Most immunoglobulin heavy chain switch mu rearrangements in B-cell chronic lymphocytic leukemia are internal deletions

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Abstract We investigated 38 cases of B-cell chronic lymphocytic leukemia (B-CLL) for the presence of non-productive rearrangements in the Sμ regions and defined for the first time the molecular nature of these rearrangements. Southern blot analysis revealed Sμ region rearrangements in 13 cases (34%) and polymerase chain reactions (PCRs) indicated that these rearrangements consisted of internal deletions of the Sμ region. Long-distance PCRs localized the Sμ deletions in the V_HDJ_H rearranged allele in most cases. We investigated if Sμ deletions were related to V_H somatic mutations that, together with isotype switch recombination, are indicative of the B-cell maturation stage. No significant correlation between the presence of Sμ deletions and V_H somatic mutations was found, indicating that the two processes are independent in B-CLL. Moreover no significant correlation between Sμ deletions and prognosis was observed. Having shown that Sμ internal deletions are not chromosome translocations rules out their involvement in the onset of malignancy, while their localization in the V_HDJ_H rearranged alleles suggests a possible role in the stabilization of the isotype of the expressed immunoglobulin. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: B-cell chronic lymphocytic leukemia; Isotype switch; Somatic hypermutation

1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most frequent form of adult leukemia, accounting for 30% of all leukemias. This leukemia is characterized by accumulation of monoclonal B-cells, with the appearance of small $CD5^+$ and $CD23^+$ mature lymphocytes [1,2]. Based on these characteristics, it had been suggested that B-CLL is a tumor of 'naive' B-cells possibly arising in the follicular mantle zone [3]. However, some B-CLL with somatically mutated immunoglobulin (Ig) variable genes have been described [4]. Since somatic mutation is believed to occur in germinal centers [5], the presence of such mutations indicates that the cell of origin has passed through the germinal center. Thus, it has been suggested that B-CLL comprises two different disease subtypes, one arising from a 'naive' B-cell, the other from a 'memory' B-cell [6]. Interestingly, patients with non-mutated variable genes have a distinctly more malignant disease and a much shorter survival than those with somatic mutations [6,7].

Under physiological conditions, somatic mutation of the variable genes occurs in temporal correlation with isotype switching in the germinal center microenvironment [8,9] and the recent discovery of AID (activation-induced cytidine deaminase) [10] has revealed an unexpected link between isotype switch recombination and somatic hypermutation.

However B-CLL cells express mostly surface Igs of the IgM or IgM/IgD isotype and only a small proportion of B-CLL is characterized by the expression of isotype-switched Igs [1,2]. Two studies reported the presence of isotype-switched transcripts in B-CLL patients [11,12] and the ability of B-CLL to proliferate and differentiate into Ig-secreting cells after in vitro stimulation has been described [2,13,14]. The major mechanism for isotype switching is a deletional recombination between switch regions, which are highly repetitive regions upstream of each cluster of constant genes, except for δ [15]. Rearrangements in the Ig Sμ regions without isotype switching have been previously observed in B-CLL cases but the molecular nature and the clinical implications of these alterations were not investigated [16,17]. We analyzed 38 cases of B-CLL to define the molecular nature of the Sμ region rearrangements, their relation to variable genes mutations and to clinical course.

2. Materials and methods

2.1. B-CLL patients, phenotypic characterization and disease course

Forty patients with clinical features of B-CLL were randomly chosen among a series of 145 cases. Peripheral blood of these patients revealed expansion of cells with the typical phenotype of B-CLL ($CD5^+/CD19^+$, $CD23^+$, $CD22^-$, low or absent CD79b expression). The disease course was assessed clinically considering the following criteria recommended in the National Cancer Institute sponsored workshop in 1996 [18]: blood cell counts (percentage of neoplastic $CD5^+/CD19^+$ cells), lymphocyte count doubling time, progression to a more advanced stage of disease, development of systemic symptoms and a downward trend of hemoglobin or platelet count. The presence of at least one altered feature was sufficient to define 'progressive disease'; patients without altered features were designated as having 'stable disease'.

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Abbreviations: Sμ, switch mu region; V_H, variable heavy chain gene; J_H, joining heavy chain region

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2.2. Southern blot analysis

Digested genomic DNA was fractionated by gel electrophoresis on a 0.8% agarose gel, denatured with 0.5 N NaOH/1.5 M NaCl, neutralized with 1 M Tris–HCl/1.5 M NaCl, and transferred overnight to nylon filters with 20× SSC. The following day, filters were air-dried and fixed with UV light (UV Stratalinker).

2.3. Probes

The Sμ region was analyzed using two ^{cb}P radiolabeled 5′Sμ and 3′Sμ probes localized upstream and downstream of the Sμ region, respectively. Probes were synthesized by polymerase chain reaction (PCR) using specific oligonucleotides reported elsewhere [19].

2.4. Hybridization conditions

Filters were hybridized overnight at 42°C in hybridization buffer (1 M NaCl, 50 mM Tris–HCl pH 7.4, 40% formamide, 10% dextran sulfate, 1% SDS and 100 μg/ml salmon sperm DNA) and 2×10^6 cpm/ml of probe. Filters were washed sequentially in solutions containing 2×SSC/0.1% SDS (room temperature), 1×SSC/0.1% SDS (42°C) and 0.1×SSC/0.1% SDS (65°C), exposed overnight, and scanned on a phosphorimager.

2.5. PCR of Sμ regions

Genomic DNA (100 or 300 ng) was used as template in PCR reactions with several oligonucleotides (Fig. 1). The reaction mixture contained dNTPs at 200 μmol/l, primers at 0.2 μmol/l, 1 U rTth polymerase (Perkin Elmer) and $Mg(OAc)_2$ at 1.2 mmol/l, in a final volume of 25 or 50 μl. PCR cycles were: one cycle at 94°C for 3 min, 34 cycles at 94°C for 1 min, 62°C for 1 min and 70°C for 5 min and a final step at 70°C for 10 min. The enzyme was added after the first cycle (Hot Start procedure).

2.6. PCR of V_HDJ_H regions

Genomic DNA (1 μg) was PCR-amplified using two consensus primers: FR1c, complementary to framework 1, and JHc, complementary to the joining segment (Fig. 1). PCR was performed in a final volume of 50 μl, with 50 pmol of each primer, 200 μM dNTPs, 1 mM $MgCl_2$, 10% of DMSO and 1 U Taq DNA polymerase (Gibco). Amplification consisted of an initial denaturation step at 94°C for 3 min followed by 34 cycles at 94°C for 1 min, at 62°C for 1 min and 72°C for 2 min, with a final extension step for 10 min at 72°C.

2.7. V_HDJ_H and Sμ sequence analysis

The V_HDJ_H sequence of each patient was analyzed using the IgBLAST database to identify the germline sequence with highest homology, followed by alignment to its closest germline to detect mutations. Based on the number of somatic mutations detected in these genes, the cases were classified as 'unmutated' or 'mutated'. Consistent with current convention, 'unmutated' genes were defined as those with $<2\%$ differences from the most similar V_H germline, while 'mutated' genes were those with $\geq 2\%$ difference from the closest germline. PCR-amplified Sμ regions were aligned to the germline sequences HSIMMDL–HSJHCMU using the BLAST database to identify deletions and/or DNA rearrangements.

2.8. PCR from the V_HDJ_H to the Sμ region

Genomic DNA (100 ng) of each patient with Sμ rearrangements was amplified using a 'touchdown' strategy. Forward primers annealing to the specific V_H family expressed by each case were used in combination with a reverse primer complementary to the 3′ of the Sμ region (3MRA or 3MRB) (Fig. 1). PCR reaction was performed in a final volume of 100 μl with 20 pmol of each primer, 200 μM of dNTPs, 1.2 mM of $Mg(OAc)_2$ and 2 U of rTth DNA polymerase (PE Applied Biosystem) with the Hot Start technique. Amplification consisted of an initial denaturation step of 1 min at 94°C followed by 24 cycles at 94°C for 15 s and 68°C for 10 min with an increasing time of 15 s in the last 10 cycles. The final extension step was performed at 72°C for 10 min.

2.9. Statistical analysis

Statistical analyses were performed with the Fisher's Exact Test.

3. Results

3.1. Southern blot and PCR analysis of Sμ regions in B-CLL cases

Forty B-CLL patients were initially screened for Ig isotype; 28 had IgM^+ B-leukemic cells, eight had IgM^+/IgD^+, two IgG^+, one IgD^+ and one had non-producing cells. DNA from peripheral blood lymphocytes of the 38 patients with 'unswitched' isotype, i.e. no evidence of productive isotype switch, was extracted and analyzed by Southern blot using two probes localized upstream (5′Sμ) and downstream (3′Sμ) of the Sμ region [19] (Fig. 1A). If the cell does not undergo isotype switch recombination, its switch regions are in germline configuration and the 5′Sμ and 3′Sμ probes cohybridize to a fragment of a given length. If the cell undergoes a physiological isotype switch recombination, the 5′Sμ probe cohybridizes with the 3′ switch probe of the correspondent isotype. If the switch recombination is 'aberrant', the 5′Sμ

Table 1
Southern blot and PCR results for the 13 cases with Sμ rearrangements

Case	Rearranged fragments size (kb)[a]		PCR fragments size (kb)	Primer pairs	PCR deletion size (kb)
	5′Sμ	3′Sμ			
1	8	8	3	5MFA/3MRA	0.9
2	8.5	8.5	3.5	5MFA/3MRA	0.4
3	7	7	1.8	5MFA/3MRA	2.1
	7.5		2.5	5MFB/CmR1	1.6
4	5.2	5.2	1.25	5′σμF/3MRB	3.75
	8				
5	7.5	7.5	2.5	5MFA/3MRA	1.4
7	7.2	7.2	2.3	5MFA/3MRA	1.6
18	6	6	2.5	5′σμF/3MRB	2.6
	8				
20	8.5	8.5	3.5	5MFA/3MRA	0.4
	6.5				
27	6.4	6.4	1.5	5MFA/3MRA	2.5
31	6.4	6.4	1 and 0.8	5MFA/3MRA and 5MFB/3MRB	2.8
	6	6	0.4	5MFB/3MRB	3.2
35	6.1	6.1	0.9	5MFA/3MRA	2.9
36		5.5	1.9	5′σμF/3MRB	3.2
40	8.5	8.5	3.5	5MFA/3MRA	0.5

[a]Only rearranged fragments smaller than the germline are indicated.

A

B

PRIMER	SEQUENCE 5'- 3'	GENBANK LOCALIZATION	
Fr1c	AGGTGCAGCTG(GC)(AT)G(GC)AGTC(AGT)GG	Framework1 consensus sequence	
JHc	ACCTGAGGAGACGGTGACC(AG)(GT)(GT)GT	Joining heavy chain consensus sequence	
5'σμF	CAGATCTGAAAGTGCTCTACTG	90885-90906	HSIMMDL
5'SμR	TCAGCTAAAGCCATCTCATTGCC	92520-92498	HSIMMDL
5MFA	GGCTCCTAAATTCTTGGTCTCA	92291-92312	HSIMMDL
5MFB	GGCAATGAGATGGCTTTAGCTGA	92498-92520	HSIMMDL
3MRB	GTGATGGGGAACGCAGTGTAGA	3803-3781	HSJHCMU
3MRA	CCGCAGGCAGCCAATAGAGT	3931-3911	HSJHCMU
CmR1	ACACGTGTCAGCCCGGTGCC	534-515	HSJHCMUDE

Fig. 1. A: Localization of the Sμ probes used for Southern blot assay and of the primers used for PCR amplification of IgH Sμ regions. B: Primer sequences and relative position in GenBank database.

probe does not cohybridize to any downstream 3′ switch probe.

On *Sph*I digestion, DNA from 25 of the 38 patients showed a Sμ region in germline configuration, while the remaining 13 patients showed additional rearranged bands (Fig. 2). Hybridization with the 5′Sμ probe, revealed seven cases (1, 2, 5, 7, 27, 36, 40) with one rearranged band and six cases (3, 4, 18, 20, 31, 35) with two rearranged bands. With the 3′Sμ probe, 10 cases (1, 2, 3, 4, 5, 7, 18, 20, 27, 40) presented one rearranged band and three cases presented two rearranged bands (31, 35, 36). Interestingly, in all but two (35, 36) of these cases, the rearranged fragments identified by the 5′ and 3′Sμ probes cohybridized and were about 0.5–4 kb smaller than the germline fragment. The different sizes of the rearranged bands (Table 1) excluded the possibility that they were polymorphic

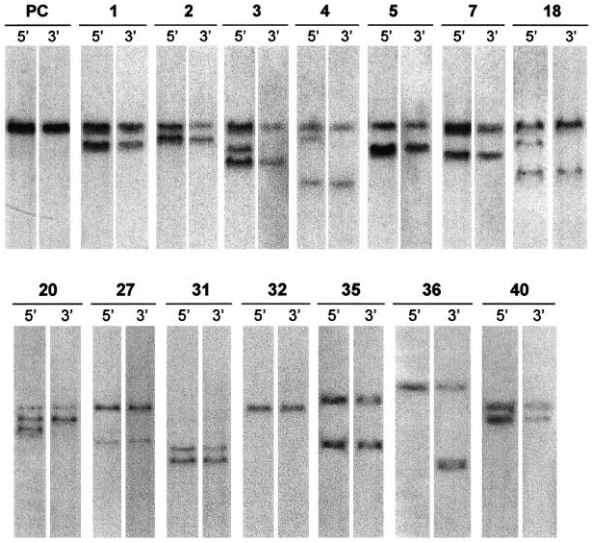

Fig. 2. Southern blot analysis of Sμ regions in B-CLL patients. Genomic DNA of B-CLL patients was digested with *Sph*I restriction enzyme, electrophoresed, blotted and probed sequentially with 5′Sμ (5′) and 3′Sμ (3′) probes. The hybridization pattern of 13 cases with rearranged Sμ bands and one case (#32) in germline configuration is shown. PC = placental genomic DNA used as germline control.

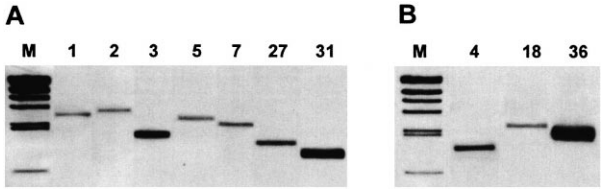

Fig. 3. PCR amplification of the Sμ region in patients with Sμ rearranged bands. A: Amplification of seven B-CLL cases with 5MFA/3MRA primer pair. B: Amplification of three cases with 5′σμF/3MRB primer pair.

alleles while the cohybridization of the two Sμ probes suggested an internal deletion in the Sμ region [20,21].

Indeed PCR using primers 5MFA/3MRA or additional primer pairs located upstream and downstream to 5MFA/3MRA (Fig. 1B) indicated the presence of deletions in the Sμ regions. In all 13 cases with Sμ rearrangements, a band of reduced size compared to the germline was amplified (Fig. 3). Cases in Fig. 3A were amplified with primers 5MFA/3MRA, which amplify a fragment of 3.9 kb when the Sμ region is in germline configuration, whereas the cases in Fig. 3B were amplified with primers 5′σμF/3MRB, which amplify a 5.1 kb fragment when the Sμ region is in germline configuration. In three cases (20, 35, 40), PCR amplification produced very faint bands (data not shown). In all cases, the reduction in size of the PCR-amplified products corresponded to the reduction observed for the rearranged bands in Southern blot (Table 1). Bands that on Southern blot were larger than the germline might contain a restriction site polymorphism or may be generated by insertions [22], or by inversions [23].

Sequencing of the PCR-amplified fragment from case 4 confirmed the presence of Sμ deletion. This case showed the germline bands and two rearranged bands of 8 kb and 5.2 kb with the 5′Sμ probe and one rearranged band of 5.2 kb with the 3′Sμ probe (Fig. 2). No reduced size bands were amplified with primers 5MFA/3MRA, whereas primers 5′σμF/3MRB amplified a 1.25 kb band (Fig. 3B). The sequence of the 1.25 kb band (Fig. 4) showed a germline configuration from the 5′σμF position in HSIMMDL (90885) to position 92021. After the breakpoint (ending at position 3671 in HSJHCMU), the sequence continued until position 3803 in HSJHCMU (3MRB position). Thus, a 3.75 kb deletion occurred on this allele between position 92021 in HSIMMDL and position 3671 in HSJHCMU. Additional PCRs did not amplify any product from the other allele, which might be involved in a translocation, or any other chromosomal rearrangement.

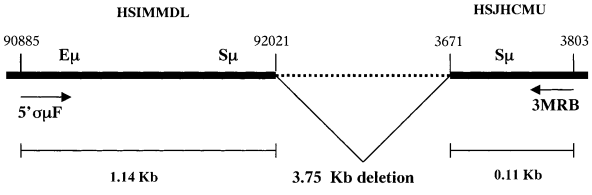

Fig. 4. Graphic representation of the breakpoint present in the Sμ fragment of case 4. The fragment was PCR-amplified using the 5′σμF/3MRB primer pair. Eμ = IgH enhancer; Sμ = switch mu region, HSIMMDL = GenBank DNA sequence of the human IgD segment locus, HSJHCMU = GenBank nucleotide sequence of the IgH Sμ region.

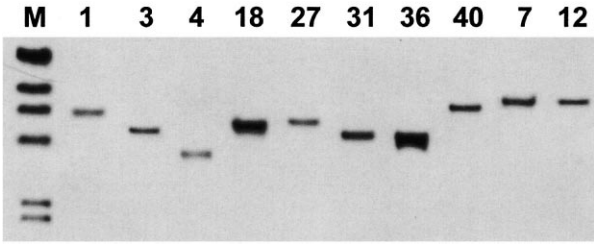

Fig. 5. Amplification of V_HDJ_H rearranged allele in B-CLL cases. V_HDJ_H rearranged allele was amplified using a forward primer annealing to a variable region in combination with a reverse primer specific for the 3′Sμ region. Case 12, that did not present rearrangements in the Sμ region, was used as control.

3.2. Localization of Sμ deletions in the V_HDJ_H rearranged allele

Sμ deletions could stabilize the isotype of the expressed Ig only if they occurred on the V_HDJ_H rearranged allele. To define the localization of Sμ deletions we amplified cases with Sμ deletions with primers in the V_H region and in the 3′ part of the Sμ region (Fig. 1A). Sizes of the V_HDJ_H rearranged fragments varied on the presence or absence of the Sμ deletion, being smaller the allele with the V_HDJ_H rearrangement and Sμ deletion. Using long-distance PCR conditions, it was possible to amplify the allele with the V_HDJ_H rearrangement and Sμ deletion in eight out of 13 cases (Fig. 5). In only one case (#7) the Sμ deletion occurred on the unrearranged allele. Case 12, that presents a V_HDJ_H rearranged allele without Sμ rearrangements, was used as control. We were not able to amplify by long-distance PCR the remaining four cases.

3.3. Analysis of somatic mutations in the variable genes

DNA of the 38 patients was then analyzed for the presence of somatic mutations in the variable heavy chain (V_H) genes. Amplification of the V_H genes using consensus primers in the framework 1 region (FR1c) and in the joining heavy chain region (JHc) resulted in a PCR fragment of about 350 bp. The number of somatic mutations was determined by comparing the V_H sequences to the germline genes with the highest homology. Sixteen cases expressed V_H1 family genes, 14 cases V_H3, five cases V_H4, two cases V_H2, and one case expressed both V_H1 and V_H3. Somatic mutations ranging from 2.3% to 11.6% were observed in 16 cases while the leukemic cells of 21 patients had V_H genes with $\geq 98\%$ sequence homology with the nearest germline gene. In the remaining case presenting a productive rearrangement of both variable regions, a V_H1–2 in germline configuration and a V_H3–48 in mutated form was observed. No correlation between the presence of Sμ rearranged bands and V_H gene mutations was observed, since four of 13 cases (32%) with a deletion in the Sμ region showed V_H mutations, and 13 of 25 of cases (52%) without deletion in the Sμ showed V_H mutations ($P = 0.13$).

3.4. Clinical observations

The clinical course of 22 patients with a follow-up period of at least 5 years was correlated with the presence of Sμ rearranged bands and V_H mutations. The presence of switch rearrangements alone was not indicative of the clinical course. Indeed, out of eight cases with switch rearrangements five underwent disease progression (62.5%) whereas out of 14 cases without switch rearrangements six underwent progression (42.8%) ($P = 0.24$). On the contrary, the presence of V_H mutations was indicative of the clinical course. Out of 11 cases with V_H mutations only three cases were in progression (27.2%) whereas out of 11 cases without V_H mutations eight cases (72%) were in progression ($P = 0.04$).

4. Discussion

Southern blot analysis of 38 B-CLL cases with IgM or IgM/IgD phenotype revealed rearrangements in the Ig Sμ region in 13 cases (34%), although no correlation between Sμ rearrangements and Ig phenotype (IgM only or IgM/IgD) was found. We show here for the first time that most of these Sμ region rearrangements, previously observed in B-CLL by other investigators [16,17,23], are genomic deletions of the Sμ region occurring in most cases in the V_HDJ_H rearranged allele. These deletions are of variable length, ranging from 0.5 kb to about 4 kb and may involve the region upstream or downstream the Sμ region but never the constant μ exons. The random distribution of these deletions might be related to the absence of a consensus-cutting site inside the Sμ region [24,25]. The frequent localization of Sμ deletions within the V_HDJ_H rearranged allele would suggest that B-CLL cells have undergone this partial deletion of the Sμ region in order to stabilize the isotype of the expressed Ig [20,21]. Indeed, although it was demonstrated that the Ig heavy chain intronic enhancer is necessary and sufficient to promote class switch recombination, a deletion of the Sμ tandem repeats in the mouse Ig heavy chain locus reduces the efficiency of class switching [26]. This event would allow the μ heavy chain-producing cells to continue producing IgM [21].

We then investigated if Sμ deletions were related to V_H somatic mutations, a marker of the B-cell maturation stage. Sequencing of the V_H regions of all 38 cases indicated the presence of somatic mutations in 17 cases (44.7%). No correlation between the presence of Sμ deletions and V_H gene mutations was observed, since 32% of cases with a deletion in the Sμ region showed V_H mutations, and 52% of cases without deletion in the Sμ showed V_H mutations. Therefore, it seems that Sμ internal deletions might occur independently of the B-cell maturation stage. Moreover, while a correlation of V_H mutations with disease progression was detected, confirming recent data on the possible use of V_H status as a prognostic factor [6], Sμ rearrangements alone did not appear to be indicative of clinical course.

Sμ internal deletions might represent 'failed switching attempts', as suggested by Q. Pan et al., who frequently detected internal deletions in the Sμ, Sγ4 and Sγ3 regions [27]. However, the frequent localization of Sμ internal deletions in the V_HDJ_H rearranged allele supports the hypothesis that Sμ internal deletions do not occur randomly, but have a precise role in stabilizing the isotype of the expressed Ig. Possibly, 'failed switching attempts' may themselves contribute to isotype stabilization. This mechanism can explain why only a small proportion of B-CLL cells undergo isotype switch and might be of great interest to fully understand the process of isotype switching in this leukemia. It is unknown if Sμ deletions occur in a B-cell before or after malignant transformation; however, having shown that these rearrangements are Sμ internal deletions and not chromosome translocations rules out their involvement in the onset of malignancy.

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