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Microsatellite instability analysis in typical and progressed mantle cell lymphoma and B-cell chronic lymphocytic leukemia

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Background and Objectives. Microsatellite instability (MSI) is characterized by tumor-associated alterations in the germline size of microsatellite repeats caused by a reduced efficacy of the DNA mismatch repair machinery. The aim of this study was to investigate the presence of MSI in a number of cases of indolent and aggressive mantle cell lymphoma (MCL) and B-cell chronic lymphocytic leukemia (B-CLL) to determine its possible role in the initial development and progression of these disorders.

Design and Methods. We examined the presence of MSI in 28 B-CLL, 24 typical and 4 transformed B-CLL (Richter's syndrome) and 29 MCL, 19 typical and 10 blastoid variants by using a panel of 10 microsatellite markers and analyzed them using an AbiPrism 310 DNA sequencer. Fisher's exact test was used to compare categorical variables and Mann-Whitney's U-test for continuous variables.

Results. MSI alterations were not observed in any case of MCL or Richter's syndrome and in only three (13%) patients with typical B-CLL. Two of these patients also had loss of heterozygosity in one of the 10 sites examined. These patients presented with a more advanced stage, diffuse bone marrow involvement, and poorer performance status than patients without these alterations.

Interpretation and Conclusions. These findings indicate that MSI is not involved in the pathogenesis or progression of B-CLL and MCL but may appear in a small subset of patients with advanced B-CLL.

Key words: microsatellite instability, B-cell chronic lymphocytic leukemia, mantle cell lymphoma, Richter's syndrome, loss of heterozygosity

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Microsatellites are short, tandemly repeated DNA sequences usually located in non-coding areas of mammalian genomes. Generally, these di-, tri-, or tetranucleotide repeat alleles are stably inherited from one generation to another and many of them exhibit length polymorphisms. Microsatellite instability (MSI) is a type of genomic instability characterized by tumor-associated alterations in the germline size of microsatellite repeats due to reduced efficacy of the DNA mismatch repair genes.[1] This genetic alteration was initially identified in the hereditary non-polyposis colorectal cancer syndrome (HNPCC).[2] The multiple replication errors in these patients are caused by the presence of hereditary and somatic mutations in different genes of the mismatch repair machinery.[3,4] MSI has also been detected in 10-15% cases of sporadic colorectal cancer[5,6] and other solid tumors.[7,8]

In hematologic malignancies, MSI seems to be relatively uncommon but has been detected in different myeloid disorders and small subsets of non-Hodgkin's lymphomas.[9-13] It has been suggested that MSI in combination with other cytogenetic changes could be associated with the progression of chronic and acute myeloid leukemias[14-16] but its possible role in the progression of lymphoproliferative disorders is not well known. Mantle cell lymphoma (MCL) and B-cell chronic lymphocytic leukemia (B-CLL) are two lymphoproliferative disorders derived from $CD5^+$ B-lymphocytes. Relatively indolent and aggressive forms of MCL may be identified; these are generally associated with a typical and blastoid morphology of the tumor cells, respectively.[17] Likewise, typical B-CLL may transform into aggressive large cell-lymphomas (Richter´s syndrome) clinically associated with a poor response to therapy.[18] Different genetic studies have observed a series of primary and secondary chromosomal and molecular alterations in these neoplasms.[19-24] However, the possible role of MSI in the pathogenesis and progression of MCL has not been previously analyzed and only a few studies have examined this type of genetic alteration in typical and transformed B-CLL.[25-27] In this study, we inves-

tigated the presence of MSI in a number of cases of indolent and aggressive MCL and B-CLL in order to determine its possible role in the initial development and progression of these disorders.

Design and Methods

Selection of cases

Paired normal DNA and tumor DNA from 29 MCL, 24 B-CLL and 4 cases of Richter's syndrome were analyzed. These cases were obtained from the Hematopathology Unit of the Hospital Clinic of Barcelona and from the University Hospital Grosshadern of Munich (10 cases of MCL).

The MCL patients were 19 males and 10 females with a median age of 61 years (range, 40 to 88 years). Twenty-four patients were in stage IV of the Ann-Arbor scale, two in stage II and one in stage I. Nineteen cases were classified as typical and 10 as blastoid variants, according to previously described criteria.[17] The immunophenotype of the tumors was analyzed using immunohistochemistry on tissue sections and/or cell suspensions by flow cytometry. All cases were positive for cyclin D1 expression by Northern blot and/or immunohistochemistry analysis.[28] Bcl-1 rearrangement was examined as previously described.[29]

B-CLL was diagnosed according to standard criteria.[30] The main characteristics of these patients are summarized in Table 1. Fifteen patients were males and 13 females. Their median age was 65 years (range, 44 to 84 years). The cases were categorized according to the Binet classification[31] (15 cases were in stage A, 5 in stage B, and 8 in stage C) and the Rai classification[32] (9 cases were in stage 0; 8 cases in stage I; 3 cases in stage II; 2 cases in stage III and 6 cases in stage IV). Cytogenetic analysis was performed on short-term cultures of bone marrow or peripheral blood cells in 12 B-CLL patients. Eight of them showed an abnormal cytogenetic pattern. Four cases showed only one cytogenetic alteration and the other 4 displayed a complex karyotype. Trisomy 12 was detected in 3 cases and deletion in the long arm of chromosome 13 at band q14q22 in one case (Table 2).

DNA extraction

Tumor DNA from MCL patients was obtained from frozen lymph nodes in 18 cases, formalin-fixed and paraffin-embedded specimens in 7 cases, and mononuclear cells from peripheral blood in 4 leukemic cases. Tumor DNA from B-CLL patients was obtained from peripheral mononuclear cells in all patients. Mononuclear cells were isolated from peripheral blood samples by centrifugation on a Ficoll/Hypaque (Seromed, Berlin, Germany) gradient. DNA was extracted from frozen lymph nodes and peripheral blood using the standard Proteinase K/RNAse treatment and phenol-chloroform extraction. DNA was extracted from paraffin-embedded tissue using the QIAamp tissue kit (Qiagen, Inc., CA, USA). Constitutional control DNA of each patient was extracted from peripheral blood granulocytes separated by density gradient centrifugation. Normal DNA was only selected after an analysis of immunoglobulin (Ig)

Table 1. Principal characteristics of the 28 B-CLL patients.

Patient	*Age/sex*	*Stage*	*WBC (x10³)*	*Cytogenetics*
B-CLL-1	57/m	A(0)	17.8	Normal
B-CLL-2	71/f	A(0)	8.8	-
B-CLL-3	49/f	A(0)	12	45, XX, -21, del(8)(p12p23)[15] 45,XX,-21,der(8)t(8;21) (p12;q11)[6] 46, XX,der(8)t(4;8)(q22;p21)[2] 46, XX[22]
B-CLL-4	59/f	A(0)	19.4	46, XX[12] 45, X, -X[4] 46, XX, del (13)(q14q22)[2]
B-CLL-5	84/f	A(0)	13.9	-
B-CLL-6	64/m	A(0)	22.7	-
B-CLL-7	74/m	A(0)	11.5	47,XY[26] 47,XY,+12,t(14;18)(q32;q21)[24]
B-CLL-8	71/m	A(0)	14.4	47, XY, +12 [2] 46, XY [48]
B-CLL-9	49/m	A(I)	51.2	Normal
B-CLL-10	84/f	A(I)	11.6	46, X, -X, +12 [42] 46, XX [8]
B-CLL-11	69/m	A(I)	23.1	-
B-CLL-12	57/m	A(I)	39.9	Normal
B-CLL-13	58/m	A(I)	33.6	Normal
B-CLL-14	60/m	B(I)	49.7	-
B-CLL-15	78/f	B(II)	3.9	-
B-CLL-16	75/f	B(II)	20.1	-
B-CLL-17	65/f	B(II)	22.6	46, X, -X, +12 [42] 46, XX [8]
B-CLL-18	67/f	C(III)	392	-
B-CLL-19	44/f	C(III)	348	-
B-CLL-20	75/m	C(IV)	119.2	46, XY [46] 46, XY, t(1;2)(q23;q37) [2] 46, XY, t(1;8) (q23;p23) [1] 46, XY, t(1;1) (p13;p36) [1]
B-CLL-21	75/f	C(IV)	14.8	-
B-CLL-22	65/h	C(IV)	334	46, XY dup (4) (q21q34) [7] 46, XY [12]
B-CLL-23	72/d	C(IV)	125	-
B-CLL-24	77/m	C(IV)	56.9	-
RS-1	53/m	A(0)	50.23	-
RS-2	44/m	A(I)	16	-
RS-3	65/f	B(I)	5	-
RS-4	59/m	C(IV)	107	-

Table 2. Microsatellites, chromosomal localization and closer gene associated with the microsatellite markers used in this study.

Marker	*Chromosome*	*Gene near marker*
D8S254	8p22	Unknown
NM23	17q21	NM23-H1
D18S35	18q21	DCC
TP53-Dint	17p13	p53
D5S346	5q21	APC
TP53-Penta*	17p13	p53
D2S123	2p16	MSH2
D1S2883	1q24	HPC1
D3S1611	3p22	MLH1
D7S501	7q31	MET

**Pentanucleotide*

rearrangement to exclude cases with contamination of tumor cells in the granulocyte cell fraction. This analysis was performed by the amplification of the CDRIII/JH region of the immunoglobulin heavy chain gene by polymerase chain reaction (PCR).

Microsatellite analysis

Normal and tumor DNA were analyzed using the RER/LOH assay kit (PE Biosystems) for detection of microsatellite instability (MSI) or loss of heterozygosity (LOH). Each DNA pair was analyzed using a panel of 10 microsatellite markers (Table 2). Nine of the markers were dinucleotide repeats (CA)n, and one was a pentanucleotide repeat-(AAAAT)n. One of each pair of PCR amplification primers was fluorescently labeled with either FAM (blue), TET (green) or HEX (yellow) dyes. PCR amplification was performed in a 20 µL reaction mixture containing approximately 25 ng of DNA using the conditions described by the manufacturers. After an initial AmpliTaq Gold activation step at 95ºC for 10 min, 45 PCR cycles were performed at 96ºC for 10 sec, 55ºC for 30 sec and 70ºC for 3 min followed by a 30 min at 70ºC of final extension. Amplified products from the ten PCR reactions were pooled and analyzed on an AbiPrism 310 DNA sequencer (PE Biosystems). Amplified products were electrophoresed for 24 min at 15.0 kV, 9.0•A, 60ºC in a 47-cm length capillary using the performance optimum polymer 4 (POP4) with the laser set at a constant power of 9.9 mW. The resulting data were analyzed for peak color and fragment size using the Genescan Fragment Analysis and the Genotyper software (PE Biosystems) according to the manufacturer's guidelines. MSI was scored if novel peaks differing in size from those seen in corresponding normal DNA were present in tumor DNA. The RER index (replication error) was calculated for each marker as the sum of alleles added together for the normal/tumor pair. A normal/tumor pair for each marker was assessed as: 1) reaction failure when the sum of alleles was 0; 2) RER negative when the RER index was <2 or equal to 4; and 3) RER candidate in the other cases. LOH index was calculated as the ratio of normal and tumor allele peak height. The normal/tumor pairs were assessed as: 1) Not informative when the marker was homozygous; 2) Reaction failure when the sum of alleles was 0; 3) LOH negative when LOH Index was between 0.67 and 1.5; and 4) LOH candidate in the other cases.

Statistical analysis

The B-CLL patients were divided according to the presence or not of MSI and LOH. The following variables were analyzed for each group: age, sex, performance status, B-symptoms, hemoglobin level, white-cell blood (WBC), lymphocyte and platelet counts, lymphocyte morphology, percentage of CD19, CD23 and CD5-positive lymphocytes, serum albumin, β_2-microglobulin and LDH levels, Rai and Binet stages, Richter transformation, cytogenetic alterations and response to therapy. Fisher's exact test was used to compare categorical variables and Mann-Whitney's U-test for the continuous variables.

Results

Normal and tumor DNA from 29 patients with MCL, 24 patients with B-CLL, and 4 cases of Richter's syndrome, before and after transformation, were examined for possible alterations in a panel of 10 microsatellites. None of the 19 typical or 10 blastoid MCL showed genetic alterations in any of the 10 microsatellites analyzed. However, replication errors were detected in 3 of the 24 (13%) typical B-CLLs (cases B-CLL-2, 18 and 20). One case (B-CLL-20) showed alterations in two microsatellites while the other two patients did so only in one locus (Table 3). None of the patients showed instability in 30% or more of the analyzed locus. Therefore, according to previous international criteria,[33] these cases showed a low level of MSI and did not fulfill the requirement to be considered as tumors with a replication error phenotype (RER+). No alterations were detected in the four cases in which the two samples from the typical B-CLL and subsequent transformation to a large cell lymphoma (Richter's syndrome) were examined.

The loci showing microsatellite instability in these three cases were D1S2883, a locus associated with the *HPC1* (hereditary prostate cancer 1) gene, in two cases (B-CLL-2 and B-CLL-20), D2S123 microsatellite located inside one intron of the *hMSH2* gene, in one case (B-CLL-20), and TP53-Dint locus located inside one intron of the *p53* tumor suppressor gene, in one case (B-CLL-18) (Figure1)(Table 3). Case B-CLL-20 with RER at two loci also showed a complex karyotype in the cytogenetic study (Table 1).

In addition to the MSI alterations, two of the 28 B-CLL patients (B-CLL-18 and 20) also had LOH at one of the 10 loci examined (Table 3). In the two cases the losses were detected at the NM23 microsatellite located at 17q21, inside one exon of the NM23 gene, a putative metastasis suppressor gene[34] (Figure1). No LOH was detected in any of the loci analyzed in patients with MCL.

The B-CLL patients with microsatellite alterations presented more frequently than the others with an advanced Binet stage: stage C 2/8 (25%) vs stages A+B 1/20 (5%), a diffuse pattern of bone marrow involve-

Table 3. Microsatellite alterations in three B-CLL patients.

	Patients		
Microsatellite	*B-CLL-2*	*B-CLL-18*	*B-CLL-20*
D8S254	–	–	–
NM23	–	LOH	LOH
D18S35	–	–	–
TP53–Dint	–	RER	–
D5S346	–	–	–
TP53–Penta	–	–	–
D2S123	–	–	RER
D1S2883	RER	–	RER
D3S1611	–	–	–
D7S501	–	–	–

RER: replication error, LOH: loss of heterozygosity.

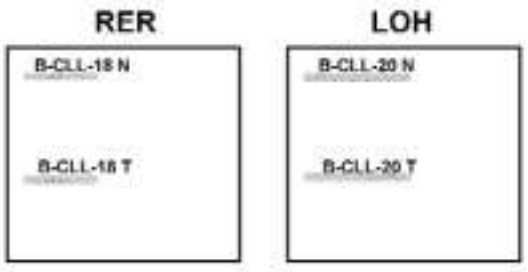

Figure 1. Microsatellite instability in paired normal and tumoral DNAs from B-CLL-18 at locus TP53-Dint and loss of heterozygosity in paired normal and tumoral DNAs from B-CLL-20 at locus NM23.

ment: 2/2 (100%) vs non-diffuse pattern 0/18 (0%) and a poor performance status: 2/3 (67%) vs 1/24 (4%) (p<0.05, in all cases). No significant differences were observed in other clinical aspects or in the survival of the patients. However, one of the patients (B-CLL-18) with MSI alterations died of progression of disease one year after the diagnosis and another (B-CLL-2) progressed to a Richter's syndrome 6 years after diagnosis.

Discussion

In this study, we have analyzed the potential role of MSI in the pathogenesis and progression of B-CLL and MCL. Replication errors were detected in only 3 of the 24 typical B-CLL (13%), whereas no alterations were observed in the 4 B-CLL transformed into large-cell lymphoma (Richter's syndrome) nor in the 19 typical and 10 blastoid MCL. The incidence of microsatellite instability in this series of tumors is relatively low and the three cases with microsatellite alterations showed instability in less than 30% of the loci analyzed. Two MSI phenotypes have been described in colorectal cancer, MSI-High (MSI-H: instability at >30% of the loci examined) and MSI-Low (MSI-L: instability at 1-30% of the loci examined).[33] The MSI-H phenotype is associated with distinct clinicopathologic characteristics of the tumors and high frequency (>95%) of inactivation of the *hMLH1* and *hMSH2* mismatch repair genes. In contrast, sporadic tumors with MSI-L phenotype do not show inactivation of these genes and seem to have features similar to those of other neoplasms without MSI.[33,35] According to these criteria, the three cases with MSI in our study should be considered as tumors with a MSI-L phenotype. Although the significance of these two phenotypes in tumors other than colorectal carcinomas is not well known, it seems unlikely that the low number of altered loci in our cases might be associated with genetic alterations in mismatch repair genes. Different studies have analyzed cytogenetic and molecular alterations in MCL.[17] However, the possible role of MSI in the pathogenesis of these tumors had not been previously investigated. In this study we have demonstrated a lack of replication errors in these tumors in both typical and blastoid variants. Similarly, in our study B-CLL cases did not have or only displayed very few microsatellite alterations. These findings are concordant with other studies on B-CLL.[25-27] However, a high level of MSI has been documented in 2 cases of B-CLL.[26] Interestingly, these 2 tumors showed replication errors in virtually all loci examined suggesting that MSI may participate in the pathogenesis of occasional cases of B-CLL.

The low level of microsatellite alterations in B-CLL and MCL observed in our series is concordant with other studies of MSI in chronic lymphoproliferative disorders.[25,26,36-38] Most of the NHL investigated in previous studies[27,36,38,39] have shown negative or low levels of MSI. Initial studies on gastric MALT lymphomas,[11,12] post-transplant lymphoproliferative disorders,[13] and NHL associated with HIV[9,10] found a high rate of MSI in a relatively large number of cases. However, these findings are controversial and have not been confirmed in recent studies using more restrictive criteria or a higher number of microsatellite loci.[27,40-43]

The absence of MSI-H in B-CLL and MCL as well as in other NHL is in contrast with the presence of these alterations in 10-20% of certain sporadic solid tumors. Inactivation of mismatch repair genes and progressive accumulation of replication errors has been postulated as a molecular pathway of oncogenesis alternative to the more common pathway involving activation of oncogenes and inactivation of tumor suppressor genes.[1] Solid tumors, particularly colorectal carcinomas, with a MSI-H phenotype have a significantly lower incidence of *p53* inactivation and *ras* mutations than microsatellite stable tumors.[1,6] The low level of MSI in MCL and B-CLL, as in other lymphoid neoplasms, is concordant with this model since cytogenetic and molecular studies have defined a number of chromosomal alterations associated with the activation of oncogenes and inactivation of tumor suppressor genes in the pathogenesis of these neoplasms.[19-24]

Several studies have suggested that MSI may participate in the progression of different types of tumors[7,8] but the relationship between replication error and tumor progression in lymphoid neoplasms has not been extensively investigated. MSI has been previously observed in a case of Richter's syndrome with alterations in 30% of the loci examined, which were not present in the indolent B-CLL phase of the patient's disease.[25] However, in our study we found no alterations in any of the loci analyzed in four cases of large cell lymphoma transformed from B-CLL. In addition, none of the 10 blastoid variants of MCL investigated showed microsatellite alterations. Similarly, Gamberi *et al.* did not observe MSI alterations in the histologic or clinical progression of 11 follicular lymphomas.[27] These findings suggest that, in contrast to some myeloid disorders,[14-16,44,45] MSI does not play a role in the progression of lymphoid neoplasms. Interestingly, our patients with replication errors had poor performance status, advanced stage and a diffuse pattern of bone marrow involvement. However, the possible relationship between these clinical characteristics and the

low level of MSI in these patients is not clear because two of them also had LOH at some loci and one had a complex karyotype, suggesting that other mechanisms could be implicated in the progression of the disease.

In conclusion, the findings of this study indicate that MSI is not involved in the pathogenesis or progression of B-CLL and MCL but a low level of MSI may appear in a small subset of patients with advanced B-CLL in association with other genetic alterations.

Contributions and Acknowledgments

LLSV participated in the design of the study and executed the experimental work, analyzed the data and wrote the first draft of the manuscript. DC was the principal author responsible for the interpretation of the experimental work. MHD and GO contributed with 10 cases of MCL and revised the paper. FB and ALG contributed to the study design, data analysis and follow-up of the patients. EM contributed to the study design and gave his final approval to the manuscript. EC conceived and co-ordinated the study and wrote the final version of the manuscript. All the authors revised the paper. The order of the authors reflects their contribution to this study in their own center, except for EM and EC who are the senior authors and heads of the departments in which the major part of the study was performed.

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Disclosures

Conflict of interest: none.

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Potential implications for clinical practice

No implications for clinical practice; this is a biological study.

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