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Detection of single and associated lesions of the Bcl-1, Bcl-2, Bcl-6, c-myc, p53 and p16 genes in B-cell non-Hodgkin's lymphomas: value of molecular analysis for a better assignment of the histologic subtype

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Abstract

Background and Objective. **Molecular genetic abnormalities have been frequently described in non-Hodgkin's lymphomas (NHL). These lesions have been associated with specific entities, allowing a better categorization of NHL. However, these abnormalities are not as specific as initially described and their association is still unknown.**

Design and Methods. **By Southern blot and polymerase chain reaction, we have simultaneously analyzed the proto-oncogenes Bcl-1, Bcl-2, Bcl-6, c-myc and MLL and the tumor suppressor genes p53 and p16, in 100 unselected B-cell NHL patients at diagnosis, to establish its incidence throughout the different NHL subtypes, defined both by Working Formulation and REAL classifications, and to assess the frequency of co-existence of two or more genetic lesions within each individual patient.**

Results. **Fifty two cases displayed some genetic abnormality. Bcl-1, altered in 12 cases, was highly specific to mantle cell lymphomas (57% of them), but 6 cases had a different histologic subtype. Bcl-2 was rearranged in 26 cases: 70% in follicular lymphomas (FL) and 20% in diffuse large cell lymphomas; these abnormalities were also present in other subtypes, i.e. marginal lymphomas (30%). Bcl-6 abnormalities were mostly found in diffuse large cell lymphomas (29%) but also found in other subgroups, like FL (14%). C-myc rearrangements were specific to Burkitt's lymphoma. MLL gene was always germline. Deletions and/or rearrangements of p53 and p16 genes were rare (4% and 8% of all cases, respectively). Finally, association of genetic lesions was a relatively common finding (13% of cases), especially in cases with adverse prognostic morphologies according to the REAL.**

Interpretation and Conclusions. **Molecular abnormalities are frequent in NHL at diagnosis, not only as unique lesions but also associated. A relative high specificity of some alterations was seen, thereby contributing to a better assessment of the histological subtype.**

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Experimental data support the view that carcinogenesis is a multistep process that involves activation of proto-oncogenes as well as inactivation of tumor-suppressor genes.[1] In past years, molecular characterization of non-random cytogenetic abnormalities has led to the identification of several genes whose alterations represent a very important mutational mechanism in non-Hodgkin's lymphomas (NHL).[2] That information has resulted in recognition of new entities and refinement of previously recognized disease entities.[3] For example, the follicular lymphomas (FL) are characterized by the t(14;18) translocation, present in 40-85%, moving the long arm of chromosome 18 to the long arm of chromosome 14.[4-6] Similarly, in about of 60% of mantle cell lymphomas (MCL), the t(11,14) translocation involves a rearrangement of the CCND1/Bcl-1 gene.[7,8] In addition, almost all Burkitt's lymphomas have a t(8;14), t(8;22) or t(2;8) in which the *c-myc* gene translocates from chromosome 8 to chromosomes that harbor the immunoglobulin heavy or light chain genes.[9-11] Bcl-6 gene rearrangements have been recently ascribed to diffuse large cell lymphomas (DLCL).[12,13] All this data has contributed to a better categorization and management of NHL, but some of these abnormalities are not as specific as initially described. For example, the t(14;18) translocation has also been found in one third of DLCL[14,15] and the t(11;14) is present in some NHL different from the MCL.[16-18] Thus, in order to define the real incidence and specificity of these molecular markers, comprehensive studies are needed in which several genes are simultaneously analyzed in unselected series of NHL. Moreover, this type of studies will help to reveal associations of genetic lesions which may contribute to the understanding of the pathogenesis of the disease.

In addition, there are some proto-oncogenes such as mixed lineage leukemia (MLL) and tumor sup-

pressor genes like p53 and p16 that have been shown to be altered in closely related disorders, such as lymphoid leukemias.[19-22] Their real incidence NHL and their possible relationship with specific histological subtypes are still unknown.

In the present study, we have simultaneously analyzed the presence of abnormalities of the proto-oncogenes Bcl-1, Bcl-2, Bcl-6, *c-myc* and MLL and the tumor suppressor genes p53 and p16 in a series of 100 untreated NHL. We then correlated them with histologic subtypes defined according to two classifications (Working Formulation and REAL classifications).

Materials and Methods

Patients

High molecular weight DNA was obtained from tumor specimens of 100 adult untreated patients consecutively admitted to the University Hospital of Salamanca between July 1989 and July 1996 with the histopathological and immunohistochemical diagnosis of B-NHL. The distribution of patients into category groups was made according to the Working Formulation criteria.[23] When the new REAL classification became available,[3] all cases were reviewed and redistributed accordingly to these new criteria.

Tissue samples

Fresh samples from lymph nodes, bone marrow or peripheral blood were selected for the analysis based on the number of tumor cells, rejecting those with less than 25% neoplastic cells. The number of tumor cells was counted by flow cytometric analysis based on their κ/λ and B cell antigen expression.[24] The presence of clonal B cells was also assessed by detection of IgH clonal rearrangements with Southern blot analysis (JH6 probe)[25] in order to confirm not only the B-cell clonality of the disease but also the presence of sufficient clonal cells in the sample.

DNA preparation and Southern blot (SB) analysis

High molecular weight DNA was isolated by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.[26] For Southern blot analysis, 10 µg of DNA were digested to completion with the Eco RI, Bam HI, Hind III, Bgl II and Xba I restriction enzymes, size fractionated in 0.8% agarose gel, denatured, neutralized, and transferred to nylon membranes (Hybond-N+, Amersham, Little Chalfont, UK). The blots were hybridized with the appropriate ready to go ^{32}P-labeled probes according to the manufacturer's specifications (Ready to go, Pharmacia Biotech, Upsala, Sweden), washed twice

Table 1. DNA probes and restriction enzymes for detection of the different gene rearrangements.

Genes	*DNA probe*	*Size of insert in Kb (restriction enzymes)*	*Restriction enzymes used for digestion of genomic DNA*	*Size of germline restriction fragments in Kb*	*Refs.*
IgH	IgHJ6	1.02 (Eco RI/Hind III)	Bgl II Bam H1/Hind III	3.8 6.0	25
Bcl-1	MTC	2.3 (Sac I-Sac I)	Eco RI Hind III Bam HI	12.5 11.0 and 2.8 21.0	4
Bcl-2 (MBR)	PFL1	1.5 (Eco RI/Hind III)	Bam HI Eco RI Hind III	20.0 3.0 4.0	5
Bcl-2 (mcr)	PFL2	4.0 (Eco RI/Eco RI)	Hind III Eco RI	13.0 3.9	27
Bcl-2 (vcr)	pB16	1.6 (Eco RI/Eco RI)	Hind III Bam HI	8.0 4.0, 1.5	28
Bcl-6/LAZ3	Bcl-6	4.0 (Sac I-Sac I)	Bam HI Xba I	11.0 14.0	12
MLL	11q23	0.8 (Bam HI-Bam HI)	Bam HI	8.3	27
c-myc	cD1A	2.0 (EcoR1-EcoR1)	Hind III Bgl II	10.0 6.0, 5.8	38
MTS1/p16	p16T (cDNA)	0.8 (Eco RI-Xho)	EcoR1 Hind III	4.2 6.0	39
p53	cDNA	1.7 (Bam HI-Bam HI)	Eco RI	15.0 & 3.7	40

in 0.5% SSC, 1% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C, and autoradiographed between 48 and 96 hours at –80°C. Bcl-1, Bcl-2 (MBR, *mcr*, vcr), Bcl-6, *c-myc*, MLL, p53 and p16 genes were studied using this methodology. Probes used to detect rearrangements and deletions on these genes, together with the restriction enzymes used for digestion of genomic DNA and size of germline restriction fragments detected with them are shown in Table 1.[4,5,12,19,25,27-31] In order to exclude the restriction fragment length polymorphisms, digestion with two or more restriction enzymes was carried out in every gene.

Polymerase chain reaction (PCR) to detect the t(11;14) and t(14;18) translocations

The amplification of the breakpoint regions of both translocations was performed by PCR with 1.5 µg of genomic DNA and the oligonucleotides as shown in Table 2,[6-8] using the specifications of Molot *et al*.[7] for the t(11;14) translocation and Gribben *et al*.[6] for the t(14;18) translocation, slightly modified. PCRs for t(11;14) translocation were performed in a 50 µL final volume containing: 100 nmol/L of oligonucleotide primers, 200 µmol each of deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$ and 1.5 U of Taq polymerase (Promega, Madison, WI, USA) in PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 1% Triton). The same conditions were used to detect the t(14;18) translocation, except for oligonucleotide primers (20 nmol/L) and $MgCl_2$ (2.25 mmol/L). For t(11;14) translocation, each amplification included a first PCR using the external primers JHc-ext and MTC-ext, followed by a re-amplification of a 5 µL aliquot of the first PCR product using the JHc-int and MTC-int primers. In both amplifications, the initial denaturation was performed over 5 minutes at 94°C and amplified for 35 cycles in a Perkin Elmer Cetus 9600 thermal cycler (1 minute of denaturation at 94°C, 1 minute of annealing at 55°C and 1 minute of extension at 72°C). The final extension period was extended to 10 minutes at 72°C. In case of t(14;18) translocation amplifications of both, MBR and *mcr*, breakpoints were carried out using a first PCR with the JH consensus external primer together with the Bcl-2 MBR-ext or Bcl-2 *mcr*-ext primers. The initial denaturation was performed for 5 minutes at 94°C followed by 30 cycles, each cycle consisting of 1 minute at 94°C, 1 minute at 55°C for the MBR or 58°C for the *mcr* amplification, and 1 minute at 72°C. The final extension was developed for 10 minutes at 72°C. In each case, a re-amplification of a 5 µL of the first PCR product was carried out for 30 cycles using oligonucleotide primers internal to the original primers (JHc-int and Bcl-2 MBR-int or Bcl-2 *mcr*-int) with 1 minute of denaturation (94°C), 1 minute of annealing at 58°C and 1 minute of extension at 72°C. The initial denaturation and final extension were again for 5 minutes at 94°C and 10 minutes at 72°C, respectively.

PCR products from the final reactions were analyzed by electrophoresis in 1.5% agarose gel (Nu Sieve, FMC, Rockland, ME, USA) in tris-borate electrophoresis buffer and visualized by staining with the ethidium bromide under UV light. In all experiments, two negative controls (sterile distilled water and normal DNA) and a positive control (genomic DNA from a positive patient diluted 10^{-3}, 10^{-4} and 10^{-5} in normal DNA) were used. The final sensitivity was $10^{-4}/10^{-5}$ in all cases.

Table 2. Oligonucleotides used for the detection of the t(11;14) and t(14;18) translocation by PCR-Nested.

Name	*Translocation*	*Localization*	*Sequence*	*Refs.*
Bcl-1 MTC-ext	t(11;14)	MTC	5'tcaggccttgatagctcg3'	7
Bcl-1 MTC-int	t(11;14)	MTC	5'gaaggacttgtgggttgct3'	8
Bcl-2 MBR-ext	t(14;18)	MBR	5'cagccttgaaacattgatgg3'	6
Bcl-2 MBR-int	t(14;18)	MBR	5'tatggtggtttgacctttag3'	6
Bcl-2 mcr-ext	t(14;18)	mcr	5'cgtgctggtaccactcctg3'	6
Bcl-2 mcr-int	t(14;18)	mcr	5'ggaccttccttggtgtgttg3'	6
JHc-ext	t(11;14) t(14;18)	JH JH	5'acctgaggagacggtgaccagggt3'	6
JHc- int	t(11;14) t(14;18)	JH JH	5'accagggtcccttggcccca3'	6

Results

Overall, 52% of the patients displayed some genetic abnormality. Tables 3 and 4 show these genetic lesions and their distribution along the WF and REAL classifications.

Bcl-1

Abnormalities of the Bcl-1 proto-oncogene were identified in a total of 12 cases, 8 of them by SB and 10 by PCR. Thus, two cases were positive by SB only, and four cases were positive by PCR only, while the remaining 6 cases were positive by both techniques. Concerning the histological subtype, four cases were diffuse small cleaved cell lymphomas (SCCL), three diffuse large cell lymphomas (DLCL) and five small lymphocytic lymphomas (SLL). According to the REAL classification, 6 of them corresponded to mantle cell lymphomas (MCL), 3 were DLCL, 2 marginal lymphomas (ML) and one small lymphocytic lymphoma (SLL). Thus, 6 out of 11 MCL (57%), 3/25 DLCL (12%) and 2/9 ML (22%) had abnormalities of the Bcl-1 proto-oncogene. Interestingly, the six MCL have both SB and PCR positive results while in the other six patients, alterations of Bcl-1 were detected either by SB or by PCR but not with both, indicating that these cases either had a different involved region

Table 3. Genetic abnormalities in NHL patients according to the Working Formulation.[23]

Type	n	Bcl-1	Bcl-2	Bcl-6	c-myc	p53*	p16
Small lymphocytic lymphoma	32	5	4	2	–	2	3
Predominantly small cleaved cell follicular lymphoma	9	–	8	2	–	–	–
Mixed follicular lymphoma	8	–	6	1	–	–	–
Predominantly large cell follicular lymphoma	4	–	1	–	–	–	–
Diffuse lymphoma small cleaved cell	11	4	1	–	1	–	–
Diffuse mixed lymphoma	5	–	–	–	–	–	–
Diffuse large cell lymphoma	15	3	3	4	–	1	2
Immunoblastic lymphoma	4	–	1	1	–	–	–
Lymphoblastic lymphoma	2	–	–	–	–	–	–
Small non-cleaved cell lymphoma	7	–	2	2	6	1	2
Unclassified	3	–	–	–	–	–	1
total	100	12	26	12	7	4	8

**p53/PCR-SSCP analysis not performed.*

Table 4. Genetic abnormalities in NHL according to the REAL classification.[3]

Type	n	Bcl–1	Bcl–2	Bcl–6	c–myc	p53*	p16
Lymphoblastic lymphoma	2	–	–	–	–	–	–
Small lymphocytic lymphoma	18	1	2	–	–	2	1
Lymphoplasmacytoid lymphoma	3	–	–	–	–	–	–
Mantle cell lymphoma	11	6	1	1	1	–	2
Follicular lymphoma	20	–	13	2	–	–	–
Follicular diffuse lymphoma	2	–	1	–	–	–	–
Extranodal marginal lymphoma	3	1	1	2	–	–	–
Splenic marginal lymphoma	6	1	2	–	–	–	–
Hairy cell leukemia	1	–	–	–	–	–	–
Diffuse large cell lymphoma	24	3	4	5	–	1	2
Burkitt's lymphoma	6	–	2	1	6	1	1
Burkitt–like lymphoma	1	–	–	1	–	–	1
Unclassified	3	–	–	–	–	–	1
total	100	12	26	12	7	4	8

**p53/PCR–SSCP analysis not performed.*

or that of the abnormality only occurred in a subset of tumor cells, respectively. All 11 mantle cell lymphoma cases were $CD19^+CD5^+CD23^-CD10^-SIg^+$, except one which had a $CD23^+$ subpopulation, and presented the t(11 ;14) translocation.

Bcl-2

Alterations of the Bcl-2 proto-oncogene were noted in 26 cases. The MBR region was involved in 20 cases, the *mcr* in 5 cases and the *vcr* in 5 cases. In four patients more than one region was involved: MBR/*vcr* in two, *mcr/vcr* in one and MBR/*mcr* in one, the last case lacking co-migration between the rearranged bands of *mcr* and JH. Of these 26 cases, 15 were follicular lymphomas (FL) (groups B, C & D of WF) and 3 were DLCL (group G). Of the remaining eight cases, two corresponded to small non-cleaved cell lymphomas (SNCCL), one to a lymphoblastic lymphoma (LBL) and one to SCCL, while the other 4 cases were initially classified as SLL (group A). Globally, the incidence of Bcl-2 abnormalities was 70% in FL and 20% in DLCL. When the REAL classification was employed, the results were similar. However, a new category included in the REAL classification, the marginal NHL, also showed a high incidence of Bcl-2 rearrangements (3/9), the mbr region being affected in all of them. Interestingly, none of the five *vcr* rearrangements corresponded to SLL, but they were observed in FL (n=3), Burkitt's NHL (n=1) and MCL (n=1).

Bcl-6

The Bcl-6/LAZ3 gene was rearranged in 12 cases: DLCL (n=4), FL (n=3), SNCCL (n=2), SLL (n=2) and IL (n=1). Rearrangements were confirmed in Bam HI, Xba I and Hind III digestions. Thus, the incidence of Bcl-6 rearrangements in DLCL was 29% but it was lower in the corresponding group of REAL classification (20%).

c-myc

Seven rearrangements of the *c-myc* proto-oncogene were observed. Six were present in cases of Burkitt's NHL and the other in an MCL, according to REAL classification. Inversely, of 7 SNCCL (group J of WF)

there was one case without *c-myc* rearrangement, but it was considered as a Burkitt-like NHL in the REAL classification. All six Burkitt's lymphoma cases were $CD19^+CD22^+CD5^-CD23^-CD10^+SIg^+$.

11Q23/MLL

No abnormalities of 11q23/MLL gene were found in any of the 100 NHL patients included in this series.

p53

Four cases showed alterations in the p53 gene using the SB methodology. There was one homozygous deletion and one hemizygous deletion, together with two rearrangements. These two rearrangements were found in high grade lymphomas (one Burkitt's and one unclassified NHL), while the two deletions were observed in two cases of SLL, representing an incidence of 6% in the WF and 9% in the REAL classification.

p16

There were 8 cases with p16 abnormalities, consisting on homozygous deletions (n=5) and clonal rearrangements (n=3). The deletions were present in two cases of SLL (group A), two DLCL (G) and one SNCCL. The rearrangements were noted in two aggressive NHL (1 SNCCL and 1 unclassified) and one SLL. There were no important discrepancies between the two classifications employed, with the exception of 3 cases of SLL according to the WF, since two of them were reclassified as MCL when the REAL classification was used (one deletion and one rearrangement).

Genetic lesion associations

Thirteen cases displayed more than one gene abnormality (Table 5). The histologic category that most frequently showed coexistence of two or more abnormalities was the SNCCL since 4 of these cases showed another genetic lesion associated to the *c-myc* rearrangement. The remaining cases did not show a particular histologic distribution. Nevertheless, upon using the REAL classification, cases with multiple genetic lesions were more frequently observed within adverse histologic categories, since only four cases corresponded to the indolent forms (2FL, 2 ML) while 9 cases corresponded to aggressive subtypes (4 Burkitt's NHL, 3 MCL and 2 DLCL) (8% of SLL+FL+ML+HCL+LPL, versus 19% in PCL+DLCL+MCL+BL+BLL+unclassified). Moreover, all 3 cases that simultaneously displayed three genetic lesions also fell into the adverse categories: two MCL and one Burkitt's lymphoma.

Discussion

Molecular characterization of non-random chromosomal translocations and other genetic abnormalities[2] has largely contributed to a better definition of NHL due to the association between some clinicohistologic pictures and specific gene alterations. Moreover, molecular analysis has helped to establish the recently published REAL classification.[3] However, most information on the incidence of these abnormalities derives from specifically-orientated studies focused on particular oncogenes and specific NHL histologic subtypes and consequently, its global incidence and real specificity remains unclear. In addition, these genetic abnormalities have not been extensively explored simultaneously and thus, the co-existence of two or more genetic lesions is not well established among the different lymphoma subtypes.

The t(11;14)(q13;q32), which results in the rearrangement of the Bcl-1 locus, is usually associated with the recently described MCL[3] corresponding to the small cleaved cell lymphomas of the Working Formulation[23] and less frequently to the diffuse mixed or large cleaved cell lymphomas.[3,17] Using molecular techniques, this translocation can be observed in approximately 40-70% of all MCL.[3,7,8] In our experience the incidence was 57% (6/11 MCL) and the abnormality could be detected by both, SB and PCR techniques. The 6 remaining cases with Bcl-1 abnormalities were not MCL and the molecular marker could only be demonstrated using either SB or PCR, but not both. The positive result using SB would indicate that the Bcl-1 abnormality could be located outside the MTC region in these cases, as other groups have previously published in prolymphocytic leukemias, chronic lymphocytic leukemias, hairy cell leukemias, marginal lymphomas and large cell lymphomas.[4,16,32-36] By contrast, the PCR positive result without rearrangement by SB could be explained by the presence of the alteration only in a minor cell subpopulation of the tumor clone.[37]

Table 5. Molecular alterations associated in NHL patients according to the Working Formulation[23] and REAL classifications.[3]

Case	WF[23]	REAL[3]	Molecular analysis						
			Bcl-1	Bcl-2	Bcl-6	c-myc	p16	p53*	JH
1	B	Follicular lymphoma	–	+	RR	GG	GG	GG	RR
2	B	Follicular lymphoma	–	+	GG	GG	GG	DD	RR
3	B	Extranod. marg. lymphoma	–	+	RG	GG	GG	GG	RG
4	A	Splenic marg. lymphoma	+	+	GG	GG	GG	GG	RR
5	A	Mantle cell lymphoma	+	–	RG	GG	RG	GG	RR
6	A	Mantle cell lymphoma	+	–	GG	GG	DD	GG	RR
7	E	Mantle cell lymphoma	+	+	GG	RG	GG	GG	RG
8	F	Diffuse large cell lymphoma	+	–	RG	GG	GG	GG	RG
9	G	Diffuse large cell lymphoma	+	+	GG	GG	GG	GG	RR
10	J	Burkitt's lymphoma	–	+	RG	RG	GG	GG	RG
11	J	Burkitt's lymphoma	–	–	GG	RG	DD	GG	RG
12	J	Burkitt's lymphoma	–	–	GG	RG	GG	RG	RG
13	J	Burkitt–like lymphoma	–	–	RG	GG	RG	GG	RR

*G: germline; R: rearranged; d: Deleted. *p53/PCR–SSCP analysis not performed.*

The presence of t(14;18) has been reported in 40-85% of patients with FL[2,3,5,6] and in 20-30% of DLCL.[14,15] In most of these cases, the breakpoint usually involves the untranslated region of the exon 2 (MBR);[5] in the remaining cases, the breakpoint is located 20 Kb downstream (*mcr*).[38] In our series, 20 cases had the abnormality in the MBR region and 5 in the *mcr* region. Most of these cases corresponded to FL (groups B, C & D) or DLCL (group G), with an incidence of 71% and 20%, respectively. In addition, it should be noted that some cases of SLL and SCCL may show this translocation.[39,40] The variant forms [t(2;18); t(18;22)], that involve the *vcr* region of the Bcl-2 gene, have been classically ascribed to CLL/SLL.[41,42] However, in our experience, this genetic lesion may also be present in other histologic subtypes, such as FL, which is concordant with the observation of Tsujimoto *et al*.[28] and Merup *et al*.[43] This indicates that FL can deregulate the Bcl-2 gene through different mechanisms that can coexist within the same case, as we were able to observe in four patients with more than one of these regions affected.

Contradictory data has been reported concerning rearrangements of Bcl-6 gene. They are present in around one third of DLCL and, although they were initially described as specific of this NHL subtype,[12] they can also occasionally be observed in cases of low grade NHL.[13] In the present study, we have found Bcl-6 rearrangements in 12 out of 100 NHL; the majority of cases belonged to the groups G & H of WF but, as in other reports,[44] some cases were found within the FL (groups B & C, n=3). It should be noted that two out of the 7 SNCCL showed Bcl-6 rearrangements, which had not been previously reported.

The t(8;14), t(2;8) and t(8;22) have been classically associated with Burkitt's lymphomas so, in fact, they can be considered as hallmarks for this NHL variant type.[9-11] In this series, only one out of 7 SNCCL did not display *c-myc* rearrangement, and this case was considered to be a Burkitt-like NHL when the REAL classification was employed. In addition, there was only one case different from Burkitt's NHL with a *c-myc* rearrangement (a SCCL of the WF; a MCL of the REAL). This data confirms the high specificity of the abnormality for this type of lymphomas.

Abnormalities of 11q23 chromosome involving the MLL gene have been shown to be very important in the pathogenesis of acute leukemias, but they seem to be rare in NHL[2] and, if present, they do not affect this gene.[2,19] In our study, MLL was normal in all the 100 cases, and therefore this gene does not seem to play a relevant role in the pathogenesis of NHL.

In addition to all these proto-oncogenes, the genetics of cancer can also involve negative regulators (tumor suppressor genes). The most frequently mutated tumor suppressor gene is p53, affected in 30% of high grade non-Hodgkin lymphomas, whereas low grade NHL and CLL rarely display abnormalities in this gene.[20,45,46] In our series, the incidence of p53 lesions was very low (4%). This is probably due to the methodology employed, SB, which can not detect point mutations, the most frequent p53 aberrations.[20] However, there were two cases of low grade lymphomas (SLL) that showed a complete deletion of the p53 gene, representing an incidence of 9% according to the REAL classification. Since these two cases would not have been detected by PCR-SSCP analysis, the methodology used most frequently in the vast majority of reports,[20] the importance of p53 alterations should be reconsidered in low grade lymphomas.

It has been suggested that the other tumor suppressor gene analyzed here, p16, is as important as p53, and several reports have shown it to have a critical role in both in T-lineage[21,47] and in B-lineage[22,48] acute lymphoblastic leukemias. In B-NHLs the reported incidence of p16 alterations was very low (6%)[22] and there is only one study that has associated the abnormalities with a particular histologic subtype (DLCL),[49] but with an incidence of only 15%. In the present series, the overall frequency of p16 aberrations was 8%, while within DLCL it was 13%. This suggests that p16 lesions, though perhaps are more frequent in DLCL, are not greatly different from other histologic subtypes. Interestingly, three of our cases with p16 abnormalities were low grade lymphomas according to the WF (group A), but two of them were reclassified as MCL when the REAL classification was used. Thus, according to these results, it appears that p16 lesions are probably associated with adverse prognostic morphologic subtypes.

Co-existence of multiple genetic events in individual NHL patients has been observed during disease progression[45,50-52] supporting the multistep hypothesis suggested for the pathogenesis of cancer. Some reports have also found more than one genetic abnormality at diagnosis, especially in those cases with high grade histology and/or bad prognosis,[53] and in some specific entities like acquired immunodeficiency syndrome-related NHL,[54] although the real incidence of this finding still needs to be established. The present work shows that the incidence of more than one genetic alteration in 100 unselected NHL is relatively high at diagnosis (13%), supporting the idea that the multistep process is present not only in the latter course of the disease, but it is already present during the initial development of the tumor. In addition, the relatively higher frequency found in cases with aggressive morphologies according to the REAL, could help to support the hypothesis that the co-existence of two or more genetic lesions may contribute to the emergence of different NHL forms, probably reflecting a higher grade of genetic instability.

In summary, the present work shows that genetic abnormalities are a frequent event in NHL, some of them being associated with specific histologic types. In addition, the presence of several associated genet-

ic lesions is a relatively common finding in this disease at diagnosis and might be associated not only with a more aggressive morphology, but also with a more aggressive course of the disease within the same morphological group. However, this kind of analysis will require a larger series to be followed up in order to establish if those cases with associated genetic lesions really have a worse evolution than the same diseases lacking on this association.

Contributions and Acknowledgments

RG-S was the main investigator and designed the study, managed the data and performed the literature revision; he wrote the article with MG, and both were responsible for the data interpretation, direct supervision, funding and day-to-day contact with participants. MV carried out the DNA digestions, transferences and final preparations of Southern blot filters, together with PCRs of all samples. MCC and AB performed all DNA extractions, probe growing and purifications, and final hybridizations. TF was the pathologist who reviewed all the morphological pictures. MDC and JH were responsible for the clinical management and clinical data acquisition. JFSM was the main coordinator of the group and reviewed the article to obtain the final form in which it was sent for submission. The order tries to take into account the time work and scientific contribution of all authors.

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