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IgV gene intraclonal diversification and clonal evolution in B-cell chronic lymphocytic leukaemia

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Summary

Intraclonal diversification of immunoglobulin (Ig) variable (V) genes was evaluated in leukaemic cells from a B-cell chronic lymphocytic leukaemia (B-CLL) case over a 2-year period at four time points. Intraclonal heterogeneity was analysed by sequencing 305 molecular clones derived from polymerase chain reaction amplification of B-CLL cell IgV heavy (H) and light (C) chain gene rearrangements. Sequences were compared with evaluating intraclonal variation and the nature of somatic mutations. Although IgV intraclonal variation was detected at all time points, its level decreased with time and a parallel emergence of two more represented V_HDJ_H clones was observed. They differed by nine nucleotide substitutions one of which only caused a conservative replacement aminoacid change. In addition, one V_LJ_L rearrangement became more represented over time. Analyses of somatic mutations suggest antigen selection and impairment of negative selection of neoplastic cells. In addition, a genealogical tree representing a model of clonal evolution of the neoplastic cells was created. It is of note that, during the period of study, the patient showed clinical progression of disease. We conclude that antigen stimulation and somatic hypermutation may participate in disease progression through the selection and expansion of neoplastic subclone(s).

Keywords: B-cell chronic lymphocytic leukaemia, somatic hypermutation, immunoglobulin variable region, antigen stimulation, clonal evolution.

B-cell chronic lymphocytic leukaemia (B-CLL) is a clonal expansion of $CD5^+$ B lymphocytes that accumulate in peripheral blood and lymphoid organs (Chiorazzi *et al*, 2005). Approximately, 50% of B-CLL cases originate from lymphocytes undergoing somatic diversification of the immunoglobulin (Ig) variable (V) gene (Schroeder & Dighiero, 1994; Fais *et al*, 1998). Importantly, the presence of *IgV* gene somatic mutations predicts a disease with better evolution in comparison with cases bearing unmutated IgV regions (Damle *et al*, 1999; Hamblin *et al*, 1999). At variance from other B-cell tumours (Jain *et al*, 1994; Chapman *et al*, 1996; Matolcsy *et al*, 1999), the B-CLL clone was considered to be incapable of accumulating somatic IgV mutations because of the lack of intraclonal diversification (Schettino *et al*, 1998). This corresponded to the idea that B-CLL was derived from pre- or postgerminal centre B cells. However, this notion has been challenged by a report describing a variable but significant degree of intraclonal IgV somatic diversification in 11/18 B-CLL cases (Gurrieri *et al*, 2002). This finding has been subsequently confirmed (Ruzickova *et al*, 2002; Degan *et al*, 2004).

A role for antigen selection in the pathogenesis of B-CLL has been suggested. Analysis of *IgV* gene rearrangements in B-CLL cells frequently identifies B-cell receptors (BCR) with nearly identical IgV regions (Tobin *et al*, 2003; Ghiotto *et al*, 2004; Widhopf *et al*, 2004). However, antigen selection is thought to play a role in early disease phases by stimulating and expanding B cells with certain BCR structures. As it appears that in many instances B-CLL cells recognise autoantigens (Sthoeger *et al*, 1989; Herve *et al*, 2005), it is likely that antigen stimulation of the leukaemic clone also persists in advanced disease stages. This is supported by the finding that B-CLL with cells capable of better Ig signalling bears a worse

prognosis (Crespo *et al*, 2003; Petlickovski *et al*, 2005; Scielzo *et al*, 2005).

Here, we report on a B-CLL case with prominent IgV intraclonal diversification that has been followed for 2 years. The degree of IgV diversification gradually decreased when evaluated at four time points during this period. Nucleotide divergence was observed both in the Ig V_H and V_L rearrangements, although in the latter diversification was less marked. Two main V_H clonal variants, which increased with time, were observed. Similarly, a more represented V_L clonal variant was characterised. Analyses of molecular clone sequences suggest that neoplastic cells evolved through somatic IgV diversification producing two main V_H variants that may have acquired further genetic alterations capable of providing growth/survival advantages. These variants showed almost identical amino acid (aa) sequences although they differed by nine nucleotides.

The characteristics of the IgV somatic mutations observed in this B-CLL case were not fully compatible with somatic hypermutation (SHM) observed in the germinal centres (GC) of secondary lymphoid follicles. In addition, analyses of partially shared and unique mutations suggest that negative selection of IgV-mutated leukaemic cells is impaired.

These findings suggest that SHM observed in B-CLL (Gurrieri *et al*, 2002), in conjunction with antigen stimulation, participates in the selection and expansion of neoplastic subclone(s). In this B-CLL case, the role of SHM and antigen stimulation may also bear clinical relevance, as disease progression occurred during the time of study.

Materials and methods

Patient

A 73-year-old Italian male was diagnosed with B-CLL in 1999, based on the clinical and immunophenotypic features. At diagnosis, the white blood cell (WBC) count was $28{\cdot}2 \times 10^9$/l (neutrophils 20%, lymphocytes 80%); platelet count, 185×10^9/l; hemoglobin 15·7 g/dl. Greater than 80% of lymphocytes were $CD19^+$, $CD5^+$, $CD23^+$, with dull surface immunoglobulin (sIg). The patient had latero-cervical and axillar adenopathies (up to 1 cm) and he was classified as stage A/I according to Binet/Rai (Rai *et al*, 1975; Binet *et al*, 1981). During the study period (May 2002–April 2004), an increased WBC count was observed (see Table I) and in January 2004, physical examination, ecography and computed axial tomography (CAT) scan revealed a slight enlargement of the spleen and the appearance of small multiple abdominal adenopathies (up to 1·7 cm). Accordingly, the patient was staged as B/II.

B-cell chronic lymphocytic leukaemia cells were examined for the expression of CD38, activation-induced cytidine deaminase (AID) and ζ-associated protein (ZAP-70). None of these markers was found at significant levels and at any time point. The chromosomal pattern of the leukaemic cells was investigated by fluorescence *in situ* hybridisation with probes specific for deletions of chromosome regions 13q14, 11q22·3, 17p13·1 and trisomy of chromosome 12 (Fabris *et al*, 2005). Deletion of 13q14 was observed in the majority of the cells without significant changes over time.

The patient was last seen in July 2005, with no further evolution of the clinico-hematological parameters and has not received any treatment. Informed consent was obtained from the patient.

Amplification and sequencing of B-CLL V(D)J rearrangements

DNA was extracted from peripheral blood mononuclear cells (PBMC) using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Milan, Italy). IgV_H and V_L rearrangements were determined by amplifying 0·5 μg of DNA with primers and polymerase chain reaction (PCR) conditions previously described (Fais *et al*, 1998; Capello *et al*, 2000). IgV_H and V_L PCR products were either sequenced directly after purification with Montage PCR (Millipore, Milan, Italy), or cloned into a TA vector (Invitrogen, Milan, Italy) and then sequenced using an automated sequenator (310 Genetic Analyzer; Applied Biosystems, Monza, Italy). PCR products to be analysed for intraclonal diversification were obtained using 1·25 U of Platinum *Taq* HiFi (Invitrogen) high fidelity DNA polymerase. The reaction was run for 30 cycles following the manufacturer's instructions with the exception of extension temperature, which was kept at 72°C in a final volume of 50 μl.

Determination of Taq error

To evaluate the rate of *Taq* HiFi nucleotide misincorporation, we used two IgV_H2 molecular clones with divergent sequences derived from the B-CLL patient under study. Five pg of DNA derived from each clone were admixed and amplified using a

Table I. Number of V_HDJ_H sequences identified by molecular cloning at various time points.

Sample	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Others	Total clones	WBC count	Binet/Rai stage
PBMC 1	11 (22·91)	7 (14·58)	2 (4·16)	2 (4·16)	0	0	0	26 (54·16)	48	64·4*	A/I
PBMC 2	17 (32·69)	11 (21·15)	1 (1·92)	0	0	0	0	23 (44·23)	52	85·7	A/I
PBMC 3	22 (40·00)	11 (20·00)	0	0	2 (3·63)	2 (3·63)	0	18 (32·72)	55	99·6	A/I
PBMC 4	24 (41·37)	12 (20·68)	0	4 (6·89)	1 (1·72)	0	3 (5·17)	14 (24·13)	58	87·4	B/II

Percentages are indicated in parenthesis.
*$\times 10^9$/l.

V_H2-J_H primer pair for 30 cycles under the same conditions and reagent concentrations used for amplification of DNA derived from the patient PBMC. The 500 bp PCR product was gel-purified using the MinElute Gel Extraction Kit (Qiagen, Milan, Italy) and cloned in TA vector (Invitrogen). Sixty molecular clones were sequenced. No nucleotide misincorporation was observed. Two clones derived from PCR cross-over were identified. The manufacturer's reported error rate of Platinum *Taq* HiFi was $0{\cdot}18 \times 10^{-5}$ base/cycle, with a prediction of 1·6 base change in the 30·000 nucleotides sequenced. In our hands, the error rate was lower and was therefore considered to be negligible.

Ig V_H and V_L sequence analyses

Sequences obtained were compared with Ig germline gene database using IgBlast (available at http://www.ncbi.nlm.nih.gov/igblast/). MACVECTOR 6 software (Accelrys; S. Diego, CA, USA) was used for further sequence analyses.

Distribution of mutations among complementary determining region (CDR) and framework (FR) gene segments was evaluated by the Chang–Casali binomial distribution model (Chang & Casali, 1994). Expected replacement (R) mutations were calculated using the formula $R = n \times$ CDR *Rf* (or FR *Rf*) $\times$ *CDRrel* (or *FRrel*), where *n* is the total number of observed mutations, *Rf* is the replacement frequency inherent to the CDR or FR, and *CDRrel* and *FRrel* are the relative sizes of these segments. *Rf* were calculated for each individual gene sequence using the INHSUSCALC 1·0 software, kindly provided by Dr Paolo Casali (Center for Immunology, University of California, Irvine, CA, USA). A binomial probability model was used to evaluate whether the excess of *R* mutations in CDR or their scarcity in FR was because of chance.

Somatic mutations were defined as 'shared' when observed in the totality of V_HDJ_H or $V_\lambda J_\lambda$ molecular clones, as 'partially shared' when observed in a proportion of the clones and as 'unique' when observed only once in the IgV rearrangements.

Statistical analysis

The two-tailed Fisher's exact test was used to determine whether partially shared and unique mutations had a different representation of *R* and silent (*S*) mutations. *P* values <0·05 were considered significant.

Results

During the characterisation of *IgV* gene rearrangements in a cohort of B-CLL patients, one case was noted to show nucleotide ambiguities in the sequence derived from the PCR product of the rearranged IgV_H2 family gene used by the neoplastic cells. Therefore, molecular clones derived from the V_H2 PCR product were generated and sequenced with the aim of defining the nucleotide composition of the rearranged IgV gene. Sequencing revealed the presence of nucleotide diversification within the rearrangement. Therefore, clonal diversification was evaluated at the different time points available.

Analysis of IgV_H sequences at multiple time points

DNA derived from PBMC was extracted at four time points (May 2002, September 2002, July 2003 and April 2004) and amplified using the V_H2 leader-J_H primer pair. Altogether, 213 molecular clones derived from these PCR products were sequenced (see Table I). All sequences were clonally related in that they shared a common CDR3 motif. The putative IgV_H germline gene assigned by analysis was the V_H2–5 gene juxtaposed to D2-15 and J_H6*02 gene segments.

At all time points, two groups of identical sequences were more represented than the others (defined as V_H groups 1 and 2 clones, see Fig 1A). At each time point, these clones ranged from 37·5% (at the 1st time point) to 62·0% (at the 4th time point, see Table I for details). Other, less represented, identical clones were occasionally observed (see Table I). Groups 1 and 2 clones differed from the putative germline V_H2–5 gene for eight (2·7%) and 10 (3·3%) nucleotides, respectively. Two additional mutations were observed in the intron region of group 1 clones and one of these mutations was shared by group 2 clones.

Overall, among molecular clones, the mutations compared with the putative germline V_H2–5 gene varied from 1·3% (corresponding to four nucleotide substitutions, shared by all molecular clones) to 4·0% (12 nucleotide substitutions). Among the 213 molecular clones, we found 97 different sequences (45·5%). The number of clones that displayed a divergent nucleotide sequence declined with time, from 30 of 48 clones (62·5%) in the 1st sample to 19 of 58 (32·7%) in the last sample analysed.

Nucleotide substitutions were represented mostly by transitions. The transition/transversion (T/T) ratio ranged from 1·27, observed at the 3rd time point to 4·14, at the 2nd time point (see Table IIa).

Analysis of nucleotide substitutions, by the Chang–Casali binomial distribution model, observed in the IgV_H gene segment in groups 1 and 2 clones indicated a significant preservation of the FR structure ($P = 0{\cdot}0095$ and 0·0020, respectively). No significant accumulation of *R* mutations was observed in the CDR1 and CDR2 regions.

The four mutations observed in the V_H2-5 coding region shared by all clones were of *R* type (three in the CDR and one in the FR). The analysis of *R versus S* of partially shared and unique mutations observed in the clones sequenced at the four different time points showed relevant differences. Eight of 48 (16·6%) of partially shared mutations were represented by *R* in comparison with 34 of 62 (54·8%) *R* mutations observed among unique mutations ($P < 0{\cdot}0001$). When CDR and FR regions were considered separately, a highly significant difference was observed in the FR region (Table III).

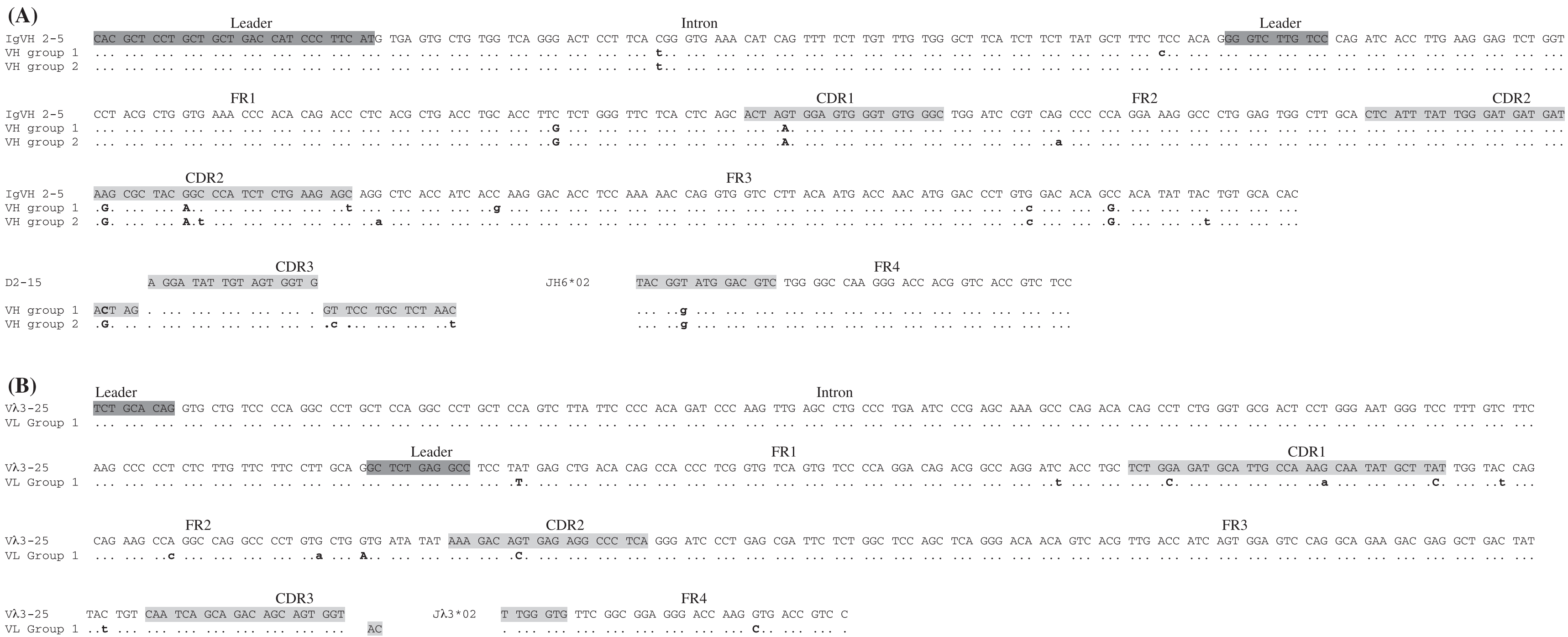

Fig 1. (A) Alignment of V_H groups 1 and 2 clone sequences with putative germline IgV segment genes. Dark grey regions indicate the V_H leader sequence. Light grey regions indicate CDR regions. *R* mutations are indicated by capital letters. (B) Alignment of V_L group 1 clone sequence with putative germline IgV_λ segment genes. Dark grey regions indicate V_λ leader sequence. Light grey regions indicate CDR regions. *R* mutations are indicated with capital letters.

Table II. Transitions and transversions observed in the (a) V_HDJ_H (b) $V_\lambda J_\lambda$ molecular clones.

Sample	Transitions	Transversions	T/T ratio
(a)			
PBMC 1	32	15	2·13
PBMC 2	29	7	4·14
PBMC 3	23	18	1·27
PBMC 4	21	8	2·62
Total	105	48	2·18
(b)			
PBMC 1	17	6	2·83
PBMC 2	14	5	2·80
PBMC 3	13	5	2·60
PBMC 4	11	4	2·75
Total	55	20	2·75

Table IV. Number of $V_\lambda J_\lambda$ sequences identified by molecular cloning at various time points.

Sample	Group 1	Group 2	Others	Total clones
PBMC 1	13 (52·0)	2 (8·0)	10 (40·0)	25
PBMC 2	13 (61·9)	0	8 (38·1)	21
PBMC 3	15 (68·1)	0	7 (31·8)	22
PBMC 4	19 (79·1)	0	5 (20·8)	24

Percentages are indicated in parenthesis.

Analysis of IgV_L sequences at multiple time points

The IgV_L chain was amplified using $V_\lambda 3$ leader-J_λ primer pair at each time point. Molecular clones derived from single PCR products were sequenced and analysed to evaluate the intraclonal diversification at the IgV_L chain level. Ninety-two molecular clones were examined (Table IV). Similar to the IgV_H2 molecular clones, all sequences shared a common CDR3 motif. The putative IgV_λ germline gene assigned was $V_\lambda 3$-25, which was juxtaposed to the $J_\lambda 3$*02 gene segment. Nucleotide divergence was observed in 35 of 92 molecular clones (38%). The number of clones that displayed divergent nucleotide sequence decreased with time, ranging from 12 of 25 clones (48%) in the 1st sample analysed to 6 of 24 (25%) in the last sample. A single, more represented sequence was tracked in every sample (defined as V_L group 1, shown in Fig 1B). The representation of this sequence varied at each time point, from 52% (at the 1st time point) to 79·1% (at 4th time point, see Table II for details). V_L group 1 sequence showed 11 nucleotide differences (4·0%) when compared with the putative $V_\lambda 3$-25 germline gene. Overall, among molecular clones, the divergence from the putative coding germline $V_\lambda 3$-25 gene ranged from seven to 12 mutations (2·4% and 4·2%, respectively). The Chang–Casali analysis of the V_L group 1 sequence showed a significant preservation of FR sequences ($P = 0{\cdot}0166$), whereas no significant accumulation of *R* mutations was observed in the CDR. Similar to the V_H sequences, transitions exceeded transversions (see Table IIb). The T/T ratio did not show significant variation among the time points (range 2·60–2·83).

H and L CDR3 analysis

HCDR3 was composed of 17 aa. Based on the aa composition, molecular clones could be clustered into four variants. The CDR3 prototypical sequence was that observed in the clone with the lowest number of mutations (3G042-CL24). Three additional variants of this CDR3 aa sequence (shown in Fig 2) were repeatedly used at the different time points. In one case, a single aa change was present (Ala at position 100b replaced with Thr). In a 3rd variant, Ala at position 100b and Thr at position 100c were replaced with Gly and Ser, respectively. Finally, a 4th group of clones displayed an additional Ser > Thr substitution at position 95 in addition to those described in the previous group. The estimated isoelectric point (pI) of all HCDR3 variants was 5·95.

An LCDR3 consisted of 10 aa. The aa sequences were preserved among all molecular clones studied. The estimated pI was 3·43.

Discussion

We report a case of B-CLL in which IgV intraclonal diversification was evaluated over a period of 2 years. Expression of CD38, ZAP-70 and AID was never observed. We thus failed to detect markers that are associated with IgV intraclonal

Table III. Partially shared *versus* uniqueV_HDJ_H mutations at various time points.

	CDR partially shared mutations		FR partially shared mutations		CDR unique mutations		FR unique mutations		Total partially shared mutations		Total unique mutations	
Sample	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
PBMC 1	1	4	1	9	4	2	6	7	2	13	10	9
PBMC 2	0	2	2	5	5	1	9	2	2	7	14	3
PBMC 3	1	3	2	7	0	3	8	7	3	10	8	10
PBMC 4	0	3	1	7	0	3	2	3	1	10	2	6
Totals	2*	12	6**	28	9*	9	25**	19	8***	40	34***	28

*P = not significant; **$P = 0{\cdot}0005$; ***$P < 0{\cdot}0001$.

SRGYCSGATCSNYGMDV	Progenitor variant 1 (from 3G042-CL24)	
.....T.....	Variant 2	
.....GS.....	Variant 3	Observed in V_H group 2 clone
T.....GS.....	Variant 4	Observed in V_H group 1 clone

Fig 2. IgV_H amino acid sequences of CDR3 that were more frequently observed among molecular clones.

heterogeneity. In addition, leukaemic cells showed a deletion of chromosome13q14.

In spite of the apparent homogeneity of the neoplastic cells, analyses of rearranged *IgV* genes showed a profound heterogeneity. Two more frequent variants of V_HDJ_H rearrangement were identified. These variants differed by nine nucleotides in the coding region (see Fig 1A). However, the nucleotide differences resulted in a unique conservative Ser > Thr substitution, located in the CDR3 region (at position 95, see Fig 2). V_H groups 1 and 2 sequences tended to increase with time. In particular, group 1 variant almost doubled its relative percentage among the clones sequenced when the first and the last time point analysed were compared (see Table I).

A similar analysis was also performed on $V_\lambda J_\lambda$ rearrangement. In this case, the nucleotide divergence was less striking. We identified a sequence that was more represented among the others. Similarly to V_H groups 1 and 2 variants, the frequency of this sequence increased with time (see Table IV).

Intraclonal diversification, defined by the number of diverse V_HDJ_H rearrangements, involved most of the clones (62·5%) at the earliest time point and reduced to 32·7% at the latest time point. Reduction of intraclonal diversification paralleled the emergence of V_H groups 1 and 2 clones. A similar pattern was observed in the $V_\lambda J_\lambda$ molecular clones. Thus, the decreased rate of intraclonal diversification seemed to reflect the emergence of a clone(s) that probably acquired survival/growth advantage and limited its ability to somatically diversify the BCR. This hypothesis is supported by the concomitant increase in the WBC count (see Table I). It has to be emphasised that this analysis does not provide an accurate estimate of the diverse leukaemic subclone composition but it may offer a reliable representation of the general trend of the clonal evolution.

Table V. Partially shared and unique nucleotide substitutions in the V_HDJ_H sequences.

	G > H	A > B	C > D	T > V
PBMC 1	12	6	10	14
PBMC 2	7	10	6	8
PBMC 3	9	6	8	13
PBMC 4	6	2	6	10
Total	35 (25·9%)	24 (17·7%)	31 (22·9%)	45 (33·3%)
Observed nucleotide frequency of IgV_H 2–5 gene	G 23·9%	A 22·1%	C 29·3%	T 24·6%

H = A, C or T; B = C, G or T; D = A, G or T; V = A, C or G.

Some considerations can be drawn on the nature of the somatic mutations observed. An SHM occurring in GC shows higher T/T ratio in comparison with randomly occurring somatic point mutations that are expected to be one-third transitions and two-third transversions (Yelamos *et al*, 1995). In the V(D)J sequences analysed, the T/T ratios observed were always higher than 1 (see Table IIa and b). In contrast, the higher frequency of A nucleotide substitutions described in SHM occurring in GC (Steele *et al*, 2004) was not observed (see Table V). Both these features are in agreement with a previous report (Gurrieri *et al*, 2002).

Analysis of partially shared mutations compared with unique mutations of IgV_H sequences shows that the latter are more often of an *R* type (see Table III). When considering the CDR and FR regions, significant numbers of unique mutations of an *R* type were observed in an FR regions (see Table III). This pattern was reminiscent of the higher number of *R* mutations observed in the FR regions in SHM occurring in non-functional V_HDJ_H rearrangements (Dorner *et al*, 1998). Thus, it may be assumed that most of these *R* mutations do not provide better antigen fitting to the BCR. This suggests that neoplastic cells do not undergo the negative selection observed in GC. An alternative possibility is that an SHM of leukaemic cells occurs in extrafollicular sites, in the absence of T-cell help, as described in hyper-IgM disease (Weller *et al*, 2001) and in an animal model (de Vinuesa *et al*, 2000). However, the mechanism of B-cell selection undergoing an SHM in the absence of T-cell help remains to be determined.

A positive selection of leukaemic cells based on an antigen recognition is suggested by the nucleotide substitutions observed in the more represented clones (V_H groups 1 and 2 and V_L group 1) as significant preservation of an FR is observed. In addition, it is of note that, although the V_H groups 1 and 2 sequences differed by nine nucleotides, only one conservative change was observed. Considering the nucleotide composition of the putative ancestor rearrangement clone 3G024-CL24, the expected number of *R* mutations generated from nine random nucleotide substitutions would be of 6·76 ($P < 0·0001$). This observation reinforces the possibility that interaction with an antigen guided the selection of these neoplastic clones. Accordingly, leukaemic cells harbouring the unique mutations would represent cells with suboptimal BCR antigen recognition and therefore would be observed only transiently in the peripheral blood. In this regard, it would be of interest to determine the antigen specificity of the diverse BCR we identified and evaluate possible changes of antigen affinity. Indeed, studies showed

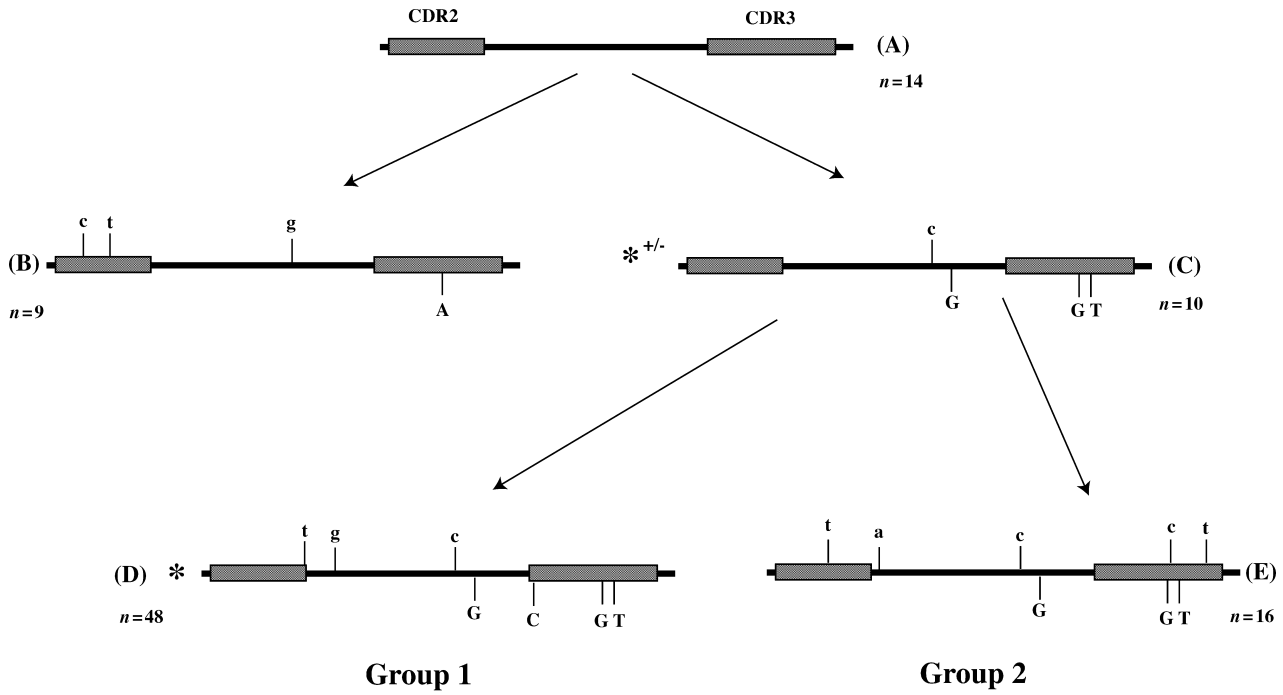

Fig 3. Genealogical tree representing clonal evolution of V_HDJ_H clones. The tree was created by considering partially shared mutations observed in the CDR2–FR3–CDR3 region, which accounts for the large majority of partially shared substitutions. Clone 3G042-CL24 was chosen as the ancestor sequence, as it showed the lowest degree of diversity in comparison with the putative germline V_H2–5 gene. Replacement mutations are indicated on the lower side of the bars as capital letters. Silent mutations are indicated on the upper side with small letters. *n* indicates the number of variants identified in each cluster (clusters are defined as A, B, C, D and E). The asterisk indicates a partially shared mutation in the intron region first observed in a proportion of cluster C clones and then stably represented in cluster D variants.

that the BCR of B-CLL cells recognise autoantigen(s) (Sthoeger *et al*, 1989; Herve *et al*, 2005) and therefore this point is worth being addressed in future studies.

Another possibility may be related to an inherent capability of this leukaemic cell clone to undergo SHM. However, the above-reported analyses suggest that the survival (and possibly the expansion) of certain clones is influenced by the interaction of their BCR with an antigen.

In order to represent the clonal evolution and diversification of neoplastic cells, a genealogical tree was created using the information derived from all V_HDJ_H molecular clones (see Fig 3). For simplicity, we considered the more informative partially shared mutations observed in the CDR2–FR3–CDR3 region of the IgV_H gene segment to be comprehensive of the vast majority of partially shared mutations observed. These mutations allowed definition of five clusters of clones (termed A, B, C, D and E). As the progenitor sequence, we used the molecular clone 3G042-CL24, which displays the lowest amount of somatic mutations. These substitutions are shared among all clones sequenced. In the 3G042-CL24 sequence, a consensus was defined by the absence of partially shared mutations identified in the other clones. This consensus sequence was present in 14 of 97 variants (cluster A) and generated two main clusters (B and C). Cluster C branched into two additional clusters (D and E). Cluster D shows most of the variants observed (48/97) and includes the V_H group 1 sequence. Cluster E included V_H group 2 sequence and was composed of 16 variants. From this tree, it was possible to deduce that somatic diversification occurred in the less mutated progenitor sequences as well as in the more mutated clones.

Evidence that antigen selection plays a promoting role in B-CLL comes from several reports (Tobin *et al*, 2003; Ghiotto *et al*, 2004; Messmer *et al*, 2004; Widhopf *et al*, 2004). However, our study may add further details on the role of antigen stimulation in disease evolution. Indeed, it must be noted that in the B-CLL case described here, the status of known disease prognostic markers (lack of expression of CD38, ZAP-70 and AID and chromosome 13q14 deletion) was in agreement with a favourable clinical course (Damle *et al*, 1999; Crespo *et al*, 2003; Guarini *et al*, 2003; McCarthy *et al*, 2003). In addition, the percent of somatic mutations of *IgV* genes observed in the more represented V(D)J clones (higher than 2%) also provides a favourable prognostic factor (Damle *et al*, 1999; Hamblin *et al*, 1999). Nonetheless, the patient progressed from stage A/I to B/II, suggesting that an SHM, together with antigen stimulation/selection, may play a relevant role in disease evolution.

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