1 cells show a Zap70 value of 7.3; TCL1 MFI was 2.0 for Jurkat E6-1 cells (negative control) and 25.0 for DoHH2-TCL1 (pos. ctrl.).

* categorized this way due to a TCL1-dim subpopulation by FACS and a weak signal in RT-PCR.

categorized this way based on a weak WB band.

Figure legends

Figure 1. *IGHV* gene rearrangement of CLL and normal B cell LCLs. (A) The I83-E95 cell line showed identical mutated *IGHV3-30/IGHD3-16/IGHJ4* gene rearrangement with the patient's blood cells. (B) The WaC3CD5⁺ cell line showed an unmutated *IGHV3-30/IGHD3-22/IGHJ6* gene rearrangement (325 bp long) and a minor unmutated *IGHV3-30/IGHD7-27/IGHJ2* gene rearrangement (310bp long). The Wa-osel cell line showed an unmutated *IGHV3-30/IGHD7-27/IGHJ2* gene rearrangement identical with Wa-patient blood cells. (C) The 232B4 cell line showed a mutated *IGHV3-48/IGHD3-10/IGHJ3* gene rearrangement also present in the patient blood cells. (D) The HG3 cell line showed an identical unmutated *IGHV1-2/IGHD6-13/IGHJ6* gene rearrangement with patient blood cells. (E) The MEC1 and MEC2 showed an identical mutated *IGHV4-59/IGHD2-21/IGHJ4* gene rearrangement as described for patient blood cells in the primary reference. (F) The CII and CI cell lines showed an identical unmutated *IGHV1-69/IGHD3-10/IGHJ6* gene rearrangement. A dot indicates homology with the top sequence.

Figure 2. Surface marker phenotype FACS analysis. The cell lines were analyzed for surface markers CD5, CD19, CD20, CD23, μ , λ , and κ by flow cytometry. The number of double positive cells is shown for each surface marker combination.

Figure 3. High resolution short tandem repeat (STR) analysis of neoplastic and normal pair CLL cell lines exemplified with the cell lines 232B4, 232A4 and patient's in vivo cells. Supplemental Figure 1 shows that all twin pair cell lines had identical STR profiles, thus considered to originate from the same patient.

Figure 4. *FMOD* and *CLLUI* expression in CLL cell-lines and normal B cell sister LCLs. (A) *FMOD* mRNA expression levels in CLL cell lines, CLL patient samples and healthy PBMC. Real-time PCR expression show that cell lines have low *FMOD* levels in comparison with primary CLL blood cells from 12 patients and 2 normal healthy persons PBMC. (B) *CLLUI* expression analyzed by real-time PCR. A pool of normal B lymphocytes (purified by CD19-positive selection of buffy coats from 10 healthy donors) were used as a calibrator, and *CLLUI* expression levels were expressed as fold up-regulation in relation to the normal B cells. The median *CLLUI* value (22.9-fold up-regulation above B cell levels) for a population

of 175 untreated CLL patients previously described is shown in the right side of the graph. LCL-3M and IARC-171 are two control LCLs derived from normal B cells.

Figure 5: *ZAP70* and *TCL1* expression in CLL and normal B LCLs. **(A)** Immunoblot analysis reveal a differential *ZAP70* expression across CLL cell lines ranging from absent (i.e. MEC1) to high-level expression (i.e. CII) being in agreement with flow cytometry expression shown below in conjunction with Jurkat E6-1 cells, as positive controls. **(B)** RT-PCR data illustrate the variable *TCL1* expression levels across the CLL-LCL and LCL cell lines that correlate with the Western blot and flow cytometry data (not shown). DoHH2 B cells and their stably transfected *TCL1*-transfected sublines DoHH2-*TCL1* served as controls.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5A

Figure 5B

B

Supplemental Tables and Figure 1.

Supplemental Table A. Detailed characteristics of CLL-derived lymphoblastoid cell lines

CLL cell line Sister LCL cell line	I83-E95 I83-LCL
Clinical data	71-year-old Caucasian male
Patient data:	Phenotype at diagnosis: CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD21 ⁺ , CD22 ⁺ , CD23 ⁺ , CD24 ⁻ , CD25 ⁺ , CD32 ⁻ , CD37 ⁺ , CD38 ⁺ , CD39 ⁺ , CD40 ⁺ , CD45 ⁻ , HLA-DR ⁺ , CD54 ⁺ , CD2 ⁻ , CD3 ⁻ , CD10 ⁻ phenotype, whereas the I83-LCL cell line had a CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD21 ⁺ , CD22 ⁺ , CD23 ⁺ , CD24 ⁻ , CD25 ⁻ , CD32 ⁻ , CD37 ⁺ , CD38 ⁺ , CD39 ⁺ , CD40 ⁺ , CD45 ⁻ , HLA-DR ⁺ , CD54 ⁺ phenotype. Conclusions based on RFLP pattern, immunophenotype, and chromosomal markers have proven that I83-E95 was of CLL and I83-LCL of normal B-cell origin.
Disease status:	at diagnosis (Binet stage A)
Source of material:	peripheral blood
Year of establishment:	1994
Availability of cell line:	A.R. and DSMZ GmbH pending
<u>CLL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV3-30*03/IGLV3-3</i> (identical rearrangement with patient <i>in vivo</i> CLL cells)
<i>IGHV/IGLV</i> homology (%)	96.9/99.6
<i>IGHD/IGHJ</i> usage	<i>IGHD3-16*01/IGHJ4*03</i>
FISH data	trisomy/tetrasomy 12 + extra copies 13q/17q
DNA STR analysis	Identical origin with I83-LCL sister cell line
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ^+ , λ^+
<u>LCL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	nt
<i>IGHV/IGLV</i> homology (%)	nt
<i>IGHD/IGHJ</i> usage	nt
FISH data	Normal
DNA STR analysis	Identical origin with I83-E95
Phenotype data	CD5 ⁺ /CD19 ⁺ /CD20 ⁺ /CD23 ⁺ /IgM ⁺ λ^+ / CD38 ⁺ /TCL1 ⁺ /ZAP70 ⁻ Ref. Wendel-Hansen V <i>et al.</i> Leukemia, 1994[5]
CLL cell line characteristics as defined by primary reference	
Karyotype data	tetraploid karyotype, reciprocal t(2;11)
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , among others
Other markers	IGHJ-RFLP pattern identical with the <i>in vivo</i> CLL clone using <i>Hind III</i> and <i>Bg/II</i>
LCL cell line characteristics as defined by primary reference [5]	
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ^{low} , CD23 ⁺ , among others
Other markers	IGHJ-RFLP pattern not identical with the <i>in vivo</i> CLL clone using <i>Hind III</i> and <i>Bg/II</i>

Comments:

I83-E95 and I83-LCL. I83-E95 had identical *IGHJ*-RFLP pattern (using *Hind III* and *Bg/II*) with the *ex vivo* patient CLL clone, whereas the I83-LCL did not.[5] I83-E95 had a tetraploid karyotype carrying a reciprocal translocation t(2;11), also found in patient's blood B cells.[5] Our present gene analysis revealed identical *IGHV3-30/IGHD3-16/IGHJ4* and *IGLV3-3/IGLJ3* gene rearrangements with 96.9 % and 99.6% identity to the corresponding germline genes, respectively, in I83-E95 and in *ex vivo* patient cells (Table 1, Fig. 1). I83-E95 CLL and the I83-LCL had identical STR-fragment profiles, proving their derivation from the same individual (Supplemental Fig. 1). The immunophenotype of I83-E95 was CD5⁺/CD19⁺/CD20⁺/CD23⁺/IgM⁺ λ^+ (Fig. 3), and CD38⁺/TCL1⁺/ZAP70^{dim}, whereas I83-LCL was CD5⁺/CD19⁺/CD20⁺/CD23⁺/IgM⁺ λ^+ /CD38⁺/TCL1⁺/ZAP70⁻ (Table 2). We conclude that I83-E95 represents the authentic neoplastic CLL clone and that I83-LCL was derived from the patient's normal B cells.

Supplemental Table B

CLL cell line Sister LCL cell line:	WaC3CD5 ⁺ Wa-osel
Clinical data	
Patient data:	Male
Disease status:	at diagnosis (Rai stage I)
Source of material:	peripheral blood
Year of establishment:	1994
Availability of cell line:	A.R. and DSMZ GmbH pending
<u>WaC3CD5⁺ characteristics</u>	
<u>IGHVDJ/IGLV gene</u>	A) <i>IGHV3-30-3*01/IGHD3-22*01/IGHJ6*01</i> and <i>IGKV3-20*01</i> (<i>IGHV</i> rearrangement homologous with HCDR3 subset 32 defined by Murray et al, 2007[6])
	B) <i>IGHV3-30*03/IGHD7-27*01/IGHJ2*01</i>
<i>IGHV/IGLV</i> homology (%)	A) 100/100 B) 100
FISH data	del(13q), del(17p)
DNA STR analysis	Identical origin with Wa-osel LCL sister cell line and Wa-patient <i>in vivo</i> CLL cells
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , CD10 ⁻ , CDw45 ⁻ , CD54 ⁺ (ICAM-1 ⁺), μ^+ , κ^+
<u>Wa-osel characteristics</u>	
<u>IGHV/D/J gene</u>	<i>IGHV3-30*03/NT*/IGHD7-27*01/IGHJ2*01</i>
<i>IGHV/IGLV</i> homology (%)	100/NT
FISH data	tetrasomy 17p-, 11q-, +12, extra copies 13q-
DNA STR analysis	Identical origin with WaC3CD5 ⁺ cell line and Wa-patient <i>in vivo</i> CLL cells
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ^+ , κ^+
CLL cell line characteristics as defined by primary reference	Ref. Wendel-Hansen V <i>et al.</i> Leukemia, 1994[5]
Karyotype data	46,XY,del(13q)
Phenotype data	CD5 ⁺ , CD23 ⁺ , μ^+ , λ^+
LCL cell line characteristics as defined by primary reference [5]	
Karyotype data	Normal LCL
Phenotype data	CD5 ⁻

Comments:

WaC3CD5⁺ and Wa-osel. WaC3CD5⁺ was established by EBV-infection of B cells isolated by anti-CD5 magnetic beads, while the Wa-osel was established from PBMC. The WaC3CD5⁺ cell line had a 46,XY,del(13)(q12q14) karyotype identical to the patient's CLL clone. Presently, we found that the WaC3CD5⁺ line had two functional *IGHV* rearrangements; one dominant, unmutated *IGHV3-30-3/IGHD3-22/IGHJ6* gene rearrangement (360 bp long) (Fig. 1) with a HCDR3 sequence, CARGPGDYDSSGYYWWNYYYGMDVW, homologous to the stereotyped CLL subset-32, and a minor unmutated *IGHV3-30/IGHD7-27/IGHJ2* gene rearrangement (310 bp long) with another HCDR3 sequence, CAKGRPLTGYW. The latter rearrangement was present in *ex vivo* CLL cells and also in the Wa-osel cell line. Interestingly, at the time of establishment, the WaC3CD5⁺ cell line had the dominant *IGHV3-30-3/IGHD3-22/IGHJ6* rearrangement only (frozen samples early after establishment), but after a culturing period, the minor *IGHV3-30/IGHD7-27/IGHJ2* gene rearrangement could also be detected. FISH data showed deletions of chromosome 13q and chromosome 17p (Table 1). The Wa-osel cell line had a tetraploid karyotype (Table 1). DNA/STR-fingerprinting showed that WaC3CD5⁺ and Wa-osel were derived from the same person (Supplemental Fig. 1). WaC3CD5⁺ was CD5⁺ and Wa-osel CD5⁻ (Fig. 3). Both lines lack CD38, ZAP70 and TCL1 (Table 2), however, a TCL1^{dim} subpopulation was detected in flow cytometry in Wa-osel. We conclude that WaC3CD5⁺ and Wa-osel are authentic CLL neoplastic clones. The WaC3CD5⁺ line is biclonal due to either a lack of allelic exclusion as reported previously,[2,3] or more likely, presence of two separate monoclonal CLL populations,[4] since the rearranged *IGHV* genes were of different size. The Wa-osel cell line is monoclonal.

Supplemental Table C

CLL cell line	232B4
Sister LCL cell line	232A4
<u>Clinical data</u>	
Patient data:	Male
Disease status:	at diagnosis (Rai stage 0)
Source of material:	peripheral blood
Year of establishment:	1994
Availability of cell line:	A.R. and DSMZ GmbH pending
<u>CLL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV3-48*01/IGKV3-11*01</i> (325bp)
<i>IGHV/IGLV</i> homology (%)	90.8/95.8
<i>IGHD/IGHJ</i> usage	<i>IGHD3-10*01/IGHJ3*01</i>
FISH data	trisomy 17p, tetrasomy 11q/13q/12
DNA STR analysis	Identical origin with sister LCL cell line 232A4
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , γ ⁺ , κ ⁺
<u>LCL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV1-46*01/NT*</i>
<i>IGHV/IGLV</i> homology (%)	94.1/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD5-5*01/IGHJ6*01</i>
FISH data	Normal LCL
DNA STR analysis	Identical origin with CLL cell line 232B4
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ ⁻ , κ ⁺
CLL cell line characteristics as defined by primary reference	Ref. Wendel-Hansen V <i>et al.</i> Leukemia, 1994[5]
Karyotype data	47,XY,+12
Phenotype data	μ ⁺ , κ ⁺
Other markers	Identical PCR-SSCP pattern with the <i>in vivo</i> CLL clone
LCL cell line characteristics as defined by primary reference [5]	
Karyotype data	Normal LCL
Phenotype data	Undefined
Other markers	Unrelated PCR-SSCP pattern with the <i>in vivo</i> CLL clone

Comments:

232B4 and 232A4. 232B4 cell line had identical clonality pattern in *IG* analysis (PCR-SSCP pattern) with the patient's *in vivo* CLL clone. It showed a trisomy 12 karyotype and had a CD5⁺/CD23⁺/ μ ⁺/ κ ⁺ phenotype. 232A4 cell line, in contrast, was karyotypically normal, CD5-negative, and had an unrelated PCR-SSCP pattern with the *in vivo* CLL clone. Here we reconfirmed the authenticity by *IGHV* sequencing of patient blood B-cells which showed a biclonal appearance with two *IGHV3-48* rearrangements, one unmutated of 310 bp and one dominating mutated of 325 bp length. The 232B4 line showed an identical *IGHV3-48/IGHD3-10/IGHJ3* rearrangement with the 325 bp rearrangement found in patient's peripheral blood (Fig. 1). The sister cell line 232A4 had an unrelated and mutated *IGHV1-46/IGHD5-5/IGHJ6* gene rearrangement, and showed a normal karyotype as described by primary reference. STR-analysis revealed identical origin of 232B4 and 232A4 cell lines (Fig. 2). The surface membrane phenotype of the 232B4 cell line was CD5⁺/CD19⁺/CD20⁺/CD23⁺/IgG⁺/ κ ⁺ whereas 232A4 were CD5⁺/CD19⁺/CD20⁺/CD23⁺/ μ ⁻/ κ ⁺ (Fig. 3). Both lines were CD38-negative, but showed ZAP70 and TCL1 expression, which tended to be higher in the 232B4 CLL line than in the 232A4 LCL (Table 2). We conclude that the 232B4 cell line is authentic with one of the CLL patient leukemic clones and confirm that the 232A4 cell line was of normal LCL origin.

Supplemental Table D

CLL Cell line	HG3
<u>Clinical data</u>	
Patient data:	70-year-old male
Disease status:	at diagnosis
Source of material:	peripheral blood
Year of establishment:	1998
Availability of cell line:	A.R., E.K. and DSMZ GmbH pending
CLL cell line characteristics	
<i>IGHV/IGLV</i> gene	<i>IGHV1-2*04/IGLV2-14</i> (identical rearrangement with patient <i>in vivo</i> CLL cells)
<i>IGHV/IGLV</i> homology (%)	100/100
<i>IGHD/IGHJ</i> usage	<i>IGHD6-13*01/IGHJ6*01</i>
FISH data	biallelic del(13q)
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ ⁺ , λ ⁺
CLL cell line characteristics as defined by primary reference	Ref. Rosén et al. OncoImmunology, 2012[7]
Karyotype data	46,XY,del(13q)
Phenotype data	CD5 ⁺ , CD14 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ ⁺ , λ ⁺
Other markers	miR15 and miR16 loss

Comments:

HG3. The cell line was CD5⁺, CD19⁺, CD20⁺, CD23⁺, CD14⁺, μ ⁺, and λ ⁺. The authenticity of HG3 was confirmed by *IG* gene sequence analysis showing that the *IGHV1-2/IGHD6-13/IGHJ6* and *IGLV2-14* gene rearrangements expressed by HG3 were identical to the patient's malignant *in vivo* CLL clone (Fig. 1). The *IGHV* and *IGLV* rearrangement showed 100% identity to germline genes. FISH analysis revealed a biallelic del(13q) (Table 1). Flow cytometry analysis of HG3 showed a CD5⁺, CD19⁺, CD20⁺, CD23⁺, CD27⁺, μ ⁺, λ ⁺ profile (Fig. 3). The cells were CD38 and ZAP70 positive and showed low-level TCL1 expression (Table 2). We conclude that HG3 CLL line is of authentic CLL origin.

Supplemental Table E

CLL in polymphocytoid transformation cell line	MEC1 and MEC2
<u>Clinical data</u>	
Patient data:	58-year-old Caucasian male
Disease status:	at diagnosis (Rai stage II), progressed into polymphocytic leukaemia
Source of material:	peripheral blood
Year of establishment:	MEC1 in 1993 and MEC2 in 1994
Availability of cell line:	DSMZ GmbH, K.N. Uppsala and E.K. Stockholm
MEC-1 and MEC-2 cell line characteristics	
IGHV/IGKV gene	IGHV4-59*01/NT
IGHV/IGKV homology (%)	94.1/NT
IGHD/IGHJ usage	IGHD2-21*02/IGHJ4*02
FISH data	NT
DNA STR analysis	NT
Phenotype data	NT
MEC-1 cell line characteristics as defined by primary reference	Ref. Staccini A <i>et al.</i> Leuk Res, 1999[1]
IGHV gene	IGHV4-59
IGHV homology (%)	94.8
Karyotype data	46,XY,del(12),del(17p), among others
Phenotype data	CD5 ⁻ , FMC7 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , δ ⁺ , μ ⁺ , κ ⁺ , among others
Other markers	Identical IGHV rearrangement with patient <i>in vivo</i> CLL clone
MEC-2 cell line characteristics as defined by primary reference [1]	
IGHV gene	IGHV4-59
IGHV homology (%)	94.8
Karyotype data	46,XY, + inv(12),del(17p), among others
Phenotype data	CD5 ⁻ , FMC7 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , δ ⁺ , μ ⁺ , κ ⁺ , among others
Other markers	Identical IGHV rearrangement with patient <i>in vivo</i> CLL clone

Comments:

MEC1 and MEC2 cell lines were previously established by Caligaris-Cappio and co-workers from a 58-year-old male diagnosed with CLL in polymphocytoid transformation.[1] The cell lines were established by spontaneous outgrowth at two subsequent occasions. They showed identical mutated *IGHV4-59* rearrangement with the patient's leukemic clone. CD5 expression was lost in both cell lines upon continued cell culturing. The karyotypes of the cell lines were abnormal with multiple aberrations, and cytogenetic studies on the parental cells were unsuccessful. We reconfirmed that both MEC1 and MEC2 cell lines had identical *IGHV4-59/IGHD2-21/IGHJ4* gene rearrangement as described in the primary reference.⁴² MEC1 cells lacked CD5 and only a dim expression was noted for CD38 and TCL1, while ZAP70 was completely absent (Table 2). We conclude that authenticity for both cell lines was certified (Fig. 1).

Supplemental Table F

CLL cell line Sister LCL cell line:	CII (Corina II) CI (Corina I)
<u>Clinical data</u>	
Patient data:	47-year-old female
Disease status:	at diagnosis (lymphadenopathy, hepatosplenomegaly, a white-blood-cell count of 152000/mm ³ with 88% lymphocytes, and increased lymphocytes in the marrow)
Source of material:	peripheral blood
Year of establishment:	1980
<u>CLL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV1-69*01/IGLV1-4</i> (<i>IGHV</i> rearrangement homologous with <u>HCDR3 stereotyped subset 5</u> defined by Murray et al. 2007 [6])
<i>IGHV/IGLV</i> homology (%)	99.5/99.6
<i>IGHD/IGHJ</i> usage	<i>IGHD3-10*01/IGHJ6*03</i>
FISH data	trisomy/tetrasomy 12 + tetrasomy 11q/13q/17p
DNA STR analysis	Identical origin with CI
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ^+ , λ^+
<u>LCL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV1-69*01/ NT*</i>
<i>IGHV/IGLV</i> homology (%)	99.5/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD3-10*01/IGHJ6*03</i>
FISH data	Trisomy 12 + del (11q)
DNA STR analysis	Identical origin with CII
Phenotype data	CD5 ⁺ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ^+ , λ^+
CLL cell line characteristics as defined by primary reference	Ref. Fialkow PJ <i>et al.</i> Lancet, 1978; Karande A <i>et al.</i> Int J Cancer, 1980; Najfeld V <i>et al.</i> Int J Cancer, 1980[8,9]
Karyotype data	47,XX,+12 or 47,XX,+inv(11)(p15.3;q22),+del(12)(q12 or q21),t(12;15)(q15 or q21;p13)
Phenotype data	μ^+ , λ^+
Other markers	A-type G6PD, the same as the <i>in vivo</i> CLL clone. Malic enzyme activity very weak or absent
LCL cell line characteristics as defined by primary reference [8,10]	
Karyotype data	46,XX
Phenotype data	μ^+ , κ^+
Other markers	B-type G6PD, not expressed by the <i>in vivo</i> CLL clone. Malic enzyme activity high

Comments:

CI and CII. Originally, the CII cell line contained two subpopulations, one with a 47,XX,+12 karyotype and a second population with a 47,XX, inv(11),+del(12) t(12;15) karyotype. The patient's normal tissue (skin and red blood cells) expressed both type-A and type-B of glucose-6-phosphate dehydrogenase (G6PD), whereas the CII cell line expressed exclusively type-A G6PD pattern, which was identical to the patient's peripheral blood CLL clone. CII and the patient's clone were "monoclonal" Ig producers with an IgM, I phenotype. The CI cell line was karyotypically normal, expressed IgM, κ , and type-B G6PD, thus Karande *et al.* assumed that CI were derived from non-leukemic normal B cells.[8] Based on G6PD, surface IgM, κ or I, chromosome markers, Fc-receptors and malic enzyme, the authors concluded that CII was of leukemic and CI of normal B cell origin. In our extended molecular characterization, we found that the CII cell line had unmutated *IGHV1-69/IGHD3-10/IGHJ6* and *IGLV1-4/IGLJ3* gene rearrangements (Fig. 1). The HCDR3 sequence of this cell line showed homology with the 'stereotyped' CLL subset-5, described

by Murray et al.[6] FISH analysis of the cell line revealed a trisomy/tetrasomy 12, in line with the primary report (Table 1). The CI cell line had identical *IGHV* gene rearrangements with the CII cell line, but noteworthy, the FISH results were not identical. The CII and CI cell lines both displayed a CD5⁺, CD19⁺, CD20⁺, CD23⁺, μ ⁺, λ ⁺ surface marker profile (Fig. 3). CII cells showed low CD38 density in the majority of cells and were characterized by high ZAP70 and TCL1 levels (Table 2). In view of the presence of a stereotyped HCDR3, which constitutes an almost unique phenomenon for CLL (scarcely described in other B cell neoplasias), a subset membership is thus an important piece of evidence for true CLL origin. Hence, the HCDR3 homology with subset-5 underscores an authentic CLL origin of the CII cell line. The trisomy/tetrasomy 12 revealed by FISH is consistent with primary reference, with trisomy 12 being one of the most common recurrent aberrations observed in patients with CLL,[11] although it is not specific for this disease. *IG* gene sequence alignment with patient *in vivo* CLL clones was not possible, since DNA from original patient's blood samples was not available. The CI (normal) cell line was collected from different laboratories (e.g. Uppsala University, Karolinska Institute, and Linköping University). All CI cells showed *IGHV1-69*/subset-5 rearrangements, identical with the CII cell line. These data together with the CD5⁺/CD19⁺ phenotype strongly indicates that this cell line early in the establishment history of the cell line was cross-contaminated with the neoplastic CII clone, alternatively, CI, which originally was described as normal, was overgrown by the neoplastic clone (based on the assumption that the original culture contained a few neoplastic cells along with normal B cells). We conclude that expression of CLL signature markers (identical to the *in vivo* clone), e.g. trisomy 12 (also in the *in vivo* clone), and a BCR belonging to the stereotyped subset-5, are strong indicators of an authentic CLL origin of both CI and CII.

Supplemental Table G

CLL Cell line	PGA1
Sister LCL cell line	PG/B95-8 (PG/EBV)
<u>Clinical data</u>	
Patient data:	Not available
Disease status:	at diagnosis
Source of material:	peripheral blood
Year of establishment:	1988
Availability of cell line:	A.R. E.K. and DSMZ GmbH pending
<u>CLL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV4-39*01/NT</i>
<i>IGHV/IGLV</i> homology (%)	91.8/NT
<i>IGHD/IGHJ</i> usage	<i>IGHD4-17*01/IGHJ5*02</i>
FISH data	trisomy 12 + del(13q)
DNA STR analysis	Identical origin with sister PG/B95-8 LCL cell line
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , γ ⁺ , λ ⁺
<u>LCL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV1-69*01/NT*</i>
<i>IGHV/IGLV</i> homology (%)	93.3/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD2-2*01/IGHJ4*02</i>
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ ⁻ , κ ⁺
FISH data	NT
Microsatellite data	Identical origin with PGA1 cell line
CLL cell line characteristics as defined by primary reference	Ref. Lewin N <i>et al.</i> Int J Cancer, 1988; Lewin N <i>et al.</i> Int J Cancer, 1991[12,13]
Karyotype data	47,XY,+12,+r(15)
Phenotype data	CD20 ⁺ , CD23 ⁺ , CD45 ⁺
Other markers	Rearranged JH pattern at 5.8 and 4.2 kb
LCL cell line characteristics as defined by primary reference [12,13]	
Karyotype data	46,XY
Phenotype data	CD20 ⁺ , CD23 ⁺ , CD45 ⁺
Other markers	No rearranged JH pattern

Comments:

PGA1 and PG/B95-8. cell lines were established from a male CLL patient.[12,13] Interestingly, the PGA1 cell line represents an unusual phenomenon of an *in vivo* EBV-infected CLL clone. The clone directly grew out *in vitro* without addition of the transforming B95-8 virus. PGA1 cell line and patient's blood CLL cells showed trisomy 12 and a ring chromosome 15 in 18/30 metaphases in the patient's blood and in all 53 metaphases in PGA1. Upon prolonged culturing, the ring chromosome 15 was lost in PGA1 (N. Lewin, personal communication). The cell line showed the same clonal *IGH* rearrangement detected by an *IGHJ* probe with the *in vivo* clone and had a CD20⁺, CD23⁺ and CD45⁺ phenotype. The PG/B95-8 cell line was derived after an *in vitro* EBV-transformation of blood cells by B95-8 virus. PG/B95-8 was karyotypically normal, had a CD20⁺, CD23⁺ and CD45⁺ phenotype with a different *IGH* rearrangement as compared with the patient's CLL clone. The authors concluded that their data provided incontrovertible evidence that the directly outgrowing cell line PGA1 represented the leukemia cell *in vivo*. In this study we found that the PGA1 cell line showed a mutated *IGHV4-39/IGHD4-17/IGHJ5* gene rearrangement with 91.8 % identity to germline (Fig.1). FISH analysis showed trisomy 12 and del(13q) (Table 1). STR-analysis revealed identity between PGA1 and its sister PG/B95-8 LCL cell line (Supplemental Fig. 1). The PG/B95-8 LCL cell line showed a mutated *IGHV1-69/IGHD2-2/IGHJ4* gene rearrangement with 93.3 % identity to germline (Fig. 1). Immunophenotype characterization showed a CD5⁻, CD19⁺, CD20⁺, CD23⁺, γ ⁺, λ ⁺ surface profile of the neoplastic PGA1 CLL cell line (Fig. 3). The PG/B95-8 normal LCL cell line had a CD5⁻, CD19⁺, CD20⁺, CD23⁺, μ ⁻, κ ⁺ phenotype (Fig. 3). Overall, both lines lacked unequivocally 'positive' CD38 and

TCL1 expression. However, for both markers a tendency of slightly higher levels in the PGA1 CLL line than in the PG/B95-8 normal LCL cells was noted. ZAP70 was absent in both lines (Table 2). CD5 expression in CLL cells *per se* does not prove neoplastic origin, since CD5 could be a marker of activated normal B cells as well. PG cells were infected *in vivo* by EBV and the CD5-downregulation observed for PGA1 cell line may be a result of the EBV-infection. The PGA1 cell line showed IgG expression, which is less common than IgM in CLL. The trisomy 12 identified by FISH confirms the original reference describing trisomy 12 and ring rearrangement of chromosome 15.[12] The loss of ring chromosome 15 observed by G-banding (data not shown) was also observed by the authors of the original report. DNA from the patient blood cells was not available for the present study, thus *IG* gene sequencing was not possible from the patient's CLL cells. The PG/B95-8 LCL sister cell line had a κ^+ surface immunophenotype and a mutated *IGHV1-69*01/IGHD2/IGHJ 4* gene rearrangement that differed from the *IG* gene rearrangement and λ^+ phenotype expressed by PGA1. We conclude that PGA1, carrying trisomy 12, and an IGHJ-RFLP pattern identical to the patient's clone, represents the clinical leukemic clone, as previously described,[12,13] and that PG/B95-8 sister cell line represents a normal LCL B cell line from the PG patient.

Supplemental Table H

CLL Cell line	EHEB
<u>Clinical data</u>	
Patient data:	69-year-old female
Disease status:	at diagnosis (adenopathy, hepatosplenomegaly, a white-blood-cell count of $580 \times 10^6/\text{ml}$, increased lymphocytes in the marrow)
Source of material:	peripheral blood
Year of establishment:	1990
Availability of cell line:	DSMZ and Dr. K. Guy
<u>CLL cell line characteristics</u>	
IGHV/IGLV gene	IGHV1-18*01/NT*
IGHV/IGLV homology (%)	82.8/NT*
IGHD/IGHJ usage	IGHD5-12*01/IGHJ1*01
FISH data	trisomy 11q
Phenotype data	CD5 ⁻ , CD19 ⁺ , CD20 ⁺ , CD23 ⁺ , μ^- , γ^+ , κ^+
CLL cell line characteristics as defined by primary reference	Ref. Saltman D <i>et al.</i> Leuk Res, 1990[14]
Phenotype data	CD5 ⁺ , CD19 ⁺ , μ^{low} , cytoplasmic κ^+
Karyotype data	46,XX
Other markers	Identical <i>IGHV</i> and <i>IGKV</i> rearrangement of patient <i>in vivo</i> CLL cells and CLL cell line, shown by southern blot analysis using restriction enzymes <i>Bgl II</i> , <i>Pst I</i> , <i>Xba I</i> , <i>SacI</i> , <i>Bam HI</i> and <i>EcoRI</i>

Comments:

EHEB cell line was established from a 69-year-old female diagnosed with CLL in Rai stage II.[14] It showed identical *IGH* and *IGL* rearrangements with the patient's *in vivo* CLL cells, as shown by Southern blot analysis using restriction enzymes *BglIII*, *PstI*, *XbaI*, *SacI*, *BamHI* and *EcoRI* with an *IGHJ* probe, *Ck* probe and *Cλ* probe. It was karyotypically normal after one year in culture (originally the authors found a chromosome 11q aberration) and had a CD5⁺, CD19⁺, μ^{low} , cytoplasmic κ^+ phenotype. Based on the identical gene rearrangements in the patient's blood and EHEB cell line, the authors concluded that the cell line was derived from the neoplastic clone. We found that the EHEB CLL cell line had a mutated *IGHV1-18/IGHD5-12/IGHJ1* gene rearrangement with 82.8% identity to germline (Fig. 1). FISH analysis showed extra copies of 11q (Table 1) and FACS analysis revealed a CD5⁻, CD19⁺, CD20⁺, CD23⁺, μ^- , κ^+ surface phenotype (Fig. 3 and Supplemental Table G). We could not conclusively re-confirm/verify a neoplastic origin of the EHEB cell line in this study due to loss of CD5 expression, lack of CLL markers, and inaccessibility to patient's blood cells.

Supplemental Table I

CLL Cell line	AI-60
Sister LCL cell line:	All and AIII
<u>Clinical data</u>	
Patient data:	54-year-old female
Disease status:	at diagnosis (a white-blood-cell count of 495,000/mm ³ with 99% lymphocytes, thrombocytopenia)
Source of material:	peripheral blood
Year of establishment:	1980
Availability of cell line:	E.K. and DSMZ GmbH pending
<u>CLL cell line characteristics</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV1-69/NT*</i>
<i>IGHV/IGLV</i> homology (%)	87.7%/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD2-2*01/IGHJ4*02</i>
FISH data	NT*
DNA STR analysis	Identical origin with sister All and AIII LCL cell lines
Phenotype data	NT*
<u>LCL cell line characteristics (All)</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV3-33*01/NT*</i>
<i>IGHV/IGLV</i> homology (%)	95.1%/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD3-16*01/IGHJ4*02</i>
FISH data	NT*
DNA STR analysis	Identical origin with CLL cell line AI-60 and AIII LCL cell line
Phenotype data	NT*
<u>LCL cell line characteristics (AIII)</u>	
<i>IGHV/IGLV</i> gene	<i>IGHV4-34/NT*</i>
<i>IGHV/IGLV</i> homology (%)	86.1%/NT*
<i>IGHD/IGHJ</i> usage	<i>IGHD3-10*01/IGHJ4*02</i>
FISH data	Identical origin with CLL cell line AI-60 and All LCL cell line
DNA STR analysis	NT*
Phenotype data	
CLL cell line characteristics as defined by primary reference	Ref. Najfeld V <i>et al.</i> Int J Cancer, 1980[10]
Karyotype data	45,X,-X
Phenotype data	κ ⁺
Other markers	Typ-A G6PD, the same as the <i>in vivo</i> CLL clone.
LCL cell lines characteristics as defined by primary reference [10]	
Karyotype data	Initially abnormal karyotype, but eventually normal
Phenotype data	Initially μ ⁺ , κ ⁺ but finally γ ⁺ and both κ ⁺ , λ ⁺
Other markers	Initially A-type G6PD, but eventually B-type G6PD only

Comments.

AI-60, All, and AIII cell lines were established from a 54-year-old female CLL patient.[10] The AI-60 cell line had monospecific kappa-light chain expression, a 45,X,-X karyotype and showed only A-type G6PD, which was identical to the neoplastic blood cell expression (IgM, kappa, G6PD-A;45,X-X). Patient's cultured skin fibroblasts showed a normal 46,XX karyotype, excluding Turner's syndrome. The All and AIII cell lines showed leukemic origin on initial examination, but the cultures rapidly evolved and were overgrown by normal cells during the first year of establishment. All and AIII expressed after one year type B G6PD only, a normal karyotype, and switched from IgM to IgG, κ⁺ light chains. We performed *IG* gene analysis of AI-60, which showed a mutated *IGHV1-69/IGHD2-2/IGHJ4* rearrangement. All had a mutated *IGHV3-33/IGHD3-16/IGHJ4* rearrangement, whereas AIII carried a mutated *IGHV4-34/IGHD3-10/IGDJ4* rearrangement. STR-analysis showed unequivocal identity between AI-60, All and AIII (Supplemental Fig,1). All and AIII were tested CD5-negative. All showed strong CD38 and TCL1

expression, while AIII was scored CD38-negative and with TCL1-positive protein levels lower than in AII (Table 2). Both lines were assigned a weak-positive ZAP70 score. We conclude that these three CLL cell lines established in 1979 have been used extensively for many years as a CLL leukemic prototype (AI-60) and normal sister cell lines (AII and AIII). Collectively these markers provided evidence that the EBV transformed cells were derived from the neoplastic clone. In the present study, we lacked patient's blood cells. Thus, we could not with certainty verify a neoplastic origin and due to retarded growth of AI-60, we could not perform further immunophenotyping and karyotyping.

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Supplemental Figure 1. High resolution short tandem repeat (STR) analysis of neoplastic and normal matched CLL cell lines.

A) I83-E95 and I83-LCL

B) Wa-patient, WaC3CD5+ and Wa-osel

C) CI and CII

Wa-osei

D) PGA1 and PG/EBV

E) A1-60, All, and AllI