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Detection of IgH variable region gene in paraffin-embedded tissues from MALT lymphomas using the polymerase chain reaction.

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Immunoglobulin heavy chain (IgH) variable regions are encoded by three groups of germline gene segments: an estimated 100 to 150 variable (VH) genes, 30 diversity (D) genes, and 6 joining (JH) genes. These segments are rearranged in pre-B cells to form a complete variable region (VHDJH) unit. Variable region contains three hypervariable regions (CDRs 1, 2, and 3). CDR1 and CDR2 are encoded within the VH segments whereas CDR3 is formed by VDJ junctional region. CDR3 directly contacts with antigen and is the most variable region of the Ig molecule. Antigen specificity is highly dependent on the composition of CDR3. CDR3 changes its structure in accordance with the antigen. The nucleotide sequence of CDR3 is developmentally regulated, with its length showing a tendency to increase as the antigenic stimuli increase with the maturation of the individuals.[1] The increase of diversity of IgH gene in response to various antigens is generated through the recombination of multiple VH, D and JH segments, the increased use of the longer DH and JH gene segments, the increased number of D-D fusions, the additions to the N region, and somatic hypermutation. Owing to this diversity, the CDR3 region is unique in each rearrangement. Analysis of the CDR3 sequence, therefore, is a useful procedure for studying the clonality of malignant B cells and the presumption of binding antigen.

Analysis of somatic mutation in VH gene is used as indicators of antigen selection with affinity maturation. A high ratio of replacement (R)/silent (S) mutations in the CDRs in comparison with the framework regions (FRs) is observed in postgerminal center B cells, but not in pregerminal B cells. Analysis of the VH sequence can determine the developmental stage of lymphoma cells. Here, we explain the sequence procedure to analyze the Ig VH and CDR3 region genes of paraffin-embedded tissues from patients with MALT lymphomas using the polymerase chain reaction (PCR). Furthermore, we describe the procedure to detect vacA gene of H. pylori, may be related to the pathogenesis of gastric MALT lymphomas.

Microdissection and DNA preparation

An H&E section was visualized under a x5 objective and marked off a rich zone of lymphoma cells by a marker pen. Nonlymphoid tissue was carefully scraped off leaving a zone as small as 25mm^2. Four serial sections 5μm thick were laid on top of the first H&E section and the corresponding zones were selected, the rest was scraped off. Microdissected areas from four sections were then scraped into one Eppendorf tube.

One ml xylene was added to dissolve the paraffin. The samples were then vortexed for 30 seconds and centrifuged at 15,000 rpm for 5 minutes. The supernatant was removed with Pasteur pipette and the xylene washing procedure was repeated. Then, 1 ml of ethanol was added to the pellet, and the mixture was vortexed for 30 seconds and centrifuged at 15,000 rpm for 5 minutes. The supernatant was discarded, and 1 ml of ethanol was added to the pellet. Vortexing and centrifugation were repeated. After removal of the supernatant, the samples were dried up and 200μl of sterile water was added to the pellet. The samples were boiled for 45 minutes and cooled to room temperature. DNA was extracted with phenol/chloroform and ethanol precipitation. The pellet was dissolved in 100ml distilled water and used as the DNA source for PCR amplification.

PCR for IgH CDR3

IgH gene was amplified according to the two-step PCR method of Wan et al.[2] The primers used were : 5'ACA CGG C[C/T][G/C] TGT ATT ACT GT3' (Fr3A), 5'TGA GGA GAC GGT GAC C3' (LJH), and 5'GTG ACC AGG GT[A/G/C/T] CCT TGG CCC CAG3' (VLJH). Fr3A is the oligomer for the third Framework portion of the V region. LJH and VLJH are based on the consensus sequence from the J region. For the first step of amplification, each 100 μl reaction mixture contained 0.25μM of each of the primers Fr3A and LJH, 1 μl of extracted DNA, 2.5 unit of Taq polymerase (Perking Elmer Cetus), 0.2 mM dNTP Mix, 10 mM Tris-HCL (pH 8.8), 1.5 mM MgCl2, 50mM KCl and 0.001%(W/V) gelatin. Denaturing was carried out for 2 minutes at 94°C, annealing for 2 minutes at 60°C, and extension for 2 minutes at 72°C for 30 cycles. The samples were initially denatured at 94°C for 5 minutes; after the last cycle, a final extension step of 6 minutes at 72°C was performed. The second step of 20 cycles with Fr3A and VLJH was performed with 10μl of a 1:1000 dilution of the first-step PCR product as template. After second round amplification, PCR product (40μl) was extracted with phenol/chloroform and precipitated with ethanol. The precipitant was then dissolved in 5 μl TE buffer, subjected to electrophoresis on 2% agar gel, and stained with ethidium bromide to visualize the DNA under short wavelength UV light.

PCR for VH genes

The two-step seminested PCR method was performed. The primer sequences were: 5'TGG [A/G]TC CG[A/C]

CAG [G/C]C[T/C] [T/C]C[A/G/T/C] GG3' (termed Fr2) for the second framework portion of the VH regions and LJH or VLJH for the JH region are already mentioned. One PCR cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. The first-step consisted of 30 cycles with primers Fr2 and LJH and 5 μl of template DNA; the second-step consisted of 20 cycles with FR2 and VLJH, with 10 μl of a 1 to 1,000 dilution of the first step PCR product as a template.

PCR for H. pylori genes[3]

The detection of the vacA gene was performed with amplification using the following primers: FT2-1187, 5'CGG TTG TCA ATA TCA ACC GC3' (upstream primer); FT2-1314, 5'ATT GGA CAG ATT GAC ACC GC3' (common downstream primer); FT3-1200, 5'CAA CCG CAT CAA CAC TAA CG3' (upstream primer). For the first step of PCR, 10 ng of genomic DNA or 10 μl of the extracted DNA from paraffin sections were added with 2.5 units of Taq polymerase and with the primers for FT2-1187 and FT2-1314 at a final concentration of 0.5 nM in 100 μl of standard buffer. The reaction mixture was first at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 55 °C for 30 seconds, and 72°C for 60 seconds. A final extension of 5 minutes at 72°C was performed after the last cycle. The second step of 35 cycles was performed with 5 μl of a 1:100 dilution of the first-step PCR product as template and with FT3-1200 and FT2-1314 primers with the same thermal profile as the first-step PCR. Agar gel electrophoresis of the final PCR product was performed. The expected size of amplified products was 115 bp.

Sequence Analysis

One μl of PCR product was ligated to PCR™ vector, and the ligation mixture was transformed into One Shot™ competent cells by using a TA Cloning Kit (Invitrogen Corporation). The transformants were plated on an LB plate containing 50 μg/ml ampicillin and X-Gal. The Plate was incubated at 37°C for at least 18 hours. White colonies were picked up at random and DNA was purified by small schale alkaline lysis methods. Sequencing was performed using a Taq Dye Primer Cycle Sequencing Core Kit and an Applied Biosystems model 737A DNA sequencing system (Perkin-Elmer). IgH V and CDR3 regions were identified by comparison with published sequences in the GenBank and EMBL databases using FASTA program and Kabat database.

Detection of IgH gene rearrangement and sequence analysis of CDR3 region

The study was performed on tissue samples obtained by surgical resection from 26 patients with a histopathologic diagnosis of primary B-cell lymphomas (13 low-grade MALT lymphomas; three from the stomach, two from the thyroid, eight from the lung; 6 high-grade MALT lymphomas, three from the stomach, three from the thyroid; 7 diffuse large lymphomas, three from the stomach, three from the thyroid, one from the lung). Clonal IgH gene rearrangements of paraffin embedded specimens by PCR showed a sharp band in 24 of 26 cases, whereas two cases from the stomach showed no clonally rearranged bands (Table 1).

The sequencing of the CDR3 regions revealed a single dominant clone in 24 cases and 2 cases showed two major sequences.

Table 1. Non-Hodgkin's Lymphoma Cases Studied

Cases	Age/Sex	Phenotype	IgH gene rearrangement		VacA expression
			Paraffin	Frozen	
Marginal zone B-cell, Low-grade MALT					
2	74/M	B	-	+	+
4	50/M	B	+		+
9	45/M	B	+		+
17	64/F	B	+		
19	62/F	B	+		
21	52/F	B	+		
22	56/F	B	+		
23	81/F	B	+		
24	23/M	B	+		
25	58/F	B	+		
26	53/F	B	+		
27	22/F	B	+		
28	48/F	B	+		
High-grade MALT					
3	72/F	B	+		-
6	?/F	B	+		-
7	44/M	B	-	+	-
11	51/M	B	+		
12	64/F	B	+		
18	?/F	B	+		
Diffuse large cell					
1	61/F	B	+		+
5	44/F	B	+		-
8	?/F	B	+		+
13	58/F	B	+		
14	47/F	B	+		
16	48/F	B	+		
29	59/M	B	+		

Case numbers 1-9 show gastric lymphoma, case numbers 11-19 thyroid lymphoma, and case numbers 21-29 pulmonary lymphoma.

The length of CDR3 region

The nucleotide sequence of CDR3 is developmentally regulated. The average length of the N-D-N region in the fetal liver lymphocytes, neonatal and adult peripheral B cells is 22 (range 12 to 45), 24 (range 9 to 56) and 31 (range 13 to 54) nucleotides, respectively, showing a tendency to increase with age.[1] In the low-grade MALT, high-grade MALT and diffuse large cell lymphomas, the mean length of the CDR3 region was 47.6±10.31 (range 21 to 60), 38.71±10.37 (range 27 to 57) and 40.86±3.34 (range 39 to 48) nucleotides, respectively. The length of the CDR3

region was significantly greater in the low-grade MALT lymphoma group than in the other two groups ($P<0.05$). The mean length of N-D-N was 30.2 ± 8.82 (range 7 to 40), 21.71 ± 7.99 (range 7 to 30) and 24.86 ± 4.10 (range 19 to 32) nucleotides, respectively. These findings indicate that the property of CDR3 in high-grade MALT lymphoma resembles that in diffuse large cell lymphoma rather than in low-grade MALT lymphoma. The maturation stage of low-grade MALT lymphoma is similar to ontogenetic mature B-cells, while high-grade MALT lymphoma and diffuse large cell lymphoma is at an ontogenetic early maturation stage.

Homology search of CDR3 region compared to published rearrangements

By homology search, the lymphoma cell clones of 14 cases (5 of 13 low-grade MALT lymphomas; 4 of 6 high-grade MALT lymphomas; 5 of 7 diffuse large cell lymphomas) showed 60 to 81% homology with autoantibody-associated lymphocyte clones. The incidence of these autoantibody-associated lymphocyte clones was higher in the high-grade MALT and diffuse large lymphomas than in low-grade MALT lymphoma. At protein level, 2 cases of low-grade MALT lymphomas showed 71 and 82% homology in CDR3 to those of autoreactive B cell clones, and 2 high-grade MALT lymphomas showed 67% homology. However in diffuse large cell lymphomas homology at protein level was 56 and 57%.

We also investigated the sequences of VH segment in 9 gastric lymphomas. Seven cases (2 from 3 low-grade MALT lymphomas; 2 from 3 high-grade MALT lymphomas; 3 from 3 diffuse large lymphomas) showed 85 to 98.3% homology with those of autoantibodies.

Thus, our findings suggest that the cells of MALT lymphoma strongly correlated with autoantigen and that some of diffuse large cell lymphomas may also derived from the selected autoreactive B-cell clones, even if they did not show histological evidence of MALT lymphoma.

References

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