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Immunoglobulin Gene Mutations and Frequent Use of *VH1-69* and *VH4-34* Segments in Hepatitis C Virus-Positive and Hepatitis C Virus-Negative Nodal Marginal Zone B-Cell Lymphoma

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Nodal marginal zone B-cell lymphoma (NMZL) is actually considered as a distinct entity that must be distinguished from extra-nodal and splenic marginal zone lymphomas. To define the cell origin and the role of antigen stimulation we determined the nucleotide sequence of the tumor-related immunoglobulin heavy chain variable genes in 10 cases of NMZL. The results were also evaluated on the basis of the presence of chronic hepatitis C virus (HCV) infection. All 10 cases harbored *VH* somatic mutations with a sequence homology compared to the closest germline gene, ranging from 83.33 to 98.28%. Interestingly, different *VH* segments were preferentially used in HCV-positive and HCV-negative patients: three of five HCV-negative NMZLs used a *VH4-34* segment joined with different *D* and *JH* segments whereas three of five HCV-positive NMZLs used a *VH1-69* gene joined with a *D3-22* and a *JH4* segment, with very strong similarities in the CDR3s among the three different cases. These data indicate: 1) NMZL is derived from B cells that have experienced the germinal center reaction; 2) the preferential usage of a *VH1-69* segment in the majority of the HCV-positive NMZL cases with similar CDR3s suggests the presence of a common antigen, probably a HCV antigen epitope, involved in the B-cell selection; and 3) the use of a *VH4-34* segment suggests a role of yet unknown B-cell superantigen(s) in the selection of tumor B-cell precursors in HCV-negative NMZL. *(Am J Pathol 2001, 159:253–261)*

Marginal zone B-cell lymphoma (MZL) is a distinct subtype of non-Hodgkin's lymphoma (NHL) that has been recently recognized and defined by REAL and World Health Organization classifications.[1–3] It can be subdivided into three varieties: extra-nodal MZL (EMZL) that arises from mucosa-associated lymphoid tissue (MALT) and is indicated by World Health Organization classification as EMZL of MALT type, splenic MZL, and nodal MZL (NMZL). Because NMZL displays no appreciable differences in its cytological, architectural, and phenotypic features with EMZL, it has been debated whether MZL with primary nodal involvement has to be considered a distinct disease or merely represents the dissemination of EMZL of MALT-type. For this reason, the REAL classification has included MZL cases with primary lymph node involvement in a provisional category. Very recently, Nathwani and colleagues[4] identified several distinct clinical features between nodal and extra-nodal MZL. NMZL has a more frequent involvement of peripheral lymph nodes and an advanced disease stage at the presentation compared with EMZL of MALT-type. Moreover, NMZL has a shorter overall survival duration than EMZL. Therefore, from a clinical point of view, NMZL seems to be more similar to other low-grade NHLs such as follicular and lymphocytic lymphoma than EMZL, suggesting the presence of still unknown biological differences between EMZL and NMZL. For these reasons the World Health Organization classification has clearly recognized NMZL as a distinct disease that must be distinguished from EMZL of MALT-type with lymph node involvement and from other small B-cell NHLs.[3]

Hepatitis C virus (HCV) is actually considered as the major etiological factor of type II essential mixed cryoglobulinemia, a disease associated with an underlying B-cell clonal proliferation that, in a minority of cases, may evolve into a frank NHL.[5,6] HCV is known to be both a hepatotropic and a lymphotropic virus and it has been suggested that it may play a role in the pathogenesis of clonal proliferation of B-cells.[7] Several studies have reported a higher prevalence of chronic HCV infection in patients with B-cell NHL compared with the general population, at least in certain geographical areas.[7–9] In particular, in a survey of HCV-positive NHL from the United

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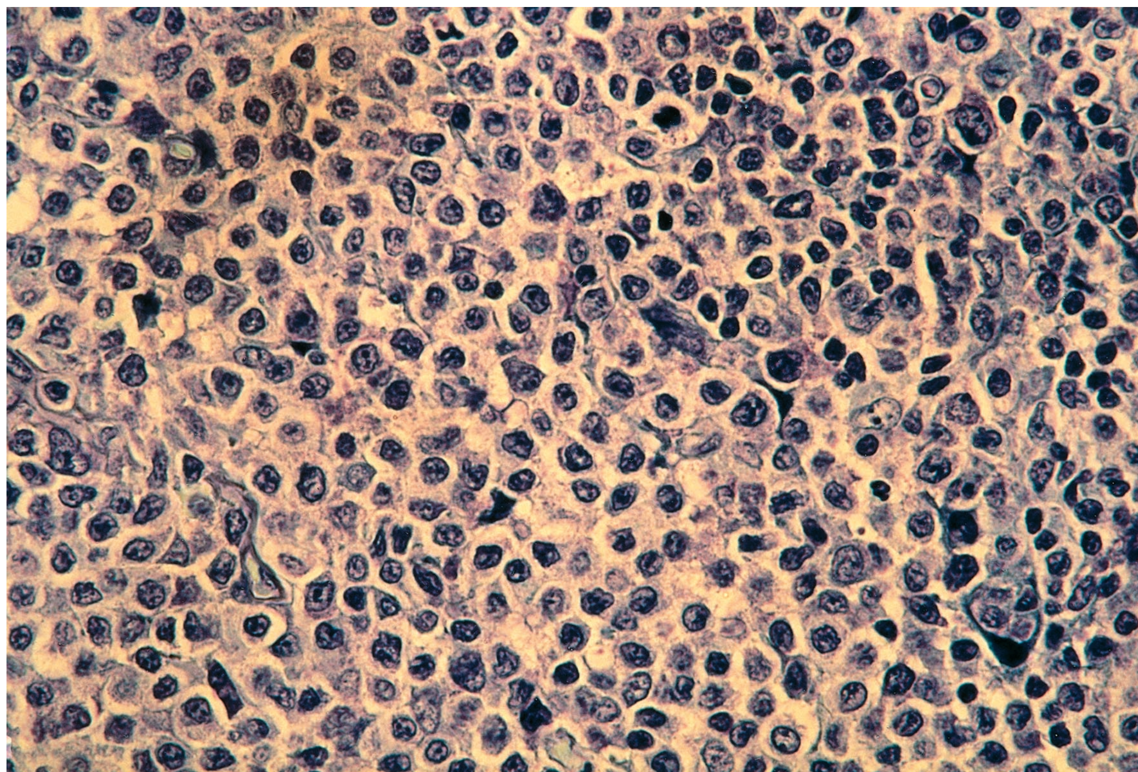

Figure 1. Lymph node from case 5 with NMZL. (Giemsa; original magnification, ×400).

States NMZL has been recognized as the most common histological type.[10]

A role of chronic immunological stimulation in the development and maintenance of MZL, and in particular of EMZL of MALT-type, has been suggested by a variety of observations. MZL frequently originates in a setting of chronic inflammation triggered by chronic infection or autoimmune disorders, such as *Helicobacter pylori* gastritis, Sjögren's syndrome, and Hashimoto's thyroiditis.[11–13] The etiological link between gastric EMZL and *H. pylori* infection has also been shown by the regression of some cases by antibiotic therapy.[14,15] A role of chronic immunological stimulation is also suggested by sequence analysis of the tumor-related immunoglobulin gene rearrangement that identified a preferential use of the immunoglobulin heavy chain variable region (*VH*) genes associated with autoimmune disorders suggesting that tumor cells may arise from autoreactive marginal zone B-cells.[16,17] Moreover, the analysis of somatic mutations of *VH* genes revealed a variable but significant level of mutations supporting the hypothesis of a germinal center (GC) of a post-GC origin of MZL.[18,19] Nevertheless, these data have been obtained in a significant number of EMZL whereas the tumor-related immunoglobulin chain gene (*IgH*) status has not yet been determined in the recently identified subset of NMZLs.

In the present study we analyzed the nucleotide sequence of the tumor-related rearranged variable immunoglobulin heavy chain genes in 10 cases of NMZL. The cases examined have been well characterized from a clinical point of view to exclude any cases with extranodal involvement. Therefore, we considered this cohort of patients represented of true NMZL in accord to World Health Organization classification. Moreover, we also analyzed the data obtained on the basis of the presence of chronic HCV infection to identify peculiar aspects of the *IgH* genes distinguishing between HCV-positive and HCV-negative cases.

Materials and Methods

Patients

The 10 cases of NMZL evaluated in this study were selected among 18 cases, collected between 1990 and 1998, for which DNA or nitrogen liquid-frozen lymph node tissues were available for molecular studies. Diagnosis of monocytoid-marginal zone B-cell lymphoma was documented in all cases by morphology and immunohistochemistry performed on formalin-fixed paraffin-embedded tissue according to the proposed "Revised European-American Classification of Lymphoid Neoplasms."[1] In particular, the presence of characteristic clear cells with a relatively abundant pale cytoplasm (positive for CD20 and CD79a and negative for CD5 antigens) recognized as marginal/monocytoid B cell was evident in all cases (Figure 1). Moreover, bone marrow and peripheral blood double-immunofluorescence labeling with anti-CD5 and anti-CD19 was performed in all cases to exclude B-cell chronic lymphocytic leukemia. Complete clinical records and follow-up were available for all patients included in the study; as long as a case had involvement of a mucosal or other extra-nodal non-

hemopoietic site by MZL it was classified as EMZL of MALT-type and discarded. On the basis of these criteria 8 out of the 18 cases initially selected were discarded from the study because of the presence of an extra-nodal localization of the lymphoma, in particular of a gastric involvement in seven cases and of the parotid gland in one case. All patients were evaluated on the basis of physical examination, endoscopy, and neck-thorax-abdominal computed tomographic scans.

Serum anti-HCV antibodies were detected in 5 out the 10 nodal MZLs examined by enzyme-linked immunosorbent assay (Chiron ELISA HCV; Chiron, Emeryville, CA) and confirmed in all cases by recombinant-based immunoblot test assay (Chiron RIBA HCV, Chiron). The serological assay was performed on the sera collected at the time of diagnosis. Patients found positive for the presence of anti-HCV antibodies underwent liver biopsy that showed the presence of a chronic liver disease in all cases. Abnormal serum levels of alanine aminotransferase were found in two HCV-positive patients. Cryocrit determinations and cryoglobulins composition were evaluated in all cases as described by Pozzato and colleagues,[6] although none of the patients presented clinical manifestations of cryoglobulinemia circulating monoclonal IgMk serum cryoglobulins (considered as positive as >1%) were present in one case (case 1).

Polymerase Chain Reaction (PCR) Amplification of the Rearranged IgH and IgK

DNA was extracted from lymph node specimens using a commercial kit (Easy-DNA; Invitrogen, Carlsbad, CA). One μg of DNA was added to PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$), containing 200 μmol/L dNTPs, 100 nmol/L of each primer and 1.5 U of AmpliTaq Gold polymerase (Perkin Elmer, Norwalk, CT) in a total volume of 50 μl. Forty cycles of amplifications were performed at the following condition: 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C with 10 minutes of an initial denaturation step at 95°C and 7 minutes of a final extension step at 72°C. Initially, PCRs were performed in separate reactions with seven family-specific FR1 sense primers together with a 3′ primer complementary to a germline *JH* consensus sequence. If the region from FR1 to *JH* could not be amplified, the amplification experiments were performed at the same conditions using 5′ oligonucleotides specific for each leader sequence of the *VH1* and *VH7* families together with the same *JH* antisense primer. For each DNA sample a DNA-free control was used to check contamination. Each amplification experiment was performed in duplicate. After agarose gel electrophoresis, a predominant band was obtained in samples 1, 4, 7, 8, 9, and 10 using the *VH* FR1-specific family primers and in samples 2, 3, 5, 6, and 9 using *VH* leader-specific family primers. The segment initially obtained with FR1 primers in sample 9, belonging to *VH3* family, was an out-of-frame rearrangement.

5′ oligonucleotides complementary to FR2 and FR3 consensus sequences were also used together with the 3′ *JH* primer at the same PCR conditions with the exception of an annealing temperature of 50°C in each cycle.

The light chain gene region was amplified using *VK* family-specific primers and a mixture of *JK* primers at the same PCR condition used for *VH* family-specific amplifications. The sequence of the oligonucleotides used for the PCR experiments is reported in Table 1.

Table 1. Primers Used for *IgH* and *IgK* Amplification

Name	Sequence (5′/3′)
VH1 FR1	CCTCAGTGAAGGTCTCCTGCAAGG
VH2 FR1	TCCTGCGCTGGTGAAAGCCACACA
VH3 FR1	GGTCCCTGAGACTCTCCTGTGCA
VH4a FR1	GGTCCCTGAGACTCTCCTGTGCA
VH4b FR1	TCGGAGACCCTGTCCCTCACCTGCA
VH5 FR1	GAAAAAGCCCGGGGAGTCTCTGGA
VH6 FR1	CCTGTGCCATCTCCGGGGACAGTG
VH1 leader	CCATGGACTGGACCTGGAGG
VH2 leader	ATGGACATACTTTGTTCCAC
VH3 leader	CCATGGAGTTTGGGCTGAGC
VH4 leader	ATGAAACACCTGTGGTTCTT
VH5 leader	ATGGGGTCAACCGCCATCCT
VH6 leader	ATGTCTGTCTCCTTCCTCAT
VH7 leader	TTCTTGGTGGCAGCAGCCACA
FR3a	ACACGGC(C/T)(G/C)TGTATTACTGT
FR2	TGG(A/G)TCCG(C/A)CAG(G/C)C(T/C)(T/C)CNGG
JH	ACCTGAGGAGACGGTGACC
VK1	ATGGACATGAGGGTCCCCGC
VK2	ATGAGGCTCCCTGCTCAGCT
VK3	ATGGAAACCCCAGC(G,T)CAGCT
VK4	ATGGTGTTGCAGACCCAGGTC
VK5	ATGGGGTCCCAGGTTCACCTC
VK6	ATGTTGCCATCACAACTCATTG
JK124	ACGTTTGAT(C,T)TCCA(C,G)CTTGGTC
JK3	ACGTTTGATATCCACTTTGGTC
JK5	ACGTTTAATCTCCAGTCGTGTC

Sequencing of PCR Products and Sequencing Analysis

PCR products were excised from 1% low-melting agarose gel, further purified with Wizard PCR prep purification kit (Promega, Madison, WI) and directly sequenced using the BigDye terminator cycle sequencing kit and a DNA sequencer (ABI prism 310; PE Applied Byosystem, Foster City, CA). Sequencing reactions were performed with the same oligonucleotides used in the PCR amplifications by both sense and antisense primers. The nucleotide sequences of the amplification products representative of the *IgH* rearrangements were compared with the VBASE directory (VBASE Sequence Directory, I.M.; Tomlinson, MRC Center for Protein Engineering, Cambridge, UK; URL: www.mrc-cpe.cam.ac.uk/imt-doc)[20] using the DNAPLOT analysis software. A diversity (*D*) germline segment was assigned to the longest stretches with the highest nucleotide homology with a minimum of six successive matches or seven matches interrupted by one mismatch.

Somatic Mutation Analysis

Mutations in the variable region were identified by comparing the nucleotide sequence of each tumor with the closest germline *VH* sequence. Two nucleotide exchanges in one codon were considered as one replacement (R) mutation. Mutations at the joining site of the *VH*

segment were not regarded as mutations. The number of expected replacement (R) mutations in the complementary determining region (CDR) or framework region (FR) was calculated using the formula: Exp R (CDR or FR) = $n \times$ (CDR Rf or Fr Rf) $\times$ (CDRrel or FRrel); where n is the total number of observed mutations, CDRrel or FRrel is the relative size of CDRs or FRs and Rf is the inherited replacement frequency to CDRs or FRs sequences. Rf value was calculated for each individual *VH* gene sequence using the InhsusCalc1.0 software, kindly provided by Dr Paolo Casali (Department of Pathology, Weill Medical College, Cornell University, NY). According to the binomial distribution model, the probability that excess or scarcity of R mutations in CDRs or FRs resulted on the basis of chance alone was calculated using the formula: $p = \{n!/[k!(n-k)!]\} \times q^k \times (1-q)^{n-k}$; where k is the number of observed mutations in the CDRs or FRs and q is the probability that a R mutation will localize to CDRs or FRs (q = CDRrel $\times$ CDR Rf or FR rel $\times$ FR Rf).[21]

Results

PCR Analysis of VH and VK Genes

IgH rearranged genes were amplified from DNAs extracted from lymph node biopsies using family-specific *VH* FR1 or, alternatively, family-specific *VH* leader primers coupled with a 3′ heavy-chain joining (*JH*) primer. After agarose gel electrophoresis and staining with ethidium bromide a predominant band was observed in all cases. All samples were analyzed in duplicate. These amplification products were directly sequenced on both strands with the same sense and antisense primers used in the PCR experiments. As anticipated by the PCR analysis, the *VH* segments involved in a productive *VH-D-JH* rearrangement in NMZLs belonged to the *VH4* family in four cases, to the *VH1* family in three cases, to the *VH3* family in two cases, and to the *VH2* family in one case. No rearranged segments of the *VH5*, *VH6*, and *VH7* family were found to be involved in this series of NMZL cases. *VH* nucleotide sequences have been deposited in the GenBank database (accession numbers AF355605 to AF355614). All three of the *VH1*-family genes identified (cases 1, 4, and 5) were found to be closely related to the *VH1-69* germline gene with a nucleotide homology ranging from 92.40 to 94.79%. Three of the four lymphoma *VH4*-family genes (cases 2, 3, and 6) seem to be related to the *VH4-34* germline gene with a nucleotide identity ranging from 83.33 to 98.28%, whereas the fourth *VH4*-family case (case 9) was assigned to the *VH4-30.4* germline segment. The two *VH3*-family lymphoma segments seemed to be related to the *VH3-30.3* (case 7) and *VH3-48* (case 10) whereas a rearranged *VH2-05* germline segment was observed in case 8 (Table 2).

Assignment of *D* gene segments was based on the homology between CDR3 sequences and the germ line *D* genes. A *D* segment could be identified with confidence in 8 of the 10 lymphoma cases examined. A *D3-22* segment was assigned in four cases. Surprisingly, the three nodal MZL cases with an involvement of the *VH1-69* fragments used a *D3-22* segment joined with a *JH4* gene. In addition, the sequence of the CDR3 region found in the three different patients were very similar, both in length and in nucleotide composition. Moreover, the *D3-22* segments were used in the same reading frame (Table 3). Nevertheless, the sequence analysis of the *VH1-69* segments revealed that the somatic mutations of the three *VH1-69* cases were different, excluding a gross contamination between the different DNA samples (see Figure 3). Moreover, to confirm these data, amplification fragments of the *VH-D-JH* rearrangements were obtained with primers specific to FR3 and FR2 consensus regions of the *VH* segments. PCR experiments performed with a degenerate oligonucleotide specific for FR3 region revealed the presence of a clear band in cases 1 and 4 and of a smear with a faint band in case 5; of note all PCR fragments obtained were of the same length. In two out of the three cases (cases 4 and 5) a band of the same length was evident also with a degenerate primer specific for the FR2 region. In the third case (case 1) a smear was present probably because of the fact that the nucleotide sequence of the FR2 region of this case was not susceptible to amplification with this set of primers (Figure 2). Sequence analysis of the DNA fragments obtained with FR2 and FR3 PCR experiments revealed a nucleotide composition identical to the sequence already determined with the family-specific amplification fragments in all cases. The three cases rearranging a *VH1-69* segment were all positive for HCV infection. The other two HCV-positive cases rearranged a *VH4-30.4* (case 9) or a *VH3-30.5* segment (case 7). The three cases using a *VH4-34* segment were all HCV-negative (Table 2).

Table 2. *VH*, *D*, and *JH* Usage and HCV Infection in NlMZL

Case no.	HCV	VH	DH	JH
1	Positive	VH1-69	D3-22	JH4
2	Negative	VH4-34	D6-19	JH3
3	Negative	VH4-34	D7-27	JH3
4	Positive	VH1-69	D3-22	JH4
5	Positive	VH1-69	D3-22	JH4
6	Negative	VH4-34	NI	JH6
7	Positive	VH3-30	D7-27	JH4
8	Negative	VH2-05	D3-22	JH4
9	Positive	VH4-30.4	NI	JH6
10	Negative	VH3-48	D3-3	JH4

NI, not identified.

VK gene analysis was performed using *VK* family-specific sense primers coupled with a mixture of *JK* antisense primers. A clear predominant band was obtained in only six cases and in particular a *VK2* segment in one case (case 7), a *VK3* segment in two cases (cases 4 and 6), a *VK4* segment in one case (case 2), and a *VK5* segment in two cases (cases 3 and 5). Because it has been pointed out a relationship between *VH1-69* heavy chain and *Vk325* light chain segments,[19] PCR products obtained with *VK3*-specific primers were purified and subject to direct sequence analysis. A mutated *Vk325* gene was identified in case 4 and a mutated *Vk305* in case 6.

Table 3. Determination of *VH*, *D* and *JH* Usage and Nucleotide Sequence of CDR3 Region in Nodal MZL

Case no.	*VH*	N	*D*	N	*JH*
1	TGT GCGAGA VH1-69	ggcccc	GATAGcAGTGGaTATTACTAC D3-22	tt	CTAC TGG JH4
2	TGT GCGAGAGGT VH4-34	agaaagggag	GCAGTGGCTtGTAC D6-19	ctgaaacat	ATGgTTTgGAcGTC TGG JH3
3	TGT GCGAGAGGT VH4-34	ggctatgatagtgatgg	CTccgTGGGGA D7-27	cgatggtc	GCTTTTGATATC TGG JH3
4	TGT GCGAG VH1-69	ggggccc	GATAGTAGTGcaTATTACTAC D3-22	ttt	TAC TGG JH4
5	TGT GCGAGA VH1-69	gggccc	GATAGTAGTGcaTATTACTA D3-22	ttt	CTAC TGG JH4
6	TGT GCG VH4-34	——	actattcgttatcggcgccgcctatctttctttcggtat	——	TACacCt TGG JH6
7	TGT GCGAAA VH3-30.5	gaagggac	TTTTTGGAGTGG D7-27	ccc	CTcTGACTgg TGG JH4
8	TGT GCACAT VH2-05	tt	GTATTAtTATGATAGTgGTGtTTATTAC D3-22	cgcc	ACTTTGACTAC TGG JH4
9	TGT GCCAGAGAT VH4-30.4	——	attcctttggtcgggagtataggtcg	——	TACTGCGGTATGGACGTC TGG JH
10	TGT GCGAGAGAT VH3-48	cgattgacggtcg	ACGATTTTTGGAGTG D3-3	cca	ACTACTTaGACTAt TGG JH4

Nucleotides of N segments and mutations with respect to the germline sequence are indicated in lowercase.
In cases 6 and 9 a germline *D* segment was not assigned.

Somatic Hypermutations and Statistical Analysis

The rearranged *VH* sequences were found to carry somatic mutations in all cases of NMZL studied, with a sequence identity rate compared with the closest germline gene ranging from 83.33 to 98.28%. To establish if the pattern of somatic mutations were indicative of antigen selection, we determined the distribution of replacement (R) and silence (S) mutations in the FRs and CDRs regions using the method described by Chang and Casali.[21] A preferential cluster of R mutations in the CDRs region ($P < 0.05$) was found only in case 7. In FRs regions the number of R mutations was significantly lower than would be expected to arise solely by chance in five cases, probably as a result of selection against R mutations within the FRs to preserve structure of immunoglobulins. Among this group of patients cases 1, 5, and 7 were HCV-positive; cases 2 and 6 were HCV-negative (Table 4).

Discussion

The status of the immunoglobulin genes provides an indicator of the cell origin and of its clonal history in NHL.[22] Somatic hypermutations seem to be restricted to B cells proliferating within the microenvironment of the germinal center (GC). As a consequence, the presence of somatic mutations in the variable region of the rearranged immunoglobulin genes is actually considered the hallmark of B cells that have participated in a GC reaction. Moreover, the pattern of the distribution of somatic mutations and a preferential usage of immunoglobulin variable, diversity, and joining segments may reveal a role of antigens in driving B-cell proliferation. Clustering of nucleotide mutations leading to an amino acid substitution in the CDRs of *VH* segments is considered to indicate that the hypermutation process is driven by an antigen and a statistical method for assessing it is available.[21]

In the cases examined we found that NMZLs harbor numerous point mutations with respect to the closest related germline gene sequences (Table 5) with a rate of homology ranging from 83.33 to 98.28% corresponding to a number of substitutions ranging from 5 to 41 nucleotides (average, 17.8). Because most of the human germline *VH* genes have been identified, it is unlikely that the majority of nucleotide substitutions found represent *VH* segments not yet identified or because of sequence polymorphism.[20] These data show that the tumor cells are likely to derive from mature B cells that had participated in a GC reaction. We cannot identify if tumor cells have to be considered of GC or of post-GC derivation because the molecular approach used (direct sequencing of PCR products) does not allow the assessment of nucleotide intraclonal variations because of an ongoing mutational process.

The distribution of R and silence S mutations of mutated *VH* sequence was analyzed by the method of Chang and Casali.[21] Somatic mutations of the our NMZLs were not distributed in a manner indicative of positive selection or of antigen-affinity maturation in the majority of cases. In fact, only one of the cases examined (case 7) showed a statistically significant evidence of accumulation of R mutations in the CDRs. Nevertheless, five cases showed evidence of negative selection of R mutations in the FRs (Table 4). This aspect is consistent with a GC selection to preserve the immunoglobulin structure providing the scaffolding for the antigen-contacting cells.

Kuppers and colleagues[23] showed in four cases defined as monocytoid B-cell lymphoma a distribution of somatic mutations indicative of a clonal antigen-positive selection driven by antigens. Our data, obtained in a larger number of cases, contrast with these findings. Nevertheless, in their cases the primary localization of the

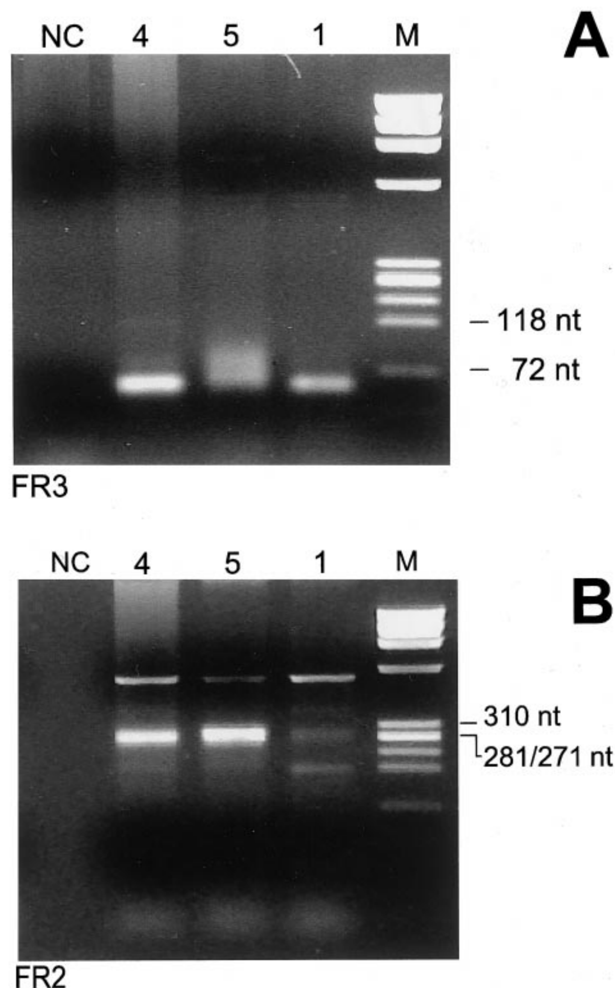

Figure 2. Gel electrophoresis of PCR amplifications of the CDR3s of cases 1, 4, and 5 obtained with primers specific for the FR3 (**A**) and FR2 (**B**) *IgH* regions. **A:** Amplicons of the same length were clearly present in patients 1 and 4 whereas in case 5 they were present as a smear with a faint band. **B:** Clonal bands of the same length were present only in cases 4 and 5. M, molecular weight marker ΦX174 *Hae*III digested; NC, DNA-free negative control.

lymphoma was not reported and at least in one case the tumor was localized in the stomach and therefore likely to be considered as an EMZL of MALT-type with monocytoid cells. Thus, the differences between our data and that of Kuppers may be because of different criteria in selecting lymphoma cases.

Zuckerman and colleagues[10] have recently reported that NMZL is the most common histological type found in a series HCV-infected NHL cases from the United States. They also found that ~50% of the NMZL cases examined were HCV-infected. Therefore, the frequency of HCV chronic infection found in our NMZL cases, selected only on the basis of the availability of biological material adequate for molecular studies, confirms Zuckerman's data. Interestingly, we also found that the *VH* gene usage among NMZL is not random. Different *VH* segments are preferentially used in HCV-positive and HCV-negative patients (Table 2). Three out of five HCV-negative NMZLs (cases 2, 3, and 6) use the *VH4-34* gene segment joined with different *D* and *JH* segments. In two cases, the *VH4-34* segments appear to be heavily mutated, with nucleotide sequence homology with the *VH4-34* germline gene of 83.33% in case 2 and 83.68% in case 6. In both these cases, despite the high frequency of somatic mutations, aspects for selection against R mutations in the FRs were present (Table 4). The conservation of the amino acid sequence of the FRs is actually considered a feature that distinguishes functional from nonfunctional sequences suggesting that the immunoglobulins encoded by these *VH4-34* MZL cells are functional.[24,25] Therefore, neoplastic cells have aspects indicating the origin from B lymphocytes that have undergone a selection process within the GC reaction. In case 3, the *VH4-34* segment was slightly mutated (98.28% of homology) without a statistical significance of the pattern of distribution of R and S mutations. Nevertheless, the low rate of mutations found in this case determines that the statistical model used may be not informative. The *VH4-34* gene is used in ~5% of healthy adult B lymphocytes[26] and is frequently found in diffuse large-cell lymphoma, primary central nervous system lymphoma, B-chronic lymphocytic leukemia, and autoimmune disorders, but never in multiple myeloma.[27–32] Interestingly, the *VH4-34* gene is

Table 4. Mutation Analysis of Rearranged *VH* Gene in Nodal Marginal Zone Lymphomas

Case no.	Germline *VH* genes	Identity (%)	Obs R/S CDR	Obs R/S FR	Exp R CDR	Exp R FR	pCDR	pFR	HCV
1	VH1-69	92.4	2/3	3/8	3.89	9.04	0.144	0.002	Positive
2	VH4-34	83.33	10/4	9/18	8.55	28.26	0.121	<0.001	Negative
3	VH4-34	98.28	2/0	2/1	0.88	2.87	0.173	0.253	Negative
4	VH1-69	92.40	2/1	8/5	3.66	8.51	0.165	0.190	Positive
5	VH1-69	94.79	3/3	4/4	2.62	8.69	0.242	0.015	Positive
6	VH4-34	83.68	10/2	15/12	8.37	26.95	0.117	<0.001	Negative
7	VH3-30.5	92.54	7/1	4/4	3.12	9.93	0.018	0.003	Positive
8	VH2-05	95.70	4/0	4/1	2.12	5.43	0.102	0.165	Negative
9	VH4-30.4	94.33	4/3	6/3	3.03	8.90	0.180	0.07	Positive
10	VH3-48	97.36	2/0	1/3	1.41	3.22	0.284	0.068	ND

VH genes, immunoglobulin heavy chain variable gene; R, replacement mutation; S, silent mutation; CDR, complementary determining regions; FR, framework regions; Obs R/S CDR and Obs R/S FR, number of the observed R and S mutations in the CDR and FR, respectively; Exp R CDR and Exp R FR, number of expected R mutation in the CDR and FR, respectively; pCDR and pFR, probability that excess or scarcity of the R mutations in the *VH* gene CDR or FR resulted from chance only; ND, not determined.

VH sequences were compared with germline *VH* sequences included in the VBASE sequence directory.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
VH1-69	CAG	GTG	CAG	CTG	GTG	CAG	TCT	GGG	GCT	GAG	GTG	AAG	AAG	CCT	GGG	TCC	TCG	GTG	AAG	GTC	TCC
N.1	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......
N.4	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......	......
N.5	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	22	23	24	25	26	27	28	29	30	31	31a	31b	32	33	34	35	36	37	38	39	40
													CDR1								
VH1-69	TGC	AAG	GCT	TCT	GGA	GGC	ACC	TTC	AGC	AGC			TAT	GCT	ATC	AGC	TGG	GTG	CGA	CAG	GCC
N.1	......	......	...-C	---	---	---	--A	---	---	--T			-**C**-	---	---	---	---	---	---	---	---
N.4	......	......	...--	---	---	---	---	---	---	-**C**-			-**C**-	---	---	---	---	---	--G	-**T**-	---
N.5	---	---	---	---	---	---	---	---	---	-**A**-			-T-	---	---	--T	---	---	---	---	---

	41	42	43	44	45	46	47	48	49	50	51	52	52a	52b	52c	53	54	55	56	57	58
																			CDR2		
VH1-69	CCT	GGA	CAA	GGG	CTT	GAG	TGG	ATG	GGA	GGG	ATC	ATC	CCT			ATC	TTT	GGT	ACA	GCA	AAC
N.1	---	---	---	--C	---	---	---	---	---	---	---	---	---			--A	---	--G	---	---	---
N.4	---	---	---	--C	---	**C**--	---	---	--C	---	---	---	---			---	---	--C	---	---	---
N.5	---	---	---	---	---	---	---	---	---	---	---	---	---			---	---	--A	-**G**-	---	--T

	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79
VH1-69	TAC	GCA	CAG	AAG	TTC	CAG	GGC	AGA	GTC	ACG	ATT	ACC	GCG	GAC	GAA	TCC	ACG	AGC	ACA	GCC	TAC
N.1	---	---	---	-**G**-	---	---	---	---	---	---	---	---	---	---	--**C**	---	---	--T	---	-**T**-	---
N.4	---	---	---	---	---	---	---	---	-**C**-	**T**--	---	---	---	---	-**G**-	---	**TT**-	-**A**-	---	-**T**-	---
N.5	---	---	---	---	--**T**	---	---	---	**A**--	--A	---	---	---	---	A--	---	---	---	--G	**A**--	---

	80	81	82	82a	82b	82c	83	84	85	86	87	88	89	90	91	92	93	94
VH1-69	ATG	GAG	CTG	AGC	AGC	CTG	AGA	TCT	GAG	GAC	ACG	GCC	GTG	TAT	TAC	TGT	GCG	AGA
N.1	---	---	T--	--**G**	---	---	---	---	--A	---	---	---	--A	---	--T	---	---	---
N.4	---	---	---	---	---	---	---	---	---	---	---	---	--A	---	---	---	---	--G
N.5	---	--**C**	---	--T	---	---	---	---	---	---	---	---	**A**-A	---	---	---	---	---

Figure 3. Sequence analysis of the *VH1-69* homologous segment in three HCV-positive NMZL cases (cases 1, 4, and 5). VH codons are numerated on the *VH1-69* germ-line sequence. **Bold letters:** replacement mutations.

found in virtually all cases of cold agglutinin disease[33–35] and the red blood cell I/i antigens bind to the FR1 domain of Ig. A restricted usage of *VH* genes, as well as binding of an immunoglobulin outside the CDRs, which are the sites that bind conventional antigens, are characteristic aspects of B-cell superantigens that are supposed to directly activate B cells.[36] In particular, certain portions of the FRs seems to be important for superantigen binding and these would be preserved in a superantigen selection pressure.[37] Interestingly, staphylococcal enterotoxins A and D, that function as a human B superantigen, are able to rescue B cell-expressing *VH3* and *VH4* (including *VH4-34*) genes inducing cell survival in *in vitro* experiments.[38,39] Therefore, the high frequency of the *VH4-34* gene usage joined with different *D* and *JH* segments as well as the preservation of the FRs sequences, suggests a possible role of yet unknown B-cell superantigen(s) in driving HCV-negative NMZL development and proliferation.

Three out five HCV-positive NMZLs revealed the usage of the *VH1-69* gene indicating a highly biased and nonrandom use of the *VH* segments in this subtype of tumors. Sequence analysis of the rearranged *VH* segments of these three cases revealed a substantial deviation from the germline *VH1-69* sequence with a degree of mutations very similar among the different cases indicating that these cells have traversed the GC activating the somatic hypermutation mechanism. Statistical analysis of the pattern and distribution of R and S mutations between CDRs and FRs revealed a statistical significance in two cases (cases 1 and 5) in which the presence of R mutations in the FRs are lower than expected by chance only (Table 4). This pattern is indicative of a selective pressure to conserve the functional structure of the immunoglobulin protein. Moreover, the *VH1-69* segments were joined with a *D3-22* and a *JH4* segment in all of the three cases. A *D3-22* segment joined with *JH4* was also found in a HCV-negative case (case 8) assembled with a *VH2-05* gene. As a consequence, the CDR3 region encoded for an amino acid sequence with very strong similarities between the three *VH1-69* different cases. We exclude that this data may be because of cross contamination because the sequence analysis of the *VH1-69* segments revealed different somatic mutations between the three samples (Figure 3) and the same CDR3 sequences were obtained with different sets of primers specific for FR3 and FR2 *VH* consensus sequences (Figure 2). Therefore, these data indicate the role of a common antigenic epitope involved in the selection and in the expansion of the B-cell clone at the origin of neoplastic cells. The *VH1-69* immunoglobulin segment is expressed in the restricted repertoire of fetal liver B lymphocytes and is thought to be involved in natural immunity.[40,41] A productive *VH1-69* rearrangement is present in ~1.6% of normal B lymphocytes in adults.[42] *VH1-69* is rearranged in ~10 to 20% of B-cell chronic lymphocytic leukemia[30,43] and a *VH1-69* monoclonal rearrangement is present in the majority of patients with type II mixed cryoglobulinemia, a typical HCV-related disorder.[44,45] A preferential usage of *VH1-69* was also found by Ivanovski and colleagues[46] in

the majority of the HCV-positive lymphoplasmocytoid lymphoma/immunocytoma NHL subtypes secreting monoclonal IgM cryoglobulins. Very recently, a frequent usage of *VH1-69* was also reported by Bahler and colleagues[19,47] in the subset of the salivary gland EMZL of MALT-type that arise in the course of lymphoepithelial scialoadenitis associated or not with Sjögren's syndrome. In one case they also reported the use of a *D3-22* joined with *VH1-69* and *JH4* segments. They also report that *VH1-69* salivary gland lymphoma often has CDR3 regions with similar characteristics, ie, a preferential use of the *JH4* segment, a length of 12 to 14 amino acids and the presence of some amino acids motifs at the *VH-D* and *D-JH* junctions, concluding that the salivary EMZL may be selected by similar antigen epitopes. Unfortunately, they did not report the status of HCV infection in the cases examined.

Therefore, in the subset of NMZLs with a chronic HCV infection we find the presence of the *VH1-69* segment in the majority of cases examined, that is frequently expressed in other subsets of lymphoproliferative disorders, mainly associated with HCV infection. The almost equal sequences of the CDR3 region of the *VH1-69* cases associated to the presence of a chronic HCV infection suggests, in first hypothesis, that a same HCV antigen epitope could be involved in the B-cell selection by a direct antigenic stimulation. This hypothesis is also sustained by the observation that B cells derived from a HCV-infected individual, immortalized as hybridomas, and selected for binding the HCV E2 envelope glycoprotein use the *VH1-69* segment in the majority of cases.[48]

Nevertheless, the possibility that the virus infects and induces the proliferation of a particular fraction of B lymphocytes cannot be excluded. Gastric EMZLs are associated with *H. pylori* infection in virtually all cases suggesting a possible *H. pylori* direct antigenic stimulation.[13] Nevertheless, in this subset of EMZL, tumor immunoglobulins seem to be autoantigen-related rather than specific to *H. pylori* and the neoplastic growth seem to be sustained by stimulation through a *H. pylori*-specific T-cell reaction.[49,50] We cannot exclude that HCV could play a role similar to that of *H. pylori* in gastric EMZL in the subset of HCV-positive NMZL.

Tierens and colleagues[18] recently reported an analysis of somatic mutations in a series of MZL occurring at different sites including four cases with primary lymph node localization in which the pattern of somatic mutations was not indicative of positive or negative antigen selection. However, they report in two out of the four cases of NMZL studied the presence of a case rearranging the *VH1-69* gene joined with a *D3-22* segment and a case with the rearrangement of a *VH4-34* segment. Unfortunately, they did not report data about HCV infection in these cases.

In conclusion, these results indicate that NMZLs are originated from GC-experienced B lymphocytes with evidence of antigen selection in half of the cases. The presence of a *VH1-69* segment in the subset of the HCV-positive NMZLs with similar CDR3 sequences strongly supports the hypothesis of a common antigen, probably a HCV antigen epitope, involved in the clonal B-cell selection. The involvement of a *VH4-34* segment with different characteristics of the CDR3 regions suggests a role of unknown B-cell superantigen(s) in the selection of HCV-negative NMZL.

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