Hypermutation in mantle cell lymphoma does not indicate a clinical or biological subentity

Margit Schraders[1,5], Sabine Oeschger[2,5], Philip M Kluin[3], Konnie Hebeda[1], Ed Schuuring[3], Patricia JTA Groenen[1], Martin-Leo Hansmann[4] and Johan HJM van Krieken[1]

[1]Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands; [2]Department of Pathology, University Hospital Giessen, Giessen, Germany; [3]Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands and [4]Department of Pathology, University Hospital Frankfurt, Frankfurt, Germany

Mantle cell lymphoma is a prime example of a well-defined entity based on morphology, phenotype, genetics and also clinical features. Although most patients have an adverse clinical course, some have a better survival than others. The most consistently reported adverse prognostic parameter is a high mitotic rate. Recently, it has been shown that hypermutation in the immunoglobulin heavy-chain gene occurs in a subset of mantle cell lymphomas. It is, however, unclear whether the mutational status is stable over time within a given case, whether hypermutation might be influenced by therapy and how it is related to other relevant biological features of mantle cell lymphoma. In this study, we analyzed 23 typical mantle cell lymphoma cases with respect to mutational status and compared the results with clinicopathological and genetic data to determine whether the presence of mutation indicates a subentity with clinical or pathological relevance. We found somatic hypermutation in 26% of our cases and, interestingly, one case showed ongoing somatic hypermutation. In tumor cells of both mutated and unmutated cases, we found a preferential usage of $V_H3\text{-}21$ (23%) and $V_H4\text{-}34$ (19%). No significant correlations were found between mutation status and the other morphological and genetic features analyzed. In conclusion, our results provide additional evidence that mutation status in mantle cell lymphoma is better interpreted as a feature within the spectrum of disease that seems to have little clinical or pathological relevance.
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Malignant lymphomas are presently considered as a series of clinicopathological entities. Each entity is defined on histological, phenotypic, genetic and, to a lesser extent, clinical features. Within a given entity, there is often a spectrum of morphology, phenotype, genetics and also clinical features. These latter aspects can be used as prognostic factor or alternatively to define a subentity. Mantle cell lymphoma is a prime example of a very well defined entity with several specific prognostic factors. The tumor is characterized by the t(11;14) translocation, which places the *BCL1* locus under the influence of the immunoglobulin heavy-chain gene enhancer, resulting in overexpression of cyclin D1.[1] Mantle cell lymphoma has rather specific morphology with monomorphic small- to medium-sized lymphoid tumor cells with a nodular, diffuse or mantle zone growth pattern; however, about 20% are of the blastoid variant.[2] The median survival for patients with mantle cell lymphoma is 3–5 years; the vast majority of patients cannot be cured. The most consistently reported adverse histopathological prognostic parameter is a high mitotic rate.[3] Other adverse prognostic factors are blastoid morphology[2,4] and the presence of p53 mutation accompanied by 17p deletion.[5–7]

Recently, it has been shown that hypermutation in the immunoglobulin heavy-chain gene occurs in a subset of mantle cell lymphomas, but the biological relevance is unclear.[8–12] In analogy with the situation in chronic lymphocytic leukemia/small lymphocytic lymphoma, it was suggested that mutated cases might be considered as a relevant subentity with different biological features and clinical behavior.[13–15] It is, however, at present unclear whether the mutational status is stable over time within a

Correspondence: Professor Dr JHJM van Krieken, Department of Pathology, Radboud University Nijmegen Medical Centre, Geert Grooteplein 24, Nijmegen 6500 HB, the Netherlands.
E-mail: j.vankrieken@pathol.umcn.nl
[5]These authors contributed equally to this work.

given case, whether it might be influenced by therapy and how it is related to other relevant biological features of mantle cell lymphoma, such as genetic imbalances, breakpoints in the BCL1/CCND1 locus and proliferative and apoptotic rate.

In this study, we analyzed a series of typical mantle cell lymphomas with respect to mutational status and compared the results with clinicopathological, morphological and genetic data to determine whether the presence of mutation indicates a subentity with clinical or pathological relevance.

Materials and methods

Case Selection

A total of 27 specimens from 23 patients were selected from the tissue banks of the departments of pathology of University Medical Centre Nijmegen, University Medical Centre Groningen, Rijnstate Hospital Arnhem and PATHAN, Rotterdam, the Netherlands. All cases were stained for cyclinD1 and CD5 and were reviewed by four experienced pathologists at a multiheaded microscope. The only inclusion criterion was the diagnosis of classical mantle cell lymphoma and the availability of frozen tissue. Morphological features were assessed including (vaguely) nodular growth, mantle zone growth and marginal zone or monocytoid differentiation of the tumor cells. Mitotic figures were counted per 15 high power fields (hpf). From two patients, serial biopsies were available, MC50, 51, 52 and 53 plus MC63 and 66, which were divided over a period of 7 years and 1 year, respectively.

Immunohistochemistry

From all cases, 4-μm-thick tissue sections of paraffin-embedded tissue were stained for CD20, CD5 (both Dako, Denmark) and cyclinD1 (Neomarkers) for diagnostic purpose. The cases were also stained for Ki-67 (mib1, DAKO, Denmark). Immunohistochemistry was performed using a standard two-step immunoperoxidase method, including development with the ABC-method (DAKO, Denmark) and DAB as chromogen. Furthermore, immunofluorescence was used for the assessment of immunoglobulin heavy and light chains. Ki-67-positive tumor cells were counted by using an automated procedure as described previously.[16]

DNA Extraction from Tissue Sections

For DNA isolation, QIAamp DNA Mini Kit (Qiagen) was used. Tissue sections with thickness of 20–30 μm were digested overnight with 40 μl proteinase K solution (20 mg/ml) and 360 μl ATL (QIAmp Tissue Lysis) buffer at 55°C. The DNA was precipitated by the addition of 6 M NaCl and dissolved in H_2O at a concentration of 100 μg/ml. A quantity of 100 ng of this extracted DNA was used in each amplification reaction.

PCR and Sequence Analysis

The clonal V_H gene rearrangements of the mantle cell lymphoma cases were identified by PCR using family-specific V_H framework region (FR) I primers together with a J_H gene consensus primer according to the BIOMED-2 protocol.[17] PCR products were purified and sequence analysis was performed on an automatic sequencing platform (ABI 3730, Applied Biosystems). Sequences were analyzed using the IMGT database (http://imgt.cines.fr:8104/). The V_H gene sequences were considered mutated when the sequences showed less than 98% homology with the corresponding germline sequence.

For light-chain gene rearrangement identification, 100 ng of genomic DNA was used in a 50 μl PCR mixture with the family specific kappa and lambda primers according to the BIOMED-2 protocol.

Southern Blot Analysis

15–20 μg of genomic DNA was digested with BglII and HindIII (Life Technologies, Rockville, MD, USA) and separated in 0.7% agarose gels. The DNA was transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany), which were subsequently hybridized with ^{32}P-labeled probes. For the determination of the IGK gene configuration probes specific for Jk (IGKJ5), Ck (IGKC) and K_{de} (IGKDE) (DAKO Corporation, Carpinteria, CA, USA) were used.[18] To examine the IGL locus, the IGLC1D and IGLJ2 probes were used.[19]

The Detection of BCL1/11q13 Breakpoints

For the detection of breakpoints in the 800-kb BCL1/11q13 region around CCND1, a previously designed interphase FISH segregation assay was performed using a probe set containing cosmids cos6.7/cCl-11-44 in combination with cos6.31/cosH1.5.[20] To identify breakpoints immediately 3′ of the CCND1 gene (breakpoint cluster 4), cosmid cos6.22/CCND1 in combination with cos6.31/cosH1.5 were used.[21] To map breaks within the 85-bp BCL1/MTC-region (cluster 2), we performed the t(11;14)-specific BCL1/JH-PCR as described previously.[17] To map breaks in the 120-kb region between MTC and CCND1 (but outside the 4000-bp MTC), an additonal segregation FISH with P1 clone 9107/11q13 and cos6.22/CCND1 was used (cluster 3).[22] Breaks detected within the 800-kb BCL1-region but outside clusters 2–4 are located centromeric of the MTC (cluster 1). Probes were labeled by Kreatech's ULS nonenzymatic labeling method (Kreatech Diagnostics, The Netherlands) with biotin-16–aUTP or digoxygenin-11–dUTP (Roche, Basel, Switzerland). Hybridization,

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Table 1 Morphological and immunohistochemical features of mantle cell lymphoma

Sample	Age/sex	Biopsy	Growth pattern	Morphological variants	Immunophenotype
MC01	ND	Tonsil	Nodular		CD5− cyclind1+
MC07	78/M	Lymph node	Nodular		CD5− cyclinD1+
MC08	69/F	Lymph node	Mantle zone, nodular	Monocytoid	CD5+ cyclinD1+
MC17	61/M	Lymph node	Nodular	Pleomorphic	CD5+ cyclinD1+
MC29	51/M	Lymph node	Nodular		CD5+ cyclinD1+
MC31	55/M	Lymph node	Nodular	Monocytoid	CD5+ cyclinD1+
MC36	67/M	Lymph node	Nodular		CD5− cyclinD1+
MC42	56/F	Lymph node	Diffuse		CD5+ cyclinD1+
MC43	49/M	Lymph node	Nodular		CD5− cyclinD1+
MC44	59/M	Lymph node	Nodular		CD5+ cyclinD1+
MC45	53/M	Lymph node	Diffuse		CD5+ cyclinD1+
MC47	67/M	Lymph node	Mantle zone, nodular		CD5+ cyclinD1+
MC48	71/M	Lymph node	Nodular	Monocytoid	CD5+ cyclinD1+
MC50[a]	54/F	Lymph node	Nodular		CD5+ cyclinD1+
MC51[a]	56/F	Lymph node	Nodular		CD5+ cyclinD1+
MC52[a]	58/F	Lymph node	Nodular		CD5+ cyclinD1+
MC53[a]	61/F	Lymph node	Nodular		CD5+ cyclinD1+
MC56	79/F	Lymph node	Diffuse		CD5+ cyclinD1+
MC57	62/F	Lymph node	Nodular		CD5+ cyclinD1+
MC58	82/M	Lymph node	Nodular		CD5+ cyclinD1+
MC59	77/M	Lymph node	Nodular		CD5+ cyclinD1+
MC61	73/M	Lymph node	Nodular		CD5+ cyclinD1+
MC62	85/F	Lymph node	Nodular		CD5+ cyclinD1+
MC63[b]	43/M	Lymph node	Nodular		CD5+ cyclinD1+
MC65	46/M	Lymph node	Nodular	Monocytoid	CD5+ cyclinD1+
MC66[b]	43/M	Adenoid	Nodular		CD5+ cyclinD1+
MC67	68/M	Spleen	Nodular		CD5+ cyclinD1+

ND, no data available.
[a]Same patient.
[b]Same patient.

immunodetection and fluorescence microscopy were performed as described previously.[20] Breakpoints are defined by the segregation of one or both probe sets and the presence of single red and/or single green hybridization signals in tissue sections. The cutoff levels for the probe sets were set at 11.0% (mean plus three times the standard deviation determined in seven negative control samples).[20]

Allelic Imbalance as Detected by CGH-Array

The genomic imbalances in mantle cell lymphoma detected by genome-wide array-based comparative genomic hybridization have been recently reported.[23] The array-CGH data have been used in this study to examine whether certain chromosomal imbalances are correlated with mutation status or V_H gene usage. In the array-CGH study, minimal common regions were defined as chromosomal regions that show copy number gain or loss in at least 5 of 14 patients. Moreover, for this study, we determined for each case the amount of BAC clones with copy number alterations (clones with a log2 ratio >0.3 or <-0.3). The number of clones with copy number alterations was divided by the amount of clones that gave a signal on the array, to calculate the percentage of clones with copy number alterations.

Results

Histology, Immunohistochemistry and Translocation Detection

The morphological and immunohistochemical features of our mantle cell lymphoma series are shown in Table 1. Four cases had monocytoid morphology of the tumor cells. In 21/23 cases, the growth pattern was (vaguely) nodular by histology. Remnants of germinal centers were seen in 2/23 cases by histology and Ki-67 staining. In these two cases, a combination of mantle zone and nodular growth pattern was present. In the two cases with serial biopsies, there was no change in cellular morphology, growth pattern or immunohistochemistry features, even in the case with a 7-year interval between the first and the last biopsy (MC50, 51, 52, 53). The great majority of cases (19/23) were CD5 positive and all of the cases showed was stained by cyclinD1. For most cases, no additional cytogenetic data are available. Therefore, there are no additional data on the CD5-negative cases in relation to cytogenetics.

Breakpoints within the *BCL1*/11q13 locus were identified in all mantle cell lymphoma cases analyzed. The breakpoints were scattered in the 800-kb region and mapped in different clusters (Table 2), 4 in the centromeric MTC-region (cluster 1), 6 in the 85-bp MTC-region, 13 between

Table 2 Immunoglobulin, proliferation and survival data of mantle cell lymphoma

| Sample | 11q13/BCL 1[a] | IGH | | | | IGK/IGL | Ki-67 (%) | Mitosis (15 hpf) | Survival (years) |
		V-gene	D-gene	J-gene	% of SH				
MC07	4	V4-b	D1-26	J4	0.00	V_K4-1	19.63	6	>5[b]
MC43	2	V3-7	D5-5	J3	0.00	V_K4-1	26.44	4	4
MC44	3	V3-21	D3-22	J6	0.00	$V_\lambda1$-47[c], V3	19.85	2	1.5
MC58	1	V3-53	D3-22	J5	0.00		57.93	51	2
MC59	3	V3-21	D6-19	J3	0.00		20.03	1	8
MC62	3	V3-7	D3-22	J4	0.00		17.66	3	
MC42	2	V2-26	D3-3	J4	0.39	V_K5	17.76	12	1.5
MC17	3	V3-21	D2-8	J6	0.43	$V_\lambda3$-19	19.28	17	>6[b]
MC31	3	V3-21	D2-2	J3	0.43	$V_\lambda3$-19	16.86	10	9
MC01	2+4	V4-34	D3-10	J3	0.44	$V_\lambda3$, $V_\lambda1$-47[c]	13.12	13	
MC47	3	V4-39	D6-13	J5	0.44	$V_\lambda8$-61	11.92	6	
MC56	3	V4-34	D2-15	J6	0.44	V_K2-30[c]	26.79	0	3
MC57	3	V3-21	D6-13	J6	1.30	V_K4-1[c], V_K5-2[c]	16.96	3	0.25
MC50[d]	2	V4-34	D5-5	J4	1.33	$V_\lambda3$-21 $V_\lambda2$-18	14.94	0	>8[b]
MC51[d]	ND	V4-34	D5-5	J4	1.33		21.42	9	>8[b]
MC52[d]	ND	V4-34	D5-5	J4	1.33		17.67	27	>8[b]
MC53[d]	ND	V4-34	D5-5	J4	1.33		29.23	9	>8[b]
MC48	3	V3-49	D5-5	J4	1.69	V_K1/OR2-0[c]	18.8	2	NA
MC67	2	V3-7	D1-1	J4	1.74		8.72	4	0.08
MC36	3	V4-34	D3-22	J4	1.76		56.84	20	1
MC63[e]	3	V3-33	D2-15	J4	2.16				>4[b]
MC66[e]	ND	V3-33	D2-15	J4	2.16		15.27	7	>4[b]
MC45	2	V3-21	D6-6	J6	2.6		39	4	1.5
		V2-5[c]	D6-6	J4	1.19				
MC29	1+4	V3-53	D3-3	J4	2.64	$V_\lambda1$-40 $V_\lambda2$-11	61.27	12	
MC08	1	V1-18	D6-6	J5	3.57		16.18	7	2.5
MC65	1	V3-33	D4-23	J5	3.91		19.99	5	1.5
		V3-33[c]	D1-1	J5	3.91				
MC61	3	V4-34	D4-23	J6	2.64		8.29	1	
					3.10				
					4.50				
		V4-39[c]	D2-2	J5	1.71				

[a]1-4 represent the clusters in the 11q13/BCL1 locus, the centromeric MTC-region (1), the 85-bp MTC-region (2), between MTC and CCND1 (3) and telomeric of CCND1 (4).
[b]Still alive.
[e]Same patient.
[c]These sequences are not in frame.
[d]Same patient.
hpf = high power field; ND = not done.
For several of the cases, the IGL or IGK were sequenced.

MTC and *CCND1* and 3 telomeric of *CCND1*. In 2 of the 23 cases, a double breakpoint was identified (MC01 and MC29). The mantle cell lymphoma cases all show variable proliferative activity (ki-67-staining) ranging from 8.29 to 61.27% and variable numbers of mitotic figures as shown in Table 2.

Sequence Analysis and Mutation Frequencies of V_H Genes

From 23 patients, 26 unique *IGH* gene rearrangements were amplified. Usage of the V_H-gene family was clearly unbalanced with preferential usage of V_H3 and V_H4 families accounting for 60 and 32% of all rearrangements, respectively. The most frequently used V_H genes were V_H3-21 (23%) and V_H4-34 (19%). There was no usage of family V_H5 and

V_H6. Mutations in the V_H gene segment were absent in six V_H gene sequences, and another six sequences carried <0.5% mutation frequencies; in 11 patients, mutated *IGH* V_H sequences were found in 6 of these to be more than 2% (Table 2).

From two patients, serial biopsies were available: cases 63 and 66 from one patient harbored identical mutations with a mutation frequency of 2.2%. The serial biopsies from another patient (cases 50, 51, 52 and 53) also showed identical mutations (mutation load was 1.3%).

The multiplex FR1 PCR (BIOMED-2) analysis showed that in three patients (MC45, MC65 and MC61) more than one *IGH* gene rearrangement was amplified (Figure 1). Case MC45 contained a V_H2-5 rearrangement, which had a mutation load of 1.2% and was out of frame. The second was a VH3-21 rearrangement, which was in frame and had a
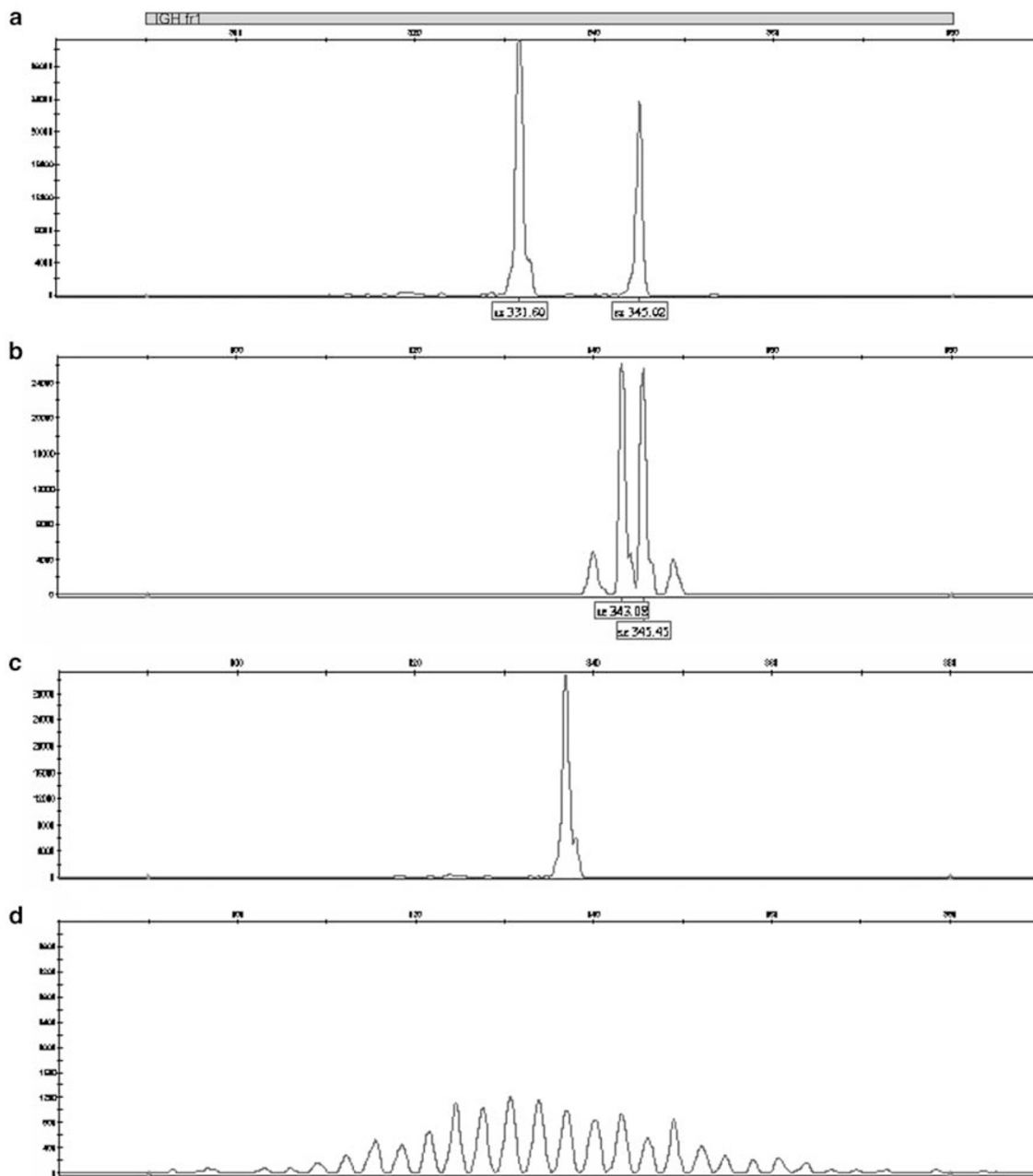
Figure 1 Genescan plots of MC45 (**a**), MC61 (**b**), a monoclonal control (**c**) and a polyclonal control (**d**). PCR was performed with BIOMED-2 multiplex IGH-VJ-FR1 forward primers and a JH consensus reverse primer. In both cases, multiple peaks were seen with the Genescan analysis.

mutation load of 2.6%. In MC65, two V3-33 rearrangements were identified; both showed a 3.91% mutation load, but different D-gene usage. MC61 showed multiple PCR products, all of the V_H4-family. To separate these products for sequence analysis, the mixture of PCR products was cloned into a PGEM-T vector. A V_H4-39 rearrangement with a mutation load of 1.71% that was an out-of-frame rearrangement was identified. Also an in-frame V_H4-34 rearrangement was identified; more specifically, VH4-34D4-23J6 in 4 of 8 PGEM-T clones, showing three different patterns of somatic hypermutation as

reported in Figure 2 and Table 2. The different mutation frequencies, in this particular case, might indicate ongoing somatic hypermutation.

Rearrangement and Sequencing of Kappa and Lambda Chains

IF data of the immunoglobulin light chains were available in 21 mantle cell lymphoma patients; of these, full analysis of kappa and lambda gene rearrangements by Southern blot was possible in

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                -----forward primer-----
MC61-seq1    CTTCGGGAGACCCTGTCGCTCRCCTGCGCTCGTCTATGGTGGGTCCTTCAGTGATTACTACT 60
MC61-seq2    CTTCGGGAGACCCTGTCGCTCRCCTGCGCTCGTCTATGGTGGGTCCTTCAGTGATTACTACT 60
MC61-seq3    CTTCGGGAGACCCTGTCGCTCRCCTGCGCTCGTCTATGGTGGGTCCTTCAGTGATTACTACT 60
VH4-34       CTTCGGGAGACCCTGTCCCTCRCCTGCGCTCGTCTATGGTGGGTCCTTCAGTGGTTACTACT 60
             ********** ****** *** ****************************** * *******
MC61-seq1    GGAGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATA 120
MC61-seq2    GGAGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATA 120
MC61-seq3    GGGCGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAGTATCAATCATA 120
VH4-34       GGAGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATA 120
             *  *********************************************  *** ** ***
MC61-seq1    GTGGAAGCACCAATTATAARCCGTCCCCCAAGAGTCGAGTCACCATATCAGTAGACACGT 180
MC61-seq2    GTGGAAGCACCAATTATAARCCGTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGT 180
MC61-seq3    GTGGGGGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCCGTAGACACGT 180
VH4-34       GTGGAAGCACCAACTACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGT 180
             ****  ***** * ** ** ******* **************** ** ***********
MC61-seq1    CCAAGAACCAGTTCTCCCTGAAGGTGAATTCTGTGACCGCCGCGGACACGGCTGTGTATT 240
MC61-seq2    CCAAGAACCAGTTCTCCCTGAAGGTGAATTCTGTGACCGCCGCGGACACGGCTGTGTATT 240
MC61-seq3    CCAARAAGCAGTTCTCCCTGAAGGTGAGCTCTGTGACCGCCGCGGACACGGCTGTGTATT 240
VH4-34       CCAAGAACCAGTTCTCCCTGAAGGTGAGCTCTGTGACCGCCGCGGACACGGCTGTGTATT 240
             **** ** ****************** *** *****************************
                           ---N1-------D------------N2---
MC61-seq1    ACTGTGCGAGAGTAACGGTCGGTTTCAACTCCGGGGCCCCCTCCTATTACTACGCTATGG 300
MC61-seq2    ACTGTGCGAGAGTAACGGTCGGTTTCAACGCCGGGGCCCCCTCCTATTACTACGCTATGG 300
MC61-seq3    ACTGTGCGAGAGTAACGGTCGGTTTCAACTCCGGGGCCCCCTCCTATTACTACGCTATGG 300
VH4-34       ACTGTGCGAGAG------------------------------------------------ 252
             ************
                              ----reverse primer----
MC61-seq1    ACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTAAG 344
MC61-seq2    ACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTAAG 344
MC61-seq3    ACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTAAG 344
VH4-34       --------------------------------------------
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Figure 2 Nucleotide sequences of the three different V_H4-34 clones found upon cloning of the FR1 amplicon of MC61. Differences in the nucleotide sequences are marked in gray. Percentage of somatic hypermutation: seq1, 3.11%; seq2, 2.67%; and seq3, 4.50%.

19 patients. In 2 additional patients, due to a limited amount of DNA, only the Lambda gene was analyzed, resulting in SB analysis of 7 kappa-positive and 13 lambda-positive cases (Table 3). When a complete in-frame $V_H-D_H-J_H$ heavy-chain rearrangement has been formed, rearrangement of the light-chain genes occurs. The Ig light-chain rearrangement process starts at the IGK locus. If no functional IGK gene rearrangements are formed, the cell can escape apoptosis by deleting the Jκ-Cκ region, followed by K_{DE} rearrangements and recombination at the IGL locus.[24,25] IGK gene rearrangements were assessed by Southern blot. Most lambda-expressing mantle cell lymphoma showed rearrangement of K_{DE} on both alleles (8 of 11). There was an overrepresentation of biallelic rearrangement of the lambda genes in unmutated cases (5 of 9), whereas mutated lambda-expressing cases retained one germline lambda gene in the majority of cases (3 of 4).

Sequence analysis of either the lambda or kappa genes was performed in 13 patients (Table 2). V_k4-1 genes were used in three of the eight sequenced kappa genes. All three of the sequences of V kappa-genes in lambda-expressing mantle cell lymphoma were out of frame. $V_\lambda3-19$ genes were used in two of nine sequenced lambda genes; in both cases, this was associated with V_H3-21 usage.

Correlation of Chromosomal Imbalances with Mutation Frequencies and V_H Gene Usage

Chromosomal imbalances that occur in mantle cell lymphoma were identified previously with array-based genome-wide comparative genomic hybridization.[23] In the array-CGH study, minimal common regions were identified, which are chromosomal regions that show copy number gain or loss in at least 5 of 14 patients. The total amount of minimal common regions varies between the cases with a minimum of 6 and a maximum of 13 minimal common regions. The mean amount of minimal common regions in cases with mutated V genes (11) did not differ from the mean of the cases with unmutated V genes (9.5) ($P=0.41$).

Cases with V_H3-21 gene usage were reported to have less chromosomal alterations than cases with other V-gene usage.[26] In our series, we also found that V_H3-21^+ cases showed a lower amount of minimal common regions(8.8) compared with V_H3-21^- cases (10.1), but the difference is not significant ($P=0.36$), which is probably because of the low number of cases. Also, the total number of clones with copy number changes did not differ ($P=0.65$) between mutated (mean of 17.3% of the clones showed gain or loss) and unmutated cases (15.2%). The same was found between cases with V_H3-21 and other V-genes. Cases with V_H3-21 had a mean of 12.1% of the clones with copy number changes, whereas cases with other V-genes had a mean of 17.1% clones ($P=0.142$).

Correlation of Mutational Status with Morphological, Clinical and Molecular Data

No difference between mutated and unmutated mantle cell lymphoma on morphological grounds
Our series of mantle cell lymphomas represent a homogenous group on the basis of morphological and immunophenotypic characteristics (Table 1); however, there were 57% unmutated and 43% mutated cases. This finding indicates that mutational status is not displayed morphologically.

No correlation of mutation frequency and survival years
Localization of the tumor biopsy, gender and age of the patient are given in Table 1. From 18 patients, clinical data about the survival years were available (Table 2). Four patients were alive when the follow-up ended; they were surviving at least 4 years after the diagnosis of mantle cell lymphoma was made. The median survival of the deceased patients was 2.7 years. No correlation was found between mutation frequency of the mantle cell lymphoma and survival years of the patients. Furthermore, no relationship between mitotic rate and survival and between mitotic rate and mutational status was detectable.

### No correlation between V gene usage or molecular characteristics and survival
A preferential usage of V_H genes was seen in mantle cell lymphoma; however no correlations were found between V_H gene usage and survival. Also no

Table 3 Southern blot analysis of the immunoglobulin light chains in mantle cell lymphoma

	IgK expressing (n = 7)						IgL expressing (n = 13)					
	Kappa genes			Lambda genes			Kappa genes			Lambda genes		
	G/G	G/R G/K_{DE}	R/R R/K_{DE} K_{DE}/K_{DE}	G/G	G/R	R/R	G/G	G/R G/K_{DE}	R/R R/K_{DE} K_{DE}/K_{DE}	G/G	G/R	R/R
Unmutated (n = 15)[a]	0	4	2	2	4	0	0	1	6	0	4	5
Mutated (n = 5)	0	1	0	0	1	0	0	1	3	0	3	1

G, germline; R, VJ-rearrangement, K_{DE}; rearrangements of K_{DE}.
[a]In two lambda expression mantle cell lymphoma, only lambda genes were analyzed.
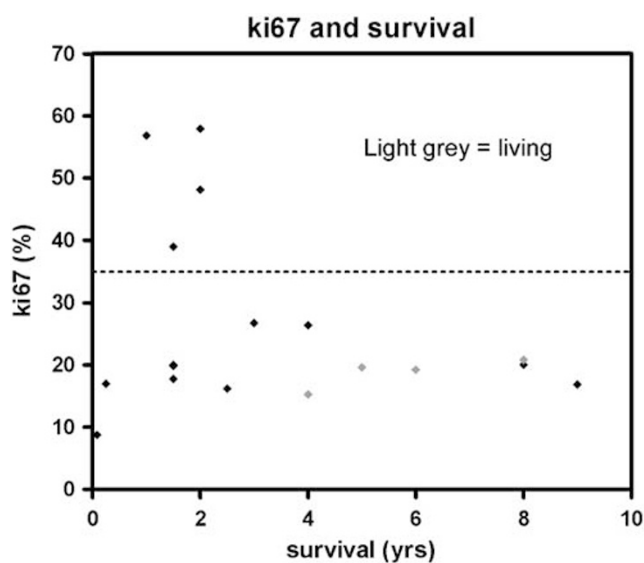
Figure 3 In our series, there is a statistically significant difference ($P = 0.015$) in survival time between mantle cell lymphoma with Ki-67-positive cells $\geq 35\%$ and mantle cell lymphoma with Ki-67-positive cells $< 35\%$, with a shorter median survival of 1.6 years in the first group compared with a median survival of 3.1 years in deceased patients of the second group.

correlations were found between chromosomal aberrations and survival. Finally, also no correlations were found between the positions of the breakpoints involved in the t(11;14), such as in the telomeric MTC-region, the 85-bp MTC-region, between MTC and *CCND1* or telomeric of *CCND1* and survival.

High proliferation activity (Ki-67 staining) is an adverse prognostic factor
In our series, there is a statistically significant difference ($P = 0,015$) in survival time between mantle cell lymphoma with Ki-67-positive cells $\geq 35\%$ and mantle cell lymphoma with Ki-67-positive cells $< 35\%$ with a shorter median survival of 1.6 years in the first group compared with a median survival of 3.1 years in deceased patients of the second group (Figure 3). Moreover, in the latter

group, there are four additional patients still living at least 4 years after diagnosis of mantle cell lymphoma was made (Welch two sample *t*-test, $t = 2.7366$, df $= 14.955$, *P*-value $= 0.01533$). No correlation of mutation frequency with Ki-67 level was detectable.

Discussion

Morphologically and immunophenotypically, neoplastic populations in mantle cell lymphoma resemble CD5 + B cells derived from the mantle zone of a lymphoid follicle. The CD5 + B cells of the mantle zone are naive lymphocytes that have not entered the germinal center of a secondary lymphoid follicle. Therefore, they lack somatic hypermutation of their V_H genes.[27] For long, it has been assumed that mantle cell lymphomas harbor unmutated V_H genes, but more recently the origin of mantle cell lymphoma from the pregerminal center cells of the mantle zone has been questioned due to investigations reporting on mutation of V_H genes in a considerable percentage of mantle cell lymphomas.[8–12,27–29]

In this study, we analyzed the mutational status of mantle cell lymphoma in 24 patients including serial biopsies taken from two patients. Both on morphological grounds and on the basis of results of immunohistochemistry, our cases of mantle cell lymphoma represent a very homogenous group, all cases being of the common type, all having the t(11;14)(q13;32) translocation and almost always CD5 positive. In our series, 26% of mantle cell lymphomas carried mutations of V_H genes with a mutation load ranging from 2.16 to 4.44%. In two cases from our series in which sequential biopsies were available for study, tumor cells displayed identical morphology and immunophenotype, that is, CD5- and cyclinD1-positivity, and similar proliferation activities. Both cases harbored mutations in the V_H genes (2.16% mutation frequency and 1.33% mutation frequency, respectively), but no change of mutation status over time was seen.

One case showed biallelic rearrangement of the *IGH* locus, a V_H2-5 and a V_H3-21 gene rearrangement, which had a mutation status of 1.19 and 2.60%, respectively. The V_H2-5 gene rearrangement was not in frame and therefore this is not a functional rearrangement. Multiple clonal *IGH* rearrangements were shown in another case. Interestingly, upon cloning the multiplex PCR products, multiple clones showed an identical VDJ usage, but different patterns of somatic hypermutation, which might indicate the presence of ongoing somatic hypermutation. As this is a feature of germinal center B cells, it is in contrast with the idea that mantle cell lymphomas arise from pregerminal center cells. Also, a subset of mantle cell lymphomas are CD5−, which is also in contrast to the idea that these lymphomas arise from CD5+ naive B cells.

Although our series is small, we observed preferential usage of V_H3-21 (23%) and V_H4-34 (19%) in tumor cells of both mutated and unmutated cases. This is in keeping with the results of other authors, who reported an overrepresentation of these two V_H gene segment usage in mantle cell lymphoma.[9,11,12] V_H3-21 and V_H4-34 both have been observed to be associated with autoimmune diseases, and the preferential usage of V_H genes in lymphomas may indicate that antigens are involved in the development of those tumors.[30–32] In our study, the five V_H genes used most frequently (V_H3-21, V_H4-34, V_H3-33, V_H3-7, V_H3-53) showed a balanced distribution in mutated and unmutated cases. Therefore, a possible antigen-dependent process with selection of V_H genes probably has no influence on the mutation status of the V_H genes.

The same seems to apply to selection of the immunoglobulin light-chain genes. Mutated and unmutated mantle cell lymphomas did not show any difference in the preferential use of Vkappa 4-1, Vlambda1-47 and Vlambda 3-1. Vkappa 4-1 is located closest of all V gene segments to the J genes (downstream) and is rarely used in peripheral B cells.[33] Increased usage of downstream Vkappa segments, including Vkappa 4-1, is reported in rheumatoid arthritis.[34] The predominant Vlambda genes are located more upstream from the J genes and are commonly used in peripheral B cells, as well as in B-chronic lymphocytic leukemia, indicating that they usually have proceeded through several receptor rearrangements.[33,35] We identified a slightly higher total number of rearranged genes in unmutated mantle cell lymphoma, resulting from more lambda rearrangements. The lambda rearrangements consisted both of biallelic lambda rearrangements in lambda-expressing cases, as well as 'illegal' lambda rearrangements in kappa-expressing cases.[25] Although 'illegal' lambda gene rearrangements occur in naive kappa-expressing B cells, their frequency (1.5%) is much lower than the 19% we observed in mantle cell lymphoma.[36] The mantle cell lymphoma light-chain rearrangement patterns also were very similar to those in mutated and unmutated B-chronic lymphocytic leukemia, which supports the idea that somatic mutation does not influence the light-chain recombination.[35]

Recently, Flordal Thelander et al[26] reported that mantle cell lymphomas with clonal V_H3-21 rearrangements exhibit fewer genomic imbalances than mantle cell lymphomas with other rearrangements. In our series, no statistical significant relationship could be established between V_H rearrangements and the occurrence of genomic imbalances. Flordal Thelander et al[26] made use of classic CGH, which has a limited resolution, and they might have missed small imbalances that could be detected with the array-CGH used to analyze our cases. Also no specific genomic alterations could be correlated with the presence or absence of hypermutation.

In chronic lymphocytic leukemia (chronic lymphocytic leukemia), it has been shown that cases with mutated IGV_H sequences have a better prognosis than unmutated cases.[14,15] Moreover, patients with V_H3-21 usage, independent of mutation status, have a poor outcome compared with cases with other VH usage.[14,15] In contrast, mantle cell lymphoma cases with V_H3-21 rearrangements seem to have a tendency toward a better prognosis as was shown by others.[8,9] In chronic lymphocytic leukemia patients, the V_H3-21$^+$ patients show a biased usage of $V_\lambda2$-14.[14,15] In this study, sequence analysis of the kappa or lambda rearrangements was performed in three of the six cases with V_H3-21 usage. $V_\lambda3$-19 usage was seen in two cases and $V_\lambda2$-14 usage was seen in the third case.

In line with other investigators, we did not identify a correlation of mutational status and survival time in our series.[8,9,12,37] In mantle cell lymphoma patients with V_H-mutations, we did not see a favorable prognosis independent of the cutoff level of mutations. This was also confirmed by a study undertaken by Walsh et al,[12] who correlated survival of patients with different cutoff percentages ranging from 1 to 5%. No significant differences in median survival times were found when comparing mutated and unmutated mantle cell lymphoma. One of the adverse prognostic parameters reported most consistently in mantle cell lymphoma is the proliferative activity of the tumor, represented histopathologically by a high number of mitoses and immunohistochemically by the level of Ki-67, a marker of cell proliferation. However, controversy continues about the number of mitotic figures and the level of expression of Ki-67 requisite to indicate a poor prognosis.[3,6,7,38] In our study, there was a statistically significant difference between mantle cell lymphoma with a Ki-67-positivity <35% and mantle cell lymphoma with a Ki-67-positivity ≥35% in regard to a longer survival time in the former, whereas mutation status of both groups did not correlate with Ki-67-positivity.

In conclusion, our results provide additional evidence that mutation status does not permit

definition of subentities in mantle cell lymphoma that are associated invariably with a certain prognosis. Mutation status in mantle cell lymphoma is better interpreted as a feature within the spectrum of disease that seems to have little clinical or pathological relevance.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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