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Primary diffuse large B cell lymphoma of the stomach: analysis of somatic mutations in the rearranged immunoglobulin heavy chain variable genes indicates antigen selection

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Gastric low grade MALT lymphomas show a pattern of somatic mutations in their rearranged immunoglobulin genes, indicative of antigen selection. This provides evidence for antigen stimulation in the lymphomagenesis. Gastric diffuse large B cell lymphomas develop secondary to low grade MALT lymphoma or *de novo*. To study whether antigen-selection is also a feature of primary diffuse large B cell lymphomas, we analysed somatic mutations in the rearranged immunoglobulin heavy chain (IgH) variable genes (VH). The rearranged VH genes of six cases of gastric primary diffuse large B cell lymphoma were amplified from genomic or complementary DNA by a VH gene family-specific polymerase chain reaction method. The PCR products were directly sequenced and were compared to published germline sequences to analyse somatic mutations. Similarly to low grade MALT lymphomas 5/6 primary diffuse large B cell lymphomas show a pattern of somatic mutation in their rearranged VH genes, indicative of antigen selection and suggesting a role for antigens in lymphomagenesis. One case showed bi-allelic VH gene rearrangements, which were nonfunctional due to extensive deletions. Antigen selection could not be demonstrated or excluded. Antigen selection is a common feature in most analysed primary diffuse large B cell lymphomas, although some heterogeneity in the mechanisms involved in the lymphomagenesis of gastric primary diffuse large B cell lymphomas has not been excluded entirely (case 4).
Keywords: diffuse large B cell lymphoma; antigen selection; mutation analysis; low grade MALT lymphoma

Introduction

Gastric lymphomas account for nearly 20% of all extranodal lymphomas.[1] The most frequent lymphomas are both low grade MALT lymphomas and diffuse large B cell lymphomas. There are data, which suggest that at least part of these diffuse large B cell lymphomas evolve from low grade MALT lymphomas. Indeed, the diffuse large B cell lymphomas may be present simultaneously with low grade MALT lymphoma in the same specimen.[2] The diagnosis of these secondary high grade lymphomas is based on specific morphologic characteristics such as the simultaneous presence of a low grade component and epitheliotropism.[2,3] In addition, the finding of similar genetic abnormalities, such as trisomy 3 is a strong indication that gastric diffuse large B cell lymphoma may evolve from a low grade MALT lymphoma.[4] However, the strongest argument for this hypothesis is the demonstration of a clonal relationship between both lymphoma subtypes within the same patient.[5,6] This progression of a low grade MALT lymphoma to a high grade lymphoma has been demonstrated to be associated with the loss of Bcl-2 expression, inactivation of p53 and c-myc rearrangement.[7–10]

It is not likely that all diffuse large B cell lymphomas represent transformed low grade MALT lymphomas, since features of a low grade component are only found in 30% of cases.[2] The finding of Bcl-6 rearrangements in an important proportion of the extranodal diffuse large B cell lymphomas, including those occurring in the gut, but not in low grade MALT lymphomas also indicates that some of these cases arise *de novo*.[11,12] Expression of Bcl-6 protein has been only observed in high grade MALT lymphomas of the stomach.[13] In addition, the detection of the t(11;18)(q21;q21) in low grade MALT lymphomas, but not in diffuse large B cell lymphomas of the gastrointestinal tract is also an indication that a subset of diffuse large B cell lymphomas arises *de novo*.[14]

Low grade MALT lymphomas of the stomach may arise through chronic *Helicobacter pylori* infection. The fact that low grade MALT lymphomas show a pattern of somatic mutations in their rearranged immunoglobulin genes, characteristic of an antigen selective pressure, is consistent with this hypothesis.[15–18]

Whether antigen selection is also a feature of primary diffuse large B cell lymphomas of the stomach, indicating a role for antigens in lymphomagenesis, is not known and is the subject of this study.

Materials and methods

Selection of cases

Six patients, who presented with a primary diffuse large B cell lymphoma of the stomach were studied. None of these patients had a previous history of non-Hodgkin's lymphoma. The patient characteristics are summarised in Table 1. All patients were treated with surgery. Only cases 5 and 6 received adjuvant chemotherapy. Cases 4 and 6 died with progressive disease 24 days and 64 months after the diagnosis, respectively. Case 3 died of a concurrent oat cell carcinoma at 39 months. Cases 2 and 5 are alive at 28 and 64 months without evidence of disease. Case 1 was lost to follow-up.

Paraffin-embedded as well as frozen tissue was available from all cases. These tissues were used for immunophenotypical analysis and molecular studies. A panel of antibodies, namely CD5, CD10, CD20, CD22, CD23, IgM, IgD, IgA, kappa, lambda and Bcl-2, was applied to phenotype and to classify these lymphomas according to the REAL classification (Table 1).[19] To detect *Helicobacter pylori* a cresyl fast violet staining or an immunohistochemical staining (Dakopatts), which recognises spiral-shape as well as coccoid forms, was performed in each case. Depending on the antibodies applied either a peroxidase anti-peroxidase method or an avidin–biotin complex technique was used.

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Table 1 Characteristics of gastric diffuse large B cell lymphomas in this study

Case	Age/ Sex	Stage at surgery[a]	CD5	CD20	CD22	CD23	CD10	Bcl-2	IgM	IgD	IgA	κ	λ
1	84/F	IIE1	–	+	+	–	–	+	+	–	ND	–	+
2	66/F	IE1	–	+	+	–	–	+	–	–	+	+	–
3	54/M	IE1	–	+	–	–	–	+	+	–	ND	+	–
4	69/M	IIIE	–	+	+	–	–	+	–	–	–	+	–
5	59/M	IIIE	–	+	+	–	–	–	+	–	ND	+	–
6	61/M	IE2	–	+	+	–	–	–	+	–	ND	+	–

ND, not determined; +, positive; –, negative.
[a]Gastric lymphomas were staged according to the Musshoff classification.

Amplification of rearranged immunoglobulin heavy chain genes

RNA was extracted from 10 20-μm frozen tissue sections, taken from a representative tissue block, using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the recommendations of the manufacturer. An amount of 1 μg of total RNA was used for reverse transcription (RT). The RT was performed using Superscript II Reverse Transcriptase (Life Technologies) following the manufacturer's instructions. Briefly, the RNA was added to 8 μl of DEPC-treated H_2O containing 150 ng random primers (Life Technologies). This mixture was heated at 70°C for 10 min and quickly chilled in ice water. Subsequently, 12 μl of a reaction mixture containing 15 mm KCL, 10 mm Tris-HCl pH 8.3, 0.75 mm $MgCl_2$, 0.1 m DTT, 10 mm dNTPs, 200 U reverse transcriptase and 2.5 U ribonuclease inhibitor (Life Technologies) was added. This mixture was incubated for 10 min at room temperature, for 50 min at 42°C and finally for 5 min at 30°C.

In case 4, in which the RT-PCR yielded no positive result, a PCR was performed on genomic DNA, extracted from frozen tissue, using the phenol/chloroform extraction procedure.[20]

A semi-nested PCR method was used to amplify the rearranged Ig heavy chain genes according to the method described by Kuppers *et al*.[21] 4 μl of a 1/100 dilution of the cDNA or 200 μg of the genomic DNA was used for the PCR. A mixture of six framework I (FR I) VH family-specific primers and a set of three consensus primers complementary to the joining region (JH) genes was applied in the first round of the PCR (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands).[21] The cDNA or DNA was added to a mixture of a PCR buffer (50 mm KCl, 10 mm Tris-HCl pH 8.4, 0.01% gelatin), 200 mm dNTPs, 2.5 mm $MgCl_2$, 100 nm of each primer and 1.5 U Taq polymerase in a total volume of 50 μl. The second round of the PCR was carried out in six separate reactions using only one of the six VH FR1 primers, respectively, and a mixture of internal JH primers (Amersham Pharmacia Biotech Benelux (Roosendaal, The Netherlands).[21] 2 μl DNA of the first round was added as template for the second-round PCR. The reaction mixture was otherwise similar to the one used in the first round of the PCR.

The PCR conditions of the first round consisted of 35 cycles for 90 s at 95°C, for 30 s at 59°C, for 80 s at 72°C and one final cycle of 5 min at 72°C. In the second round of PCR 45 cycles were performed. The annealing temperatures were 65°C and 61°C for the PCRs using VH3, VH4 and VH1, VH2, VH5, VH6 primers, respectively. The denaturation and extension steps were the same as for the first round of PCR. The PCR reactions were performed using a hot start procedure and included appropriate positive and negative controls. Each sample was analysed in duplicate. The PCR products were electrophoresed through a 6% poly-acrylamide gel (PAGE) and visualised by ethidium–bromide staining.

PCR products were gel-purified prior to sequencing. Direct sequencing of the PCR products was performed using the Sanger's chain termination method and fluorescent dideoxynucleotide chain terminators.[22] Sequencing primers were identical to the primers used for the second round of the semi-nested PCR of the VH genes. The products of the sequencing reaction were analysed using the Applied Biosystems 373A sequenator (Applied Biosystems, Foster City, CA, USA).

Analysis of the sequences was performed using the Mac Vector 5.0 sequence analysis software (Oxford Molecular Group, Campbell, CA, USA) and the V Base database, which is a comprehensive database of human immunoglobulin germline sequences compiled from published sequences (V BASE sequence directory, Tomlinson *et al*, MRC Centre for Protein Engineering). The germline VH genes, diversity region genes (DH) and JH genes and the presence of somatic mutations were identified by comparison to the closest germline sequences present in the March 1997 update of V Base. The DH genes were identified when seven or more contiguous bases were homologous to the germline gene or when six contiguous and homologous bases separated by one nucleic acid from at least one other homologous nucleic acid were present.

Somatic mutation analysis

The probability that an excess of replacement mutations in the CDR region or FR region is only the result of chance was calculated applying the binomial distribution model according to Chang and Casali, using the formula:

$$P = \{n!/[k!(n - k)!]\} \times q^k \times (1 - q)^{n-k}$$

where n = total number of observed mutations; k = number of observed R mutations in CDR or FR regions; q = probability that a R mutation will occur in CDR or FR regions ($q = CDR_{rel} \times CDR\ Rf$ or $FR_{rel} \times Rf$). Rf corresponds to the inherent susceptibility of replacement mutations in the CDR or FR region, resulting in an amino acid replacement.[23] Two mutations occurring in one codon are considered to represent one replacement mutation. CDR and FR regions were determined according to Kohot *et al*.[24]

Southern blot analysis

Southern blot analysis was performed on gastric samples obtained from the same biopsy material that was processed

for sequence and mutation analysis. Eight μg of tumour DNA and control placental DNA were digested with the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Rst*I, respectively, and size fractionated on a 0.7% agarose gel. After alkali blotting on to Hybond N+ membranes (Amersham, Buckinghamshire, UK) hybridisation was carried out using DNA probes labelled with ^{32}P-dCTP using the random printing method. The Bcl-2 gene was analysed using the probe Bcl2B (a 2.8 kb *Eco*RI–*Hin*dIII fragment, kindly provided by Dr Y Tsujimoto, Wistar Institute, Philadelphia, PA, USA) and the minor cluster region probe pFL2 (a 1.4 kb *Eco*RI fragment, kindly provided by Dr M Cleary, Stanford University, Stanford, CA, USA).

The Bcl-6 gene was investigated with probe Bcl6 (a 4 kb *Sac*I fragment, kindly provided by Dr R Dalla-Favera, University Medical Center, New York, NY, USA).

Results

Morphology and immunophenotype

All cases presented with a large, nodular mass in the stomach, the diameter of which varied between 5 and 12 cm. The mucosal surface of the tumour was invariably ulcerated. The lymphomas were deeply infiltrating and invaded the serosal fat. In cases 4 and 5 the tumour invaded adjacent organs such as the pancreas, the spleen and the colon. Involved contiguous and non-contiguous lymph nodes were found in cases 1 and 4.

All cases showed a similar histology and were composed of large cells. These cells displayed an irregular, vesicular nucleus, characterised by a prominent nucleolus, and a fair amount of cytoplasm. Features, which may suggest the presence of a concomitant low grade MALT lymphoma, such as, for example, the presence of lympho-epithelial lesions, were not found (Figure 1).

The immunohistochemical data are summarised in Table 1. The lymphoma cells in all cases expressed CD20 but not IgD, CD5, CD10 or CD23. IgM expression was documented in four cases and IgA in one. Kappa immunoglobulin light chain restriction was documented in five cases whereas lambda immunoglobulin light chain restriction was present in one case. *Helicobacter pylori* was detected in two of the six cases (33%).

RT-PCR and sequence analysis

The RT-PCR of the rearranged VH genes yielded products in five out of the six cases. In case 4, in which the RT-PCR was not successful, the PCR was performed on genomic DNA. This case showed a bi-allelic rearrangement. Each sequence was compared with the closest IgH germline sequence in the V BASE directory. The results of this sequence analysis are given in Table 2. VH3 and VH4 family genes were found to be rearranged in our cases. DH sequences were identified with certainty in only four cases. The most frequently rearranged JH genes were JH4 and JH6.

Mutation analysis (Figures 2 and 3)

The results of the mutation analysis are summarised in Table 2.

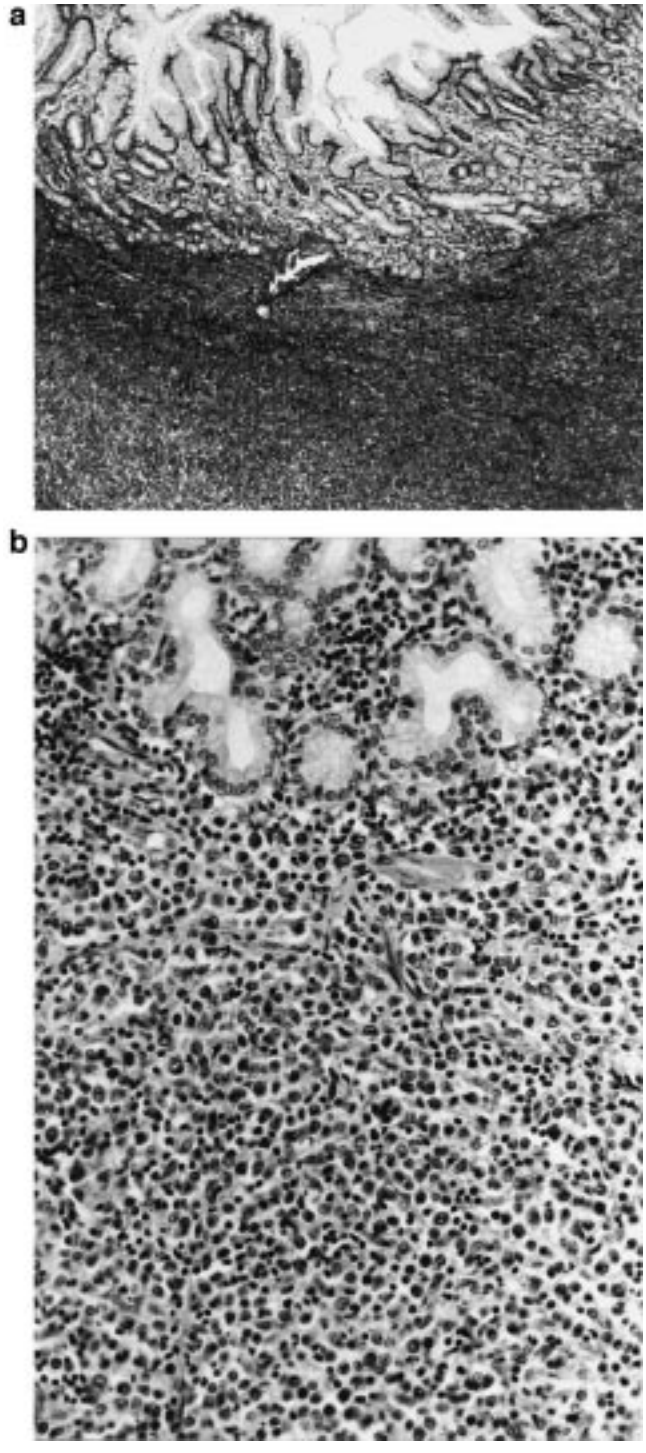

Figure 1 Primary diffuse large B cell lymphomas are extending into the deeper layers of the gastric wall, as shown in this picture (a, H/E-stain, 125×). These lymphomas are composed of large cleaved cells. These cells do not infiltrate glandular structures, causing no lympho-epithelial lesions (b, H/E-stain, 315×).

Mutation analysis was performed in each case with the exception of case 4, which displayed non-functional bi-allelic gene rearrangements. In case 5 the closest germline gene was DP47. However, the rearranged VH sequence of case 5 had two additional codons in the CDR2 region with comparison to the germline sequence. It is very likely that these extra codons in the CDR2 region represent a polymorphism of the

Table 2 Mutation analysis of the gastric diffuse large B cell lymphomas

Case	*Germline VH gene*	*VH gene family (Ref.)*	*% Identity*	*n*	*CDR*	*FR*	*R/S CDR*	*R/S FR*	*ER CDR*	*ER FR*	*pCDR*	*pFR*	*Germline DH gene*	*Germline JH gene*	*In frame*
1	DP-77	VH3[a]	98	5	3	2	3/0	2/0	1.07	2.77	0.09	0.034	D1/D2	JH4	Yes
2	V2-1+	VH4 (25)	94	14	7	7	7/0	4/3	3.38	5.62	0.042	0.044	D2	JH4	Yes
3	DP-54	VH3 (26)	94	14	6	8	4/2	4/4	3.6	5.29	0.224	0.039	DK4/D4	JH6	Yes
4A[b]	DP-54	VH3 (26)											NI	JH2	—
4B	DP-63	VH4 (26)											NI	JH6	—
5	DP-71	VH4[a]	93	20	8	12	7/1	7/5	4.94	10.54	0.109	0.05	D3	JH4	Yes
6	DP-47	VH3 (26)	84	38	15	23	14/1	11/12	9.73	19.84	0.041	0.002	NI	JH4	Yes

VH gene, variable gene of the IgH; DH gene, diversity gene of the IgH; JH gene, junction gene of the IgH; n, total number of mutations observed; R, replacement mutation; S, silent mutation; CDR, complementarity-determining region; FR, framework region; RE, replacement mutation expected; pCDR, probability that excess or scarcity of R mutations in the VH gene CDRs resulted from chance only; pFR, probability that excess or scarcity of R mutations in the VH gene FRs resulted from chance only; NI, not identified.

[a]These germline sequences have not been published but are included in the V Base sequence directory.

The sequences, submitted to Genbank, have as accession numbers: U92706, AF 030493, U92707, U92708, U92709, U92710, AF030494.

[b]Both alleles of this case contain one or more extensive deletions.

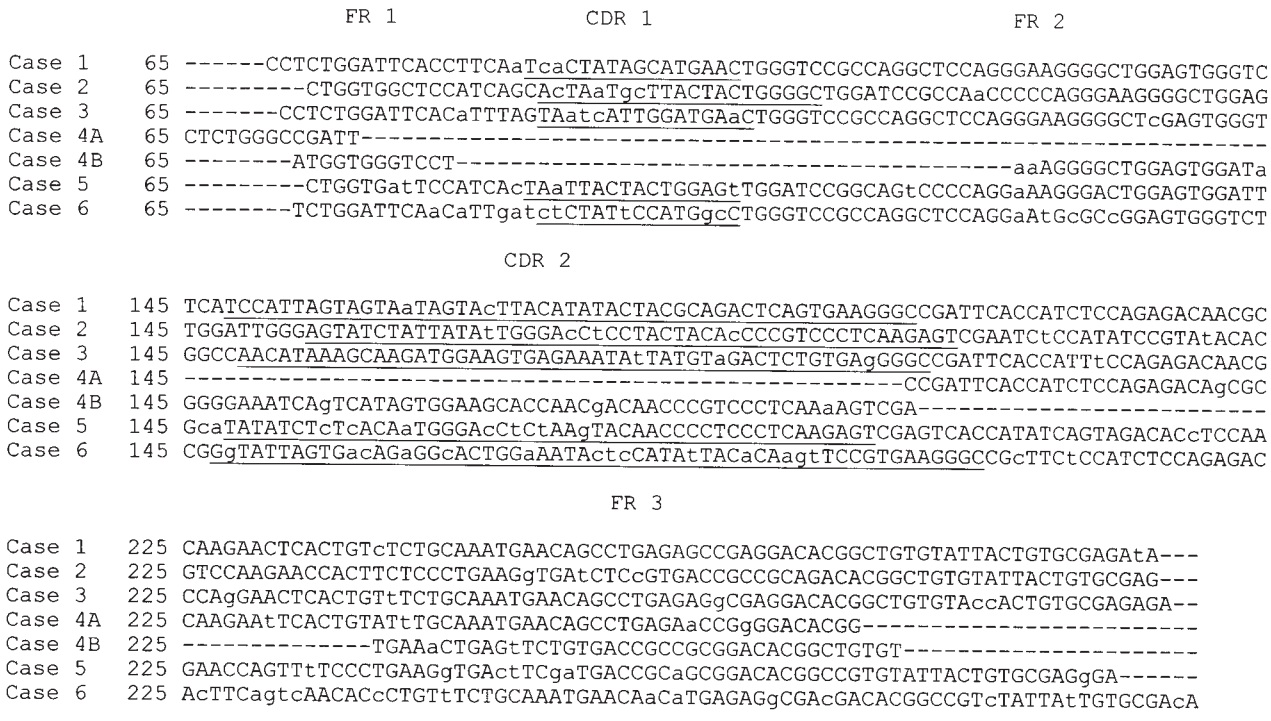

Figure 2 Nucleotide sequences of the rearranged VH genes of the cases of diffuse large B cell lymphomas. Lower case indicates replacement mutations compared to the germline sequences. The underlined parts of the sequences correspond to the CDR regions. FR, framework; CDR, complementary-determining region.

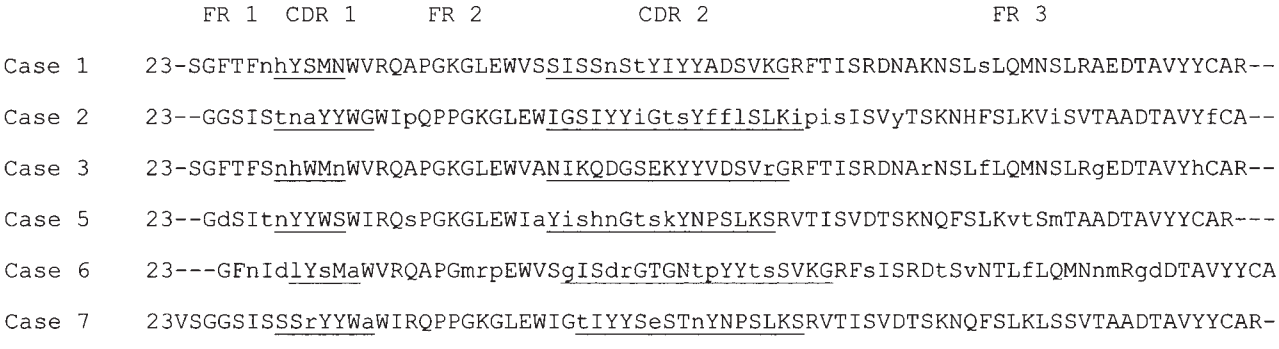

Figure 3 Deduced protein sequences of rearranged VH genes of cases of diffuse large B cell lymphomas. Replacement mutations, comparing to the germline sequences, are indicated by lower case. The underlined parts of the sequences correspond to the CDR regions. FR, framework; CDR, complementarity-determining region.

DP47 germline gene.[24] Therefore, the mutation analysis in case 5 was performed by comparison to the DP47 germline gene to which the two extra codons were added.

The rearranged VH genes of our cases of gastric lymphomas showed a variable number of somatic point mutations (between 5 and 38) in the rearranged VH genes. The pattern of replacement (R) and silent (S) mutations in the rearranged VH genes of the five cases with productive gene rearrangements was not randomly distributed: each case had a higher R/S ratio in the CDR regions compared with the FR regions. Either the pCDR or the pFR revealed statistically significant differences of the R/S ratio indicating antigen selection.

Case 4 showed bi-allelic IgH gene rearrangements, which are both non-functional due to large deletions. One allele showed a deletion stretching from nucleic acid position 78 up to 197. In the other allele three deletions were found: from bp 86 up to 125, bp 198 up to 239 and finally from bp 278 up to 293. Somatic mutations were present in both of these alleles.

Southern blot analysis

Southern blot analysis revealed no Bcl-2 or Bcl-6 gene rearrangements in any of our cases.

Discussion

During the differentiation of B cells in the germinal centre, somatic mutations accumulate in the rearranged immunoglobulin genes. Those B cells which have acquired mutations yielding high affinity antibodies will be selected through the binding of antigen exposed by follicular dendritic cells and

allowed to leave the germinal centre as antibody secreting plasma cells or memory cells. This process is called affinity maturation of the humoral immune response. Affinity maturation is molecularly characterised by the predominant occurrence of replacement mutations in the CDR regions of the rearranged V gene, the antigen-binding sites. Thus, analysis of the occurrence and pattern of somatic mutations in the rearranged V genes may yield information on the differentiation stage of B lymphocytes. Similarly, this analysis can also be used to gain information on the differentiation of B cell non-Hodgkin's lymphomas. Moreover, this analysis may answer the question whether or not antigen stimulation has preceded or is still involved in the clonal evolution of the lymphoma.[23,27,28]

In our study we analysed the occurrence and pattern of somatic mutations of the VH genes in a series of primary gastric diffuse large B cell lymphomas. We studied six patients with diffuse large B cell lymphoma of the stomach, who did not have a previous clinical history of non-Hodgkin's lymphoma, including low grade MALT lymphoma. In addition, macroscopic examination revealed only a distinct tumour in the specimen and there was no small cell lymphoma identified by microscopic examination. Therefore, we argue that these cases represent primary diffuse large B cell lymphomas of the stomach. Five of our cases displayed functional IgH gene rearrangements, which show a high number of somatic mutations. Mutation analysis shows a statistically significant difference between the observed and expected replacement to silent mutation ratios in either the CDR or the FR. This is strong evidence for antigen selection or affinity maturation. Hence, these lymphoma cells most likely originated from antigen-stimulated memory B cells.[29] Case 2, in which the tumour cells express IgA, may have originated from cells in an even later stage of maturation; these cells are possibly related to the classical isotype-switched memory cell.[30] Case 5 may represent a polymorphism of the DP47 germline gene since two codons are missing in the CDR2 region. However, a recent study has shown that deletions as well as insertions have been acquired during the somatic hypermutation process in the germinal centre.[31]

Antigen selection, documented in five of our six cases, is also a feature of low grade MALT lymphomas.[15–18] Thus, gastric low grade MALT lymphoma as well as gastric primary diffuse large B cell non-Hogkin's lymphoma may have a common origin, namely antigen-selected memory B cells. Interestingly, primary nodal diffuse large B cell lymphomas also show features of antigen selection and therefore may originate from memory cells too.[32,33] Because low grade MALT lymphoma and primary gastric diffuse large B cell lymphoma likely arise from antigen-selected memory cells, it might be argued that primary gastric diffuse large B cell lymphoma represents a progressed low grade MALT lymphoma that is no longer present at diagnosis. Based on our molecular data we cannot exclude this possibility, as we did not find Bcl-6 gene rearrangements in our cases. Our results are, however, in contrast to the study of Offitt *et al*,[11] which has found these gene rearrangements in 23% of the diffuse large B cell lymphomas, mostly extranodal situated. Some of these extranodal diffuse large B cell lymphomas were situated in the stomach.[11] In contrast to these lymphomas, Bcl-6 gene rearrangements are not observed in low grade MALT lymphomas. In contrast to Bcl-6 gene rearrangements, Bcl-6 gene hypermutations do not contribute to a different histogenesis of diffuse large B cell lymphomas and low grade MALT lymphomas, as although these hypermutations are frequently found in association with diffuse large B cell lymphomas, they are not confined to this type of lymphoma.[34,35] Moreover, it is not clear if these Bcl-6 gene hypermutations represent a tumor-associated phenomenon since, due to the process of somatic hypermutation of immunoglobulin genes, these hypermutations are also found in follicular center cells and memory cells of normal individuals.[36,37]

The absence of Bcl-2 gene rearrangements and Bcl-2 expression makes it less likely that our diffuse large B cell lymphomas are derived from follicular center cell lymphomas.[10]

Based on our molecular analysis we cannot exclude, although there is no morphological evidence, that our cases are secondary high grade lymphomas, derived from low grade MALT lymphomas. At this moment, it is not clear if antigen selection is only present in those secondary high grade lymphomas. Therefore, additional molecular data should be acquired by performing similar studies on a larger series of gastric diffuse large B cell lymphomas.

Four of our cases of gastric diffuse large B cell lymphomas used germline genes, which have been reported to encode for auto-antibodies: V2-1, DP-54, DP-47 (VH-26), DP-77.[38–42] The usage of the germline genes which may encode auto-antibodies is also a distinctive feature of low grade MALT lymphomas of the gastrointestinal tract and salivary glands.[15,17,43,44] It has been well documented that low grade MALT lymphomas may produce antibodies that bind self-antigens.[45,46] This strongly suggests that MALT lymphomas arise from auto-reactive memory B cell subsets. It has been postulated that chronic *Helicobacter pylori* infection of the stomach may stimulate auto-reactive B cells secondary to tissue damage.[47] The usage of similar VH genes frequently involved in auto-antibody production in four of our cases, might indicate that primary diffuse large B cell lymphoma of the stomach arises from auto-reactive B cells as well. Whether chronic *Helicobacter pylori* infection might underlie this presumed auto-reactive response is highly speculative. The mucosa of only two of our cases of primary diffuse large B cell lymphomas were colonised by *Helicobacter pylori*. However, the *Helicobacter pylori* infection rate may be underestimated because of the presence of an ulcerated mucosa and technical reasons, eg operation specimen.

Case 4 showed a bi-allelic non-functional gene rearrangement. Both alleles showed extensive deletions. The mechanism by which these may have occurred is not clear. Because of the presence of extensive deletions it cannot be definitely excluded that antigen selection did not occur in this case, however, mutation analysis only showed a limited number of mutations. In this case antigen stimulation may possibly not have contributed to the lymphomagenesis. This may point to some heterogeneity with respect to the biology of gastric primary diffuse large B cell lymphomas.

In accordance with a very recent study of Hallas *et al*,[48] our investigation shows that some primary gastric diffuse large B cell lymphomas show somatic hypermutation of the rearranged IgH genes with features of affinity maturation. Therefore, it is likely that primary diffuse large B cell lymphomas arise from memory B cells. As low grade MALT lymphomas diffuse large B cell lymphomas are antigen-selected, which suggest that both types of lymphomas may arise through chronic antigen stimulation. However, affinity maturation may not be a feature of all cases of primary diffuse large B cell lymphomas of the stomach, as illustrated by one of our cases displaying bi-allelic non-functional VH gene rearrangements.

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