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Direct DNA Sequence Analysis of Immunoglobulin Heavy Chain Gene Variable Region in Chinese Gastric Lymphomas

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Introduction

A close relation between gastric mucosa-associated lymphoid tissue (MALT) type lymphoma and infestation of *Helicobacter pylori* (HP) has been elucidated epidemiologically and clinically[1-5)]. It is also well-known that MALT type lymphoma is of post-germinal center (GC) B-cell. MALT type lymphoma cells have somatic mutation in immunoglobulin heavy chain (IgH) gene variable region[6-9)], although the coresponding antigen is thought to be of an autoantibody[7, 10)]. And on-going somatic hypermutation is observed in IgH gene variable region of MALT type lymphoma cells[11)]. However, pathogenesis of gastric MALT type lymphoma under HP infestation has not yet be clearly explained.

On the other hand, we analyzed Chinese gastric lymphomas. The clinicopathological features of these gastric lymphomas were followings. Developed cases, diffuse large B-cell lymphomas (DLBL), dominated in the examined cases. Some cases of DLBL included giant lymphoma cells. In a case of MALT type, cytomegalovirus inclusion was noted in the glandular epithelial cells in the lymphoma lesion. Then, we thought that giant cell formation may associate on-going somatic hypermutation in IgH gene variable region even in gastric DLBL cells[12)].

Therefore, this study investigated DNA sequence of IgH gene variable region of Chinese gastric lymphoma cells.

Material and method

Cases were 26 cases of stomach with malignant lymphoma, which were resected in the First University Hospital of China Medical University. The tissue of these gastric malignant lymphomas (gMLs) was routinely fixed and processed. Pathological subtypes of these gMLs were determined, based on histology in the H.E. stained specimen and on paraffin-immunohistochemistry employing CD3, CD5, CD79a, CD68, anti-S100 protein, anti-thymidine phosphorylase and anti-inducible nitric oxide synthase (iNOS) antibodies[13)]. And these gMLs comprised 10 cases of MALT type, 14 cases of DLBL and two cases of T-cell neoplasms (Table 1).

Paraffin sections for DNA extraction were prepared in Department of Pathology, China Medical University. The following extraction of DNA from the paraffin sections, polymerase chain reaction (PCR) analysis and direct DNA sequencing were performed without information of these gML cases in Kagoshima University.

Table 1, Cases and samples examined, and results of PCR for human β-globin (HBG) gene and immunoglobulin heavy chain (IgH) gene variable region (CDR3)

Case a)	ML Histology b)	HBG PCR c)	IgH CDR3 seminestic PCR d)
48 (s33)	Early MALT	a band	a band in smear
19 (s6)	MALT	a band	a band
35 (s20)	MALT	a band	a band in smear
38 (s23)	MALT	not-amplified	a band
50 (s35)	MALT	a band	a band
39 (s24)	MALT	a band	a band in smear
36 (s21)	Plasmacytoma	a band	a band in faint smear
24 (s9)	High MALT	a band	a band
29 (s14)	High MALT	a band	a band in smear
32 (s17)	High MALT	a band	two bands in smear
1 (s1)	DLBL	a band	a band in faint smear
2 (s2)	DLBL	not-amplified	a band in faint smear
21 (s7)	DLBL	a band	a band in faint smear
27 (s12)	DLBL	a band	a band in smear
30 (s15)	DLBL	a band	a band in smear
37 (s22)	DLBL	a band	a band
40 (s25)	DLBL	a band	a band in faint smear
41 (s26)	DLBL	not-amplified	a band
49 (s34)	DLBL	not-amplified	a band
25 (s10)	DLBL	a band	oligoclonal bands in smear
31 (s16)	DLBL	not-amplified	two bands
33 (s18)	DLBL	a band	two and concatamer bands
46 (s31)	DLBL	not-amplified	two bands
47 (s32)	DLBL	not-amplified	two bands
11 (s3)	T-ML	not-amplified	a band
14 (s4)	T-ML	not-amplified	a band

a) Number of cases (Sample number of sections, form which DNA was extracted.)
b) Early MALT: Early phase of MALT type lymphoma MALT: MALT type
Plasmacytoma: MALT type (plasmacytoma) High MALT: MALT type with areas of DLBL
DLBL: Diffuse large B-cell lymphoma T-ML: T-cell neoplasm
c) and d)
a (two, oligoclonal, concatamer) band(s): PCR product indicated a (two, oligoclonal, concatamer) band(s) of amplified DNA on the agar-gel electrophoresis.
a (two, oligoclonal) band(s) in (faint) smear: Amplified DNA indicated a (two, oligoclonal) band(s) in (faint) smear pattern on the agar-gel electrophoresis.
not-amplified: Amplified DNA was not recognized on the agar-gel electrophoresis.

PCR analysis of IgH gene CDR3 region

After deparaffinized by incubatng sections in xylen for five min three times, sections were rinsed by 100% ethanol for five min three times in a microtube. Sections were digested in the microtube by proteinase K at 37℃ overnight. Supernatant fluid in the microtube of the digested sections was removed to the next microtube and the DNA was extracted by means of enthanol sedimentation. The extracted DNA was dissolved in TE buffer. This extracted DNA solution was employed as the template DNA solution for the following PCRs[14].

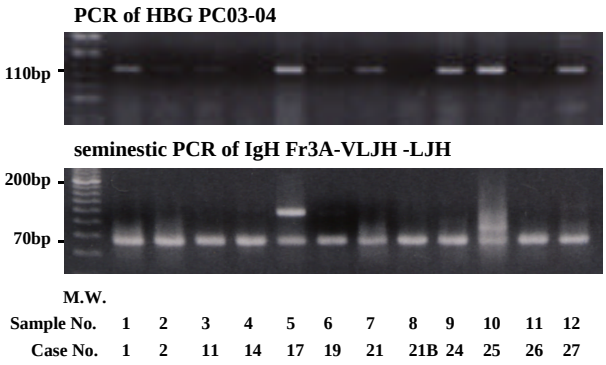
In order to see whether the extracted DNA had enough quality for PCR analysis, PCR for human β-globin (HBG) gene was performed, employing PC01-02 primers. The PCR comprised predenature at 94℃ for five min, 30 cycles of denature at 94℃ for one min, annealing at 55℃ for one min, and extension at 72℃ for one min, and final extension at 72℃ for five min[14].

IgH gene CDR3 region was amplified by means of seminestic PCR employing Fr3A and LJH primers for the first PCR and Fr3A and VLJH primers for the second PCR[15]. Each of the first and second PCRs comprised predenature at 94℃ for five min, 25 cycles of denature at 94℃ for one min, annealing at 58℃ for one min, and extension at 72℃ for one min, and final extension at 72℃ for five min[16].

Figure 1. Agar-gel electrophoresis of PCR products

Upper: PCR for HBG, PC01 and 02 primers
Lower: Seminestic PCR for IgH gene CDR3, Fr3A-LJH primers for the first PCR and Fr3A-VLJH primers for the second PCR

Number of samples and cases were listed in Table 1. Even in cases, of which the extracted DNA did not show a band of amplified DNA of HBG on the agar-gel electrophoresis, the seminestic PCR for IgH gene CDR3 region showed several patterns of amplified DNA on the agar-gel electrophoresis. In these cases, it was unknown whether the amplified DNA was from lymphoma cells or intermingling/residual B-cells.



The PCR products were examined by the agar-gel electrophoresis.

Direct DNA sequencing of IgH gene CDR3 region

A band of amplified DNA in the products of the seminestic PCR for IgH gene CDR3 region was removed from the agar gel to a microtube with a microfilter (SUPREC^{TM}-01, Takara Biochemicals), when a band of amplified DNA was recognized clearly on the agar-gel electrophoresis. The band of DNA was extracted in the microtube by means of centrifuge.

Cycle sequencing of the extracted DNA form a band of the agar-gel was performed. DNA sequence was analyzed by ABI PRESM 310/377 genetic analyzer[17].

Homology analysis of the analyzed DNA sequences of IgH gene CDR3 region was performed by means of the web DNA database[18].

Result

Products of the PCR for HBG gene indicated a band of amplified DNA at 110 bp in 17 cases but not in 9 cases (Table 1) on the agar-gel electrophoresis (Fig. 1). In more cases of DLBL than cases of MALT type there was no bands of amplified DNA on the agar-gel electrophoresis (Table 1).

Products of the seminestic PCR for IgH gene CDR3 region indicated several patterns of amplified DNA on the agar-gel electrophoresis (Fig. 1). Even in cases, of which the products of the PCR for HBG did not show a band of amplified DNA, amplification of DNA was noted (Table 1). Two or more bands of amplified DNA with or without smear was seen in 5 cases of DLBL and only in one cases of MALT type with areas of DLBL (Table 1).

From the agar-gel a band of amplified DNA was extracted in 17 cases comprising six cases of MALT type, nine cases of DLBL and two cases of T-cell neoplasm. In three out of 17 cases, DNA sequence was analyzed by means of cycle sequencing from Fr3A and in one case it was performed from VLJH.

The mean rate of unreadable nucleic acid base was significantly lower in the output of the analysis from VLJH (0.06) than form Fr3A (0.17) (student t-test, $p<0.001$). In each case the unreadable nucleic acid base rate was less from VLJH than from Fr3A (Table 2).

Homology analysis of the DNA sequences by means of program (BLASTIN) in the web DNA database informed that there were several hits with E value less than 0.001.

The hit DNA information was summarized in Table 2. In the DNA sequence from VLJH in 11 cases, of which the DNA extracted from paraffin sections was enough to show a band of amplified DNA in the products of the PCR for HBG, homology to human genomic DNA or cDNA was noted in five cases. In each one case of MALT type (35/s20) and DLBL (1/s1), homology to bacterial DNA was noted. And in two cases, homology to mouse or rat DNA was

Table 2. Results of the cycle DNA sequencing of the PCR products for IgH gene CDR3 region (Rate of unreadable nucleic acid base and homology of the DNA sequences)

Case a)	ML Histology b)	form Fr3A Rate of unreadable bases	Homology	from VLJH Rate of unreadable bases	Homology
19 (s6)	MALT	0.17	-	0.06	Homo sapiens cDNA etc.
35 (s20)	MALT	0.17	Homo sapiens chromosome 19	0.06	Chlamydophila pneumoniae
50 (s35)	MALT	-	-	0.13	Home sapiens genomic DNA
36 (s21)	Plasmacytoma	0.13	-	0.08	Human DNA sequence
24 (s9)	High MALT	0.14	-	0.11	Mus musculus genomic clone
1 (s1)	DLBL	0.19	-	0.07	Pasteurella multocida etc.
21 (s7)	DLBL	0.13	-	0.12	-
27 (s12)	DLBL	0.13	-	0.1	R.catesbeiana CCK gene etc.
30 (s15)	DLBL	0.17	-	0.08	Homo sapiens clone
37 (s22)	DLBL	0.12	-	-	-
40 (s25)	DLBL	0.13	-	0.08	Bos taurus, Human ,Drosophila etc.
#38 (s23)	MALT	0.13	Mouse 10kb plasmid	0.07	Pasteurella multocida etc.
#2 (s2)	DLBL	0.2	-	-	-
#41 (s26)	DLBL	0.13	-	-	-
#49 (s34)	DLBL	0.17	-	0.13	-
#11 (s3)	T-ML	0.16	-	0.06	-
#14 (s4)	T-ML	0.13	-	0.09	Human DNA sequence etc.

a) Number of cases (Sample number of sections, form which DNA was extracted.)
#: In the PCR for HBG amplification of DNA of the expected length was not seen on the agar-gel electrophoresis.
b) MALT: MALT type Plasmacytoma: MALT type (plasmacytoma) High MALT: MALT type with areas of DLBL
DLBL: Diffuse large B-cell lymphoma T-ML: T-cell neoplasm

seen. In two cases no homology was returned.

On the other hand, in six cases, of which the DNA extracted from paraffin sections was not enough to show a band of amplified DNA in the PCR for HBG, homology to human DNA in one case and homology to mouse and bacterial DNA in one case was noted.

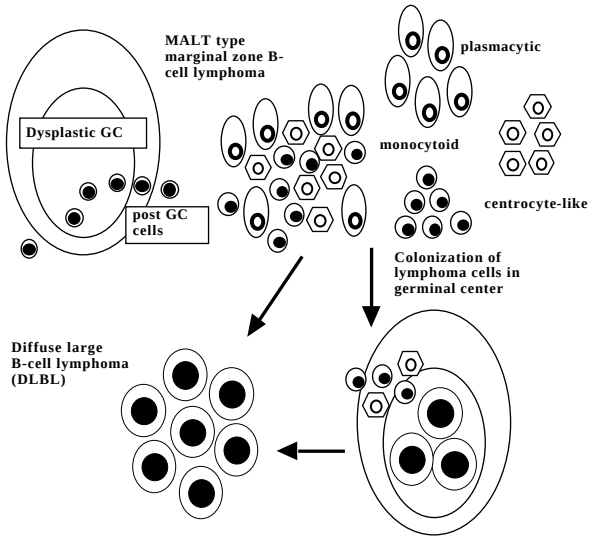
Discussion

Selection of one neoplastic clone ends in expansive clonal proliferation in the oncogenesis. But in the gMLs the agar-gel electorphoresis of IgH gene CDR3 region suggested that oligoclonality developed as lymphoma cells became larger in MALT type with areas of DLBL, and in DLBL (Table 1). A band in smear of amplified DNA also suggested various degrees of reconstruction of IgH gene CDR3 region even in obvious lymphoma. IgH gene is one of genes[19] that suffer from somatic mutation in GCs of the lymph follicle and that can be a gene with mutation in the GCs to yield neoplastic cells. But IgH gene variable region gets somatic hypermutation to recognize antigens. Developed oligoclonality in IgH gene variable region in the gMLs should be regarded from a view-point different from antigen recognition, although disordered antigen recognition may occur in the transformation from MALT type to DLBL.

It has been reported that lymphoma cells of MALT type have the IgH gene variable region against human, in the other words, are of auto-reactive B-cells[7, 10]. In this study, the homology analysis of DNA sequence of IgH gene CDR3 region indicated that the IgH gene variable region was oriented against human in a half of cases and was against the others in the other half of cases. And the homology to human genomic DNA or cDNA was seen more frequently in MALT type than in DLBL. The changes in IgH gene CDR3 region from MALT type to DLBL suggested that on-going somatic hypermutation in the transformation of MALT type lymphoma cells to those of DLBL[11] would lose specificity against antigen. Because the transformation of

Figure 2. Processes in the oncogenesis of gastric MALT type lymphoma

It is unknown where the premalignant or candidate cells of MALT type lymphoma cells come from. However, because MALT type lymphoma cells are of post-germinal center (GC) B-cells, the premalignant/candidate cells yield in the GC and indicate neoplastic proliferation in the marginal zone/areas of the lymphatic tissue. The MALT type lymphoma cells show various apperances including those of centrrocyte-like cells, plasma cells and monocytoid cells. The MALT type lymphoma cells colonize in the GC and transform to large cells. Then, diffuse large B-cell lymphoma (DLBL) appears. It is unknown whether MALT type lymphoma cells transform to those of DLBL in the extra-GC areas. At least, there are two intra-GC processes in the oncogenesis of MALT type lymphoma; one process is of the appearacne of the premalignant/candiate cells of MALT type lymphoma and the other is of the transformation of the MALT type lymphoma cells to those of DLBL.

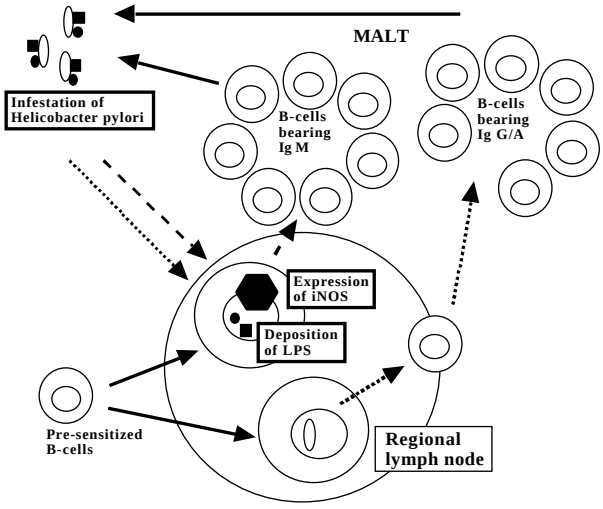


MALT type lymphoma cells occur in GC colonization[5], on-going somatic mutation ended to lose of the antigen-specificity would reflect disordered processes in the GC.

In our hypothesis of oncogenesis of MALT type lymphoma (Fig. 2), molecular events in the GCs are expected to concern with oncogenesis in the early phase (premalignant phase), because MALT type lymphoma cells are of post-GC B-cells. We thought that it might be the result of the disordered processes in the antigen recognition that MALT type lymphoma cells were of autoreactive B-cells[7, 10]. In our hypothesis, lipopolysaccharides of HP bodies[20-22] deposit in the GCs of the regional lymph node and induce iNOS in the GCs[13, 23]. Nitric oxide (NO) produced by the iNOS disturbs antigen recognition in the GCs and injuries DNA of B-cells, which are ready to be sensitized against an antigen, by disturbing receptor signaling[24-26]. On the other hand, iNOS is induced in the GCs with colonization of MALT type lymphoma cells[27]. The disordered antigen recognition and DNA injury may play a role in the transformation of MALT lymphoma to DLBL. Then, we thought that the disordered antigen recognition and DNA injury, which were induced by the NO, may be the HP-related essential events in the concogenesis of MALT type lymphoma and one of factors concerning with the transformation of MALT type

Figure 3. Hypothesis of the appearance of candiate MALT type lymphoma cells under the infestation of Helicobacter pylori

Lipopolysaccharides (LPSs) of Helicobacter pylori (HP) body deposit in the germinal center (GC) of the region lymph nodes and induce inducible nitric oxide synthase (iNOS) in the GC. The iNOS produces nitric oxide (NO), which is a mutagen and can disturb two of three kinds of cell membrane receptor signaling to nuclei and to the other intracytoplasmal molecular events. In the MALT of the stomach with HP-related peptic ulcer, IgM-positive B-cells dominate even in the long period infestation, suggesting no class switch of immunoglobulin heavy chain in the anti-HP immunity. The GC with high concentration of NO is thought to be the place where premalignant/candidate cells of MALT type lymphoma appear because of DNA injury by the NO. We found atypical nodular growth of IgM-positive B-cells in the gastric MALT of the HP-associated peptic ulcer and we looked it as the first expansive growth of premalignant/candiate cells of MALT type lymphoma.



lymphoma. Development of oligoclonality from MALT type to DLBL may be under the effects of the NO.

On the other hand, in cases, of which the extracted DNA did not show a band of amplified DNA in the PCR for HBG, it is unknown whether the amplified IgH gene CDR3 region is of lymphoma cells or not[28]. At least in one case of T-cell neoplasm the amplified IgH CDR3 region was considered to be of intermingling or residual B-cells.

At last, homology to *Chlamydophila pneumoniae* and *Pasteurella multocida* in IgH gene CDR3 region of MALT type lymphoma cells might suggest that co-infestation of these bacteria play a role in the oncogenesis of MALT type lymphoma in China.

Summary

This study aimed to see somatic hypermutation in immunoglobulin heavy chain (IgH) gene CDR3 region by means of polymerase chain reaction (PCR) and direct DNA sequencing in 26 cases of Chinese gastric lymphomas (10 cases of MALT type, 14 cases of DLBL and 2 cases of T-cell neoplasm). Template DNA was extracted from sections of the paraffin-embedded lymphoma tissue. A part of IgH CDR3 was amplified by means of seminestic PCR of Fr3A and LJH primers, and of Fr3A and VLJH primers. In the agar-gel electrophoresis of the PCR products, a band (9 cases), a band in smear (11 cases), and oligobands (6 cases) of amplified DNA were recognized. Oligoclonality in IgH CDR3 region developed, as lymphoma cells became larger from MALT type to DLBL. Even in one of 2 cases of T-cell neoplasm, the seminestic PCR amplified a band of DNA probably of intermingling or residual B-cells. In the 17 cases with a band or a band in faint smear on the agar-gel electrophoresis, DNA was extracted from the band in the agar gel. In direct DNA (cycle) sequencing by using VLJH primer the rate of unreadable nucleic acid bases was significantly lower than by using Fr3A primer (student t-test, $p<0.001$). Homology analysis of the read DNA sequences was performed at the web-site DNA databases. In eleven of 17 cases, homology to DNA sequences of bacteria (Chlamydophila pneumoniae and Pasteurella multocida), human and the other mammalian animals was recognized. In a half of cases, homology to human DNA or cDNA was noted. And in the other half cases, homology to the other mammalian animal DNA was seen. Homology to any antigens was more frequently recognized in MALT type lymphoma than in DLBL. The homology to bacteria suggested that these bacteria might play a role in oncogenesis of MALT type lymphoma in China. These findings about clonality and homology of IgH gene CDR3 region suggested that oncogenesis of MALT type associated disordered antigen recognition. We thought that the autoreactive features of MALT type lymphoma cells should be explained by the concerning abnormal processes in the disordered antigen recognition.

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