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ORIGINAL ARTICLE

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Analysis of microsatellite instability in chronic lymphoproliferative disorders

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Abstract Microsatellite instability (MSI) represents one specific pattern of genomic instability and is one of the genetic lesions most frequently detected in human neoplasia. Although MSI has been found to be associated with a wide variety of solid cancers, its involvement in lymphoid malignancies is virtually unexplored. In this study, we have investigated the presence of MSI in chronic lymphoproliferative disorders by comparing the pattern of nine microsatellite repeats (two tetranucleotides, two trinucleotides, and five dinucleotides) on autologous germline and tumor DNA of 23 patients, including 17 with B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL), four with hairy cell leukemia, one with lymphoplasmacytoid lymphoma, and one with T-cell chronic lymphocytic leukemia. All samples at diagnosis displayed a germline pattern of the microsatellites examined, thus suggesting that MSI is not involved in the pathogenesis of these lymphoproliferations. Also, no microsatellite alterations were observed in consecutive samples of B-CLL/SLL obtained from the same patient at various stages of the disease both before and after chemotherapy. Conversely, alterations in 3/9 microsatellite repeats were detected in one case of Richter's syndrome which had evolved from a pre-existent B-CLL/SLL phase. Overall, the low frequency of MSI among chronic lymphoproliferative disorders adds further weight to the common view that the mechanisms and patterns of genomic instability in lymphoid neoplasia differ markedly from those commonly observed in solid cancers.

Key words Microsatellite instability · Genomic instability · Chronic lymphocytic leukemia · Richter's syndrome · Lymphoma

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Introduction

Chronic lymphoproliferative disorders represent a relatively heterogeneous group of lymphoid maligancies of mature lymphoid cells which include chronic lymphocytic leukemia/small lymphocytic lymphoma, hairy cell leukemia, and lymphoplasmacytoid lymphoma [14]. Overall, the molecular pathogenesis of chronic lymphoproliferative disorders is poorly defined and, at present, no genetic lesion has been found to selectively and consistently associate with this group of lymphoproliferations [9]. On the one hand, genetic lesions specifically clustering with other malignancies of mature lymphoid cells, including rearrangements of *BCL*-1, *BCL*-2, *BCL*-6, and c-*MYC*, are consistently absent in chronic lymphoproliferative disorders [12, 20]. On the other hand, the involvement of the genetic lesions most commonly detected in human neoplasia, including *TP53* and *RAS* mutations as well as microsatellite instability [4, 8, 16], either is restricted to a small fraction of cases (in the case of *TP53* and *RAS* mutations [10, 12, 24] or has not been tested thus far (in the case of microsatellite instability).

Small DNA tandem repeat sequences known as microsatellites represent a very common and highly polymorphic class of genetic elements within the human ge-

nome. Amplification of microsatellites by polymerase chain reaction (PCR) has proved to be a fundamental tool for human gene mapping investigations, linkage studies as well as analysis of heterozygosity in human tumors [19, 30, 31]. Recently, it has been shown that the size of microsatellites in human cancers may undergo somatically acquired alterations, includinc, both expansions and deletions of repeat units [8]. Alterations in the number of units of microsatellites are likely due to a defect in DNA replication/repair mechanisms and are a peculiar type of genomic instability [8], conventionally defined as microsatellite instability (MSI).

At present, MSI represents one of the most common genetic alterations in several types of sporadic solid cancers, including carcinomas of the lung, gastrointestinal tract, bladder, skin, breast, and endometrium [1, 2, 6, 7, 13, 17, 22, 25, 26, 32, 33]. Among hematopoietic neoplasms, studies of MSI have been performed in chronic myeloid leukemia, suggesting a potential role of MSI in the transition from chronic to blastic phase [29]. Knowledge of the involvement of MSI in the pathogenesis of other hematopoietic tumors, including lymphoid malignancies, is presently lacking [8]. In this study, we have investigated the involvement of MSI in a panel of chronic lymphoproliferative disorders representative of the various clinical subtypes and stages of the disease.

Materials and methods

Pathologic samples and DNA extraction. Twenty-three cases of chronic lymphoproliferative disorders were studied, including 17 cases of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL), four cases of hairy cell leukemia (HCL), one case of lymphoplasmacytoid lymphoma, and one case of T-cell chronic lymphocytic leukemia. All patients were studied at diagnosis. Cases of B-CLL/SLL were representative of the various stages of the disease classified according to Binet's staging system: seven were stage A, six were stage B, and four were stage C. For one B-CLL/SLL patient who had progressed from stage A to stage C, multiple consecutive samples were available both before and after courses of chemotherapy. An additional B-CLL/SLL patient, who had developed Richter's syndrome in the final stages of the disease, was studied both at diagnosis and after clinical and histologic progression. Mononuclear cells were separated by density gradient centrifugation from involved organs, including peripheral blood, bone marrow, and lymph nodes. All tumor samples tested contained more than 80% neoplastic cells, as evaluated by immunophenotypic and immunogenotypic analysis. For all patients, autologous germline DNA was obtained from peripheral blood granulocytes separated by density gradient centrifugation. Genomic DNA from either tumor samples or peripheral blood granulocytes was extracted using the "salting-out" technique [23].

Analysis of immunoglobulin (Ig) gene rearrangement. Clonal analysis of Ig gene rearrangement in the pathologic specimen and peripheral blood granulocyte fraction was performed by Southern blotting, as previously described [11]. The configuration of the J_H locus was investigated by Southern hybridization of a J_H-specific DNA probe [18] to *Hind*III, *Eco*RI, and *Bam*HI genomic digests. The configuration of the J_κ locus was investigated by Southern hybridization of a J_κ-specific DNA probe [27] to *Bam*HI genomic digests. The U937 monoblastic cell line was used as a source of germline DNA.

Analysis of microsatellite instability (MSI) and oligo To determine the presence of MSI in chronic lympho disorders, we tested several nucleotide repeats, inclu tranucleotide repeats (FGA and ACTBP2), two trin peats (AR and D14S50), and five dinucleotide repea D6S265, D7S684, D5S404, D8S255). The inclusion o tranucleotide repeats is relevant, since MSI in other man cancers appears to involve these types of micros a peculiarly high frequency [21]. For all patients, th tion of each microsatellite was assessed both in t germline DNA, which had been obtained from aut ripheral blood granulocytes, and in the DNA deriv tumor sample(s). Each microsatellite was PCR amplif jected to electrophoresis. Alterations in the size of m in the tumor sample(s) were revealed as differences trophoretic migration of the PCR products derived DNA as compared with PCR products amplified fro line DNA of the same patient.

All the oligonucleotides used in this study were using an Applied Biosystem synthesizer. Sequences cleotides used as primers were derived from the G Base, Baltimore, Md., USA, and were as follows: TCCGCGAAGTGATCCAGAAC-3′, and A CTTGGGGAGAACCATTCTCA-3′ (for AR); D1 AACACCCCTAATTCACCACT-3′, and D14S5 GATTCCACAAGATGGCAG-3′ (for D14S50); CCATAGGTTTTGAACTCACAG-3′, and F CTTCTCAGATCCTCTGACAC-3′ (for FGA); AC AATCTGGGCGACAAGAGTGA-3′, and ACT ACATCTCCCCTACCGCTATA-3′ (for ACTBP2) 5′-GCCCTATAAAATCCTAATTAAC-3′, and D GAAGGAGAATTGTAATTCCG-3′ (for D6S105) 5′-ACGTTCGTACCCATTAACCT-3′, and D6 ATCGAGGTAAACAGCAGAAA-3′ (for D6S265 5′-GCTTGCAGTGAGCCGAC-3′, and D7S GATGTTGATGTAAGACTTTCCAGCC-3′ (fo Primers for D5S404 and D8S255 were obtained fr Genetics (Huntsville, Ala., USA).

Polymerase chain reaction of microsatellite repeats. P formed with 100 ng of genomic DNA, 10 pmol of eac μM dNTPs, 1 μCi of [(α-^{32}P]dCTP (Amersham, UK tivity, 3000 Ci/mmol; 1 Ci = 37 GBq), 10 mM Tris-H 50 mM KCl, 1–2.5 mM $MgCl_2$, 0.01% gelatin, 0.5 polymerase (Perkin-Elmer, Norwalk, Conn., USA) i ume of 10 μl. Thirty cycles of denaturation (94 °C), a nealing temperatures were optimized for each pair and extension (72 °C) were performed in a temperat (DNA Thermal Cycler Perkin-Elmer, Norwalk, CT reaction (2 μl) was diluted 1:25 in 0.1% NaDodS mM EDTA, and further mixed 1:1 with sequencing Samples were heated at 95 °C for 5 min, chilled on trophoresed onto a 6% acrylamide-TBE denaturing ing temperatures were 56 °C for D6S105; 57 °C for D for D14S50, FGA, D7S684, D5S404 and D8S255; and ACTBP2.

Analysis of TP53 mutations. Mutational analysis of 5–8) in the case of Richter's syndrome (case 457) w as previously reported [10]. Briefly, genomic DNA the PCR-single-strand conformation polymorphism analysis with *TP53*-specific primers [10]. For each P analyzed by SSCP, at least three samples known to tion within the fragment tested were included as pos [10].

Results

Clonal analysis. Clonal analysis of Ig gene ment in the pathologic specimens and perip

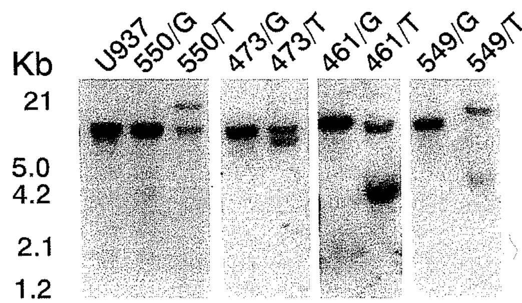

Fig. 1 Clonal analysis of immunoglobulin heavy-chain genes (Ig_H) configuration of the peripheral blood granulocyte fraction (*550/G, 473/G, 461/G, 549/G*) and tumor sample (*550/T, 473/T, 461/T, 549/T*) of patients with chronic lymphoproliferative disorders. Patients 550, 473, 461, and 549 represent cases of B-CLL/SLL. Genomic DNA was digested with *Hind*III and subjected to Southern blot hybridization with a DNA probe representative of the J_H locus 18]. *U937,* a monoblastic cell line, was used as a control of the germline configuration of Ig_H. All B-CLL/SLL tumor samples showed a monoclonal Ig_H rearrancrement, whereas only the germline Ig_H band was present in the autologous peripheral blood granulocytes

granulocyte cell fractions from patients with B-cell chronic lymphoproliferative disorders was performed by Southern blotting with a J_H and a J_κ probe tested on multiple digests. A band of clonal rearrangement was observed in all pathologic specimens (peripheral blood mononuclear cells, bone marrow, or lymph nodes). Conversely, the peripheral blood granulocyte cell fraction of all patients displayed only the germline Ig gene configuration (see Fig. 1 for representative results).

Analysis of microsatellite instability (MSI). All samples of chronic lymphoproliferative disorders tested at diagnosis displayed an identical pattern of microsatellites in the germline and in the tumor DNA (Fig. 2 and Table 1). Similarly, no alterations in the size of microsatellites were observed in one B-CLL/SLL patient who had progressed from Binet's stage A to stage C, and from whom multiple samples were studied at various stages of the disease both before and after chemotherapy. Conversely, in one B-CLL/SLL patient who had progressed to Richter's syndrome (case 457), 3/9 microsatellites displayed a size alteration in the sample of Richter's syndrome when the DNA of the Richter's phase was directly compared in the same experiment with both germline DNA and DNA derived from the previous B-CLL/SLL phase of the patient. These data suggest that the microsatellite alterations observed in case 457 had occurred during the clinical and histologic transformation of the disease (Fig. 2 and Table 1).

Analysis of the TP53 mutations. Analysis of *TP53* mutations was performed in the case of Richter's syndrome (case 457) by PCR-SSCP, as previously reported [10]. No *TP53* mutations were detected in this patient.

Discussion

Ever since the studies of Boveri [5], genomic instability has been thought of as one of the major molecular mechanisms of the neoplastic conversion of human cells. Distinct patterns of genomic instability may be potentially identified in human tumors, leading to (a) gross deletions and/or amplifications of variably sized DNA fragments; (b) recurrent chromosomal translocations consistently involving specific genetic loci; and (c) alterations of microsatellite repeats, otherwise defined as microsatellite instability [8, 15, 28]. Putatively, as already demonstrated for MSI, distinct primary genetic defects are responsible for each different pattern of genomic instability [8, 15, 28]. It is notable that lymphoid neoplasia distinctively associates at high frequency with chromosomal translocations, generally involving an antigen receptor locus, whereas the presence of gross deletions and/or amplifications, commonly observed in solid cancers, is a relatively rare event in these tumors [28]. At present, the involvement of MSI in lymphoid neoplasia is virtually unexplored [8].

In this study, we have investigated the involvement of MSI in a specific category of lymphoid malignancies, i.e., chronic lymphoproliferative disorders. Our analysis of nine microsatellite loci, including tri-, tetra-, and dinucleotide repeats, indicates that MSI is a rare event in the molecular pathogenesis of these disorders. All but

Table 1 Alterations of microsatellite repeats in chronic lymphoproliferative disorders (*B-CLL/SLL* B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, *HCL* hairy cell leukemia, *LPC NHL* lymphoplasmacytoid lymphoma, *T-CLL* T-cell chronic lymphocytic leukemia, *Richter's* Richter's syndrome)

Diagnosis	Microsatellite repeats								
	AR[a,b] (X)	D14S50[a,b] (14)	FGA[a,b] (4)	ACTBP2[a,b] (6)	D6S105[a,b] (6)	D6S265[a,b] (6)	D7S684[a,b] (7)	D5S404[a,b] (5)	D8S255[a,b] (8)
B-CLL/SLL	0/17	0/17	0/17	0/17	0/17	0/17	0/17	0/17	0/17
HCL	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
LPC NHL	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
T-CLL	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Richter's	1/1	0/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1

[a] Microsatellite repeat locus; the chromosome to which each locus maps is indicated in *brackets.*
[b] No. positive of no. tested.

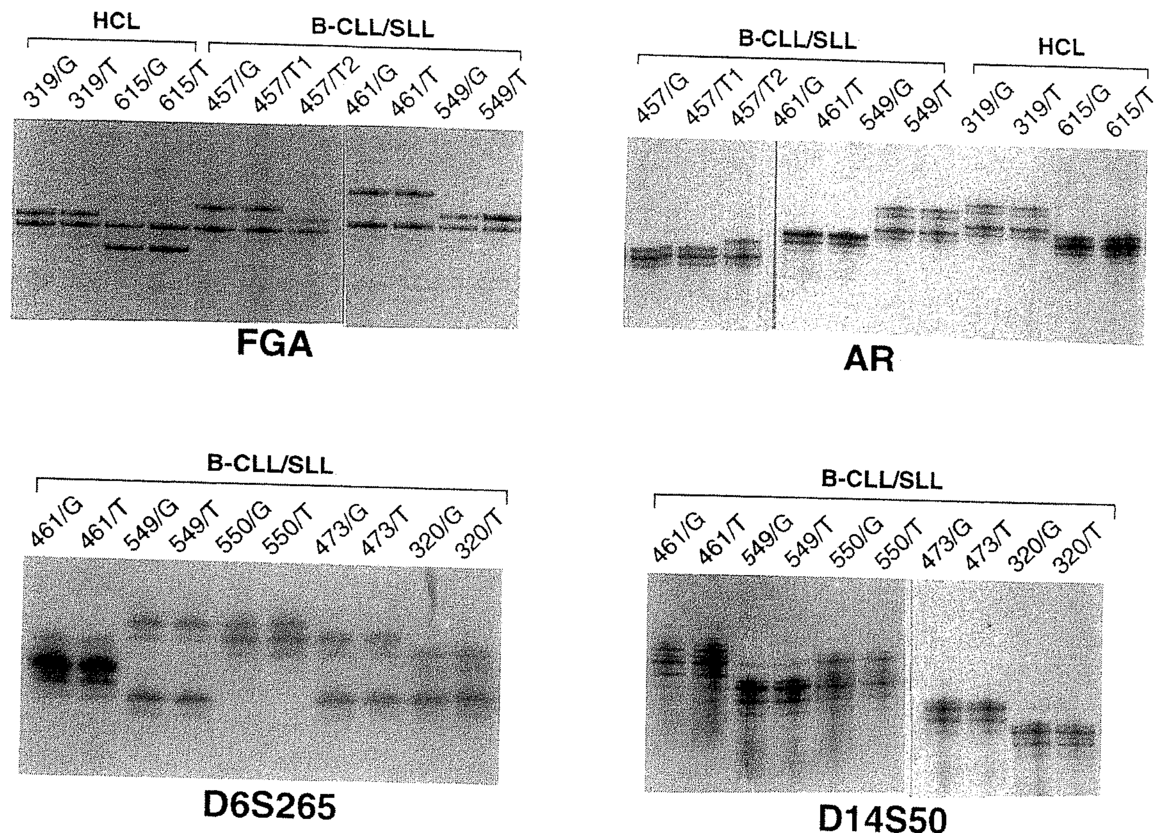

Fig. 2 Analysis of microsatellite instability in chronic lymphoproliferative disorders. Representative microsatellite repeats are shown, including *FGA, AR, D6S265,* and *D14S50.* Each patient is indicated by a *numbered code,* followed by "*G*" (in the case of germline DNA), or "*T*" (in the case of tumor DNA). No alterations of the microsatellites tested were observed in cases at diagnosis (selected cases of *B-CLL/SLL* and *HCL* are shown). Conversely, patient 457, who had processed from B-CLL (sample *457/T1*) to Richter's syndrome (sample *457/T2*), displayed size alterations at selected microsatellites (*FGA* and *AR* are shown), as derived by the different migration pattern of the microsatellites in the tumor DNA (sample *457/T2*) as compared with the autologous germline DNA (sample *457/G*). The microsatellite alterations of patient 457 were present only in the sample of Richter's syndrome (sample *457/T2*), but not in the B-CLL sample (sample *457/T1*), suggesting that MSI had accumulated during tumor progression. For details, see text. (*B-CLL/SLL* B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, *HCL* hairy cell leukemia)

one case displayed only a germline pattern at the microsatellites tested. The one case which scored positive for MSI involved a patient with Richter's syndrome, representing the clinical and histologic progression of a pre-existent B-CLL phase. Since the B-CLL sample of this same patient displayed a germline pattern at the microsatellites examined, it is likely that MSI had developed during tumor progression. Whether MSI is a general feature of the transition from B-CLL to Richter's syndrome will be clarified by future studies on larger numbers of patients for whom consecutive DNA samples, including germline, B-CLL, and Richter's syndrome DNA, may be available.

The virtual absence of MSI in chronic lymphoproliferative disorders is in contrast to the high frequency of MSI in most solid cancers tested to date [1, 2, 6–8, 13, 17, 22, 25, 26, 32, 33]. MSI appears to be also consistently absent in other neoplasms of mature cells of the general population, as derived fro of Bedi et al. on high-grade B-cell lymphoma our preliminary analysis of the clinical and spectrum of non-Hodgkin's lymphomas [3, a published observation]. Overall, these data a weight to the common view that the mecha patterns of genomic instability in lymphoid markedly differ from those commonly observ other types of human tumors, namely solid

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