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Molecular pathological comparison of primary gastric B-cell lymphomas in China and Japan: Polymerase chain reaction (PCR) analysis of immunoglobulin heavy chain gene variable region and direct sequencing of the product

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Summary

In order to see whether there are any differences in primary gastric B-cell malignant lymphoma(s) (pGBML(s)) between China and Japan, Chinese 9 and Japanese 6 pGBMLs and 2 extranodal B-cell lymphomas were examined, were categorized according to the new WHO classification of lymphoid and hematopoietic cells malignancy, and were analyzed in immunoglobulin heavy chain (IgH) gene variable region by means of polymerase chain reaction (PCR). DNA employed for the PCR was obtained by means of one of the following three methods; a) micro-resection of a small number of lymphoma cells and proteinase K digestion, b) resection of dewaxed section and proteinase K digestion, and c) direct extraction by means of TAKARA DEXPAT™. Two times PCR of the primes, Fr3A and LJH, was performed. In Chinese pGBMLs, only one case of low-grade mucosa-associated lymphoid tissue (MALT) type showed infestation of Helicobacter (H.) pylori, and one case of diffuse large B-cell lymphoma revealed Hodgkin cell-like giant cells. A band of amplified DNA of the PCR was noted in one Chinese and three Japanese cases of pGBML and in one case of extranodal B-cell lymphoma. A broad smear of amplified DNA of the PCR was noted in two Japanese cases of low grade MALT type pGBML. One Japanese MALT type pGBML did not show amplified DNA in the PCR. DNA sequence of the amplified variable region of IgH gene could be analyzed in two Japanese cases of high grade pGBML and indicated high homology to substances that are not usual target antigens in vivo. Further cases of Chinese and Japanese pGBMLs must be analyzed to see the exact difference in the pGBMLs between China and Japan in points of infection of H. pylori, distribution of peroxynitrite and NO-related substances, distribution of MHC molecules on antigen-presenting cells especially in germinal centers with or without colonization of lymphoma cells, and infection of Epstein-Barr virus and human T-cell leukemia virus type-1, although this study suggested that there were more alteration in IgH gene variable region in Chinese cases than in Japanese cases.

Introduction

It is accepted that most cases of pGBML comprise MALT type lymphoma(s) (MALToma(s)) and its secondary transformed high-grade cases[1]. The postulated relationship among various neoplastic cells in MALTomas is presented in Fig. 1 according to Issacson[1].

It is also accepted that pathogenesis of MALToma has a close relation to infestation of H. pylori[2]. Free radical oxygen produced by H. pylori induces pooling of peroxynitrite (ONCO-) that can make mutation on genes. It is thought to be an essential mechanism in pathogenity of H. pylori[3]. Under the infestation of H. pylori in gastric

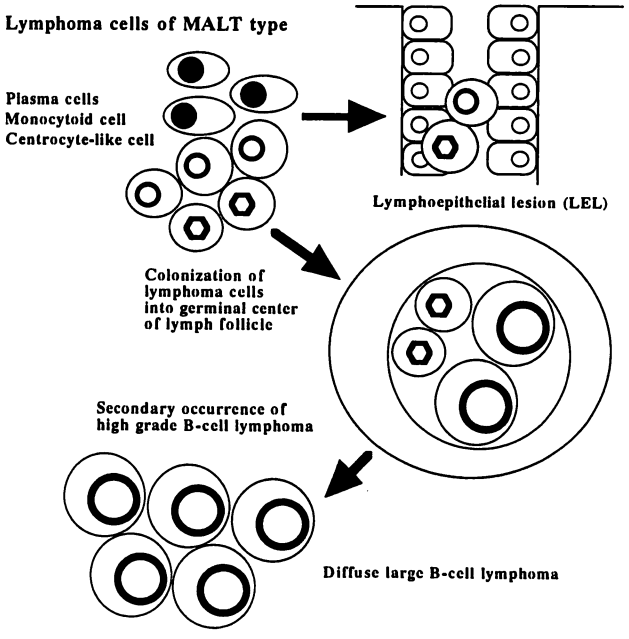


Figure 1. Neoplastic cells in mucosa-associated lymphoid tissue (MALT) type lymphoma

The lymphoma cells in low-grade MALT type lymphoma comprise centrocyte-like cells, monocytoid cells and plasma cells. The centrocyte-like cells would transform to monocytoid cells and large cells in the geminal center. On the other hand, lymphoma cells of MALT type lymphoma infiltrate into glands, forming lymphoepithelial lesion (LEL).

Table 1. Cases studied

Chinese primary gastric B-cell lymphomas	
1, 69321	Low-grade MALT type
2, 52953	Low-grade MALT type
3, 104176	Low grade MALT(Immunocytoma) type
4, 95470	Early phase of High grade MALT type
5, 108605 -1	High grade MALT type
6, 93461 -1	DLBCL
7, 47699-5	DLBCL
8, 54764	DLBCL
9, 99907	DLBCL (Anaplastic,HD-like)
Japanese primary gastric B-cell lymphomas	
1, II962778-1,.2	Low-grade MALT type (Early phase)
2, II951468-4	Low-grade MALT type
3, II896485-14	Low-grade MALT type
4, S95-1309-16	DLBCL
5, II950587-6	DLBCL
6, S910069-2	DLBCL
Japanese extranodal B-cell lymphomas	
7, P85-5063	Multiple myeloma (BM)
8, S902376	Immunocytoma (LN)

Mucosa-associated lymphoid tissue type: MALT type
Diffuse large B-cell lymphoma: DLBCL
Bone marrow: BM
Lymph node: LN

mucosa an abnormal immunological environment would be induced[4], in which MALToma might appear. It is suggested by studies[5,6] analyzing DNA sequence of immunoglobulin heavy chain gene variable region.

On the other hand, we experienced a human T-cell leukemia virus type 1 (HTLV-1) carrier with metachronous occurrence of gastric B-cell lymphoma and nodal T-cell lymphoma[7]. We thought that HTLV-1 infection might influence on the immunological microenvironment in the gastric mucosa, as in human immunodefficiency virus type 1 (HIV-1) carrier[8]. This possibility might be suggested by a fact that gastric lymphoma seemed to be more numerous in Kagoshima district than in the other areas in Japan, considering the number of gastric lymphomas reported previously[9], although an exact epidemiological study has not yet been performed.

This study aimed to be skillful in direct analysis of DNA sequence of immunoglobulin heavy chain (IgH) gene variable region that was amplified by means of polymerase chain reaction (PCR) in comparing a small number of pGBMLs in China and in Kagoshima with prevalence of HTLV-1 infection, and tried to see what HTLV-1 infection influences on in the occurrence of pGBMLs.

Material and method

Cases

Cases of malignant lymphoma studied were listed in table 1. Nine cases of Chinese pGBMLs were diagnosed in Department of Pathology, General Hospital of Armed Force, and in Department of Pathology, Liaoning Tumor Hospital, Sheng Yang in China. Six cases of Japanese pGBMLs and two extranodal B-cell lymphomas were diagnosed in Second Department of Pathology, Kagoshima University Faculty of Medicine, Kagoshima in Japan. Immunological phenotype of these lymphomas were examined by means of avidin-biotin complex (ABC) method with or without antigen retrieval, employing anti-CD3, CD4, CD8, CD5, MB-1, L26, LN-1, LN2, LN-3, S100 protein, muramidase, immunoglobulin kappa type and lambda type light chains antibodies. Based on the immunological phenotype, these lymphomas were categorized according to the new WHO classification of hematological malignancy[10].

Extraction of DNA from paraffin sections

DNA was extracted from paraffin sections of these lymphomas by means of one of the following methods.

The first method (micro-resection method): Paraffin sections dewaxed were stained by Hematoxylin. Under stereoscopic wide field microscope, a small number of lymphoma cells were removed from the section by cutting with glass probe that was prepared by heating and elongation of Pasteur pipette, into a microtube containing 10μl 200mg/ml proteinase K Tris buffer solution. After incubation of the microtube for 3 hours at 37°C, the microtube was heated for 10 min at 94°C in order to destroy activity of proteinase K. The solution was employed as a template DNA solution for the PCR.

The second method: After the removing a small number of lymphoma cells in the first method, the residual lymphoma tissue were removed by a razor into a microtube containing 100μl 200mg/ml proteinase K Tris buffer solu-

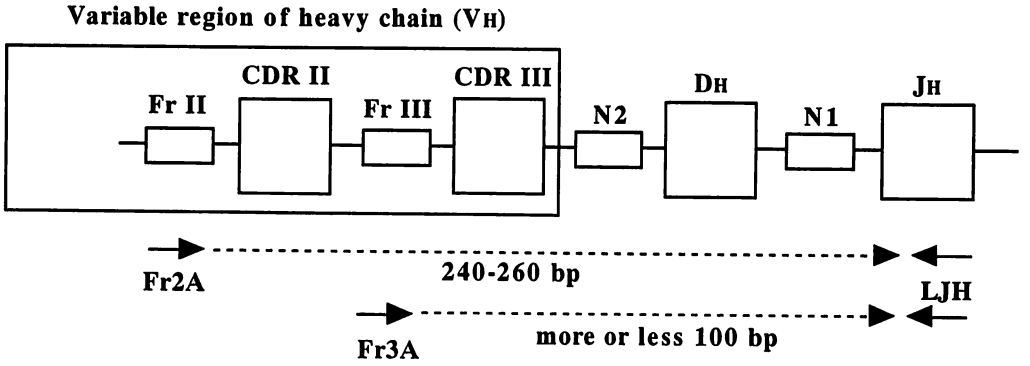


Figure 2. Primers employed in the polymerase chain reaction (PCR) for immunoglobulin heavy chain (IgH) gene
The variable region (Vh) of IgH comprises CDR I, CDR II and CDR III. The primers corresponding Fr II and Fr III are Fr2A and Fr3A. DNA amplified in the PCR of a pair of primers Fr2A and LJH is 240 to 260 bp long. That of the PCR of a pair of primers Fr3A and LJH is about 100bp long.

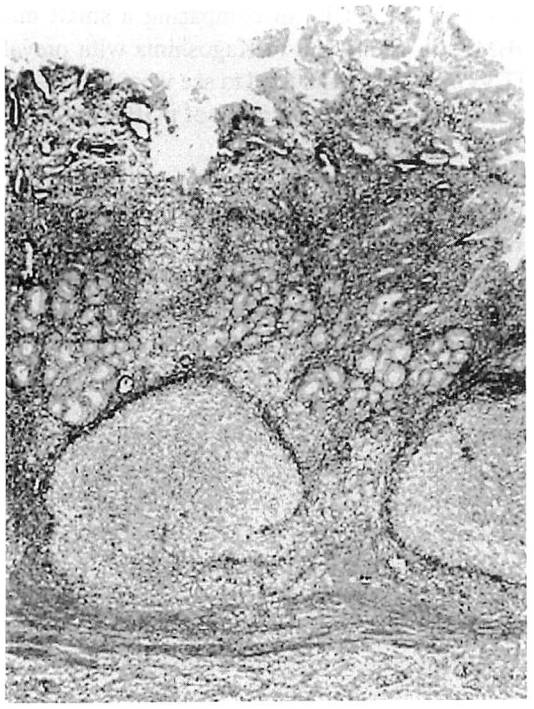
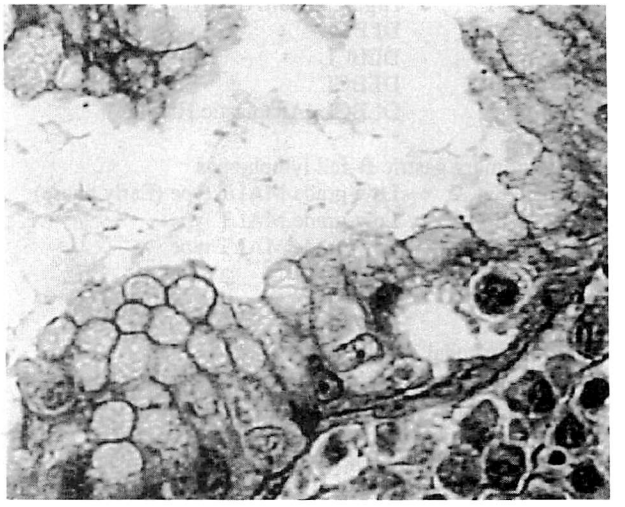


Figure 3. A case of Chinese primary gastric B-cell lymphoma (Case 1, MALT type lymphoma)
a) Lymphoma cells are seen around the enlarged lymph follicle and in the submucosal layer.
b) Infestation of a small number of H. pylori in a foveolar gland.

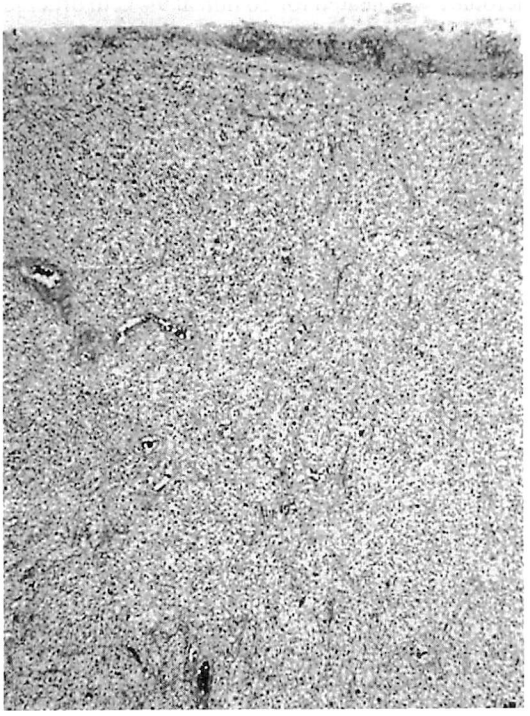


tion. After incubating the microtube at 37℃ overnight, activity of proteinase K was destroyed by heating for 3 to 5 min at 100℃. Five μl of the solution was employed as a template DNA solution for the PCR.

The third method: Several 3 μthick paraffin sections were cut into a 1.5 ml microtube. By means of the TAKARA DEXPAT™, about 400 μl DNA solution was sampled. Five μl of the solution was employed as a template DNA solution for the PCR.

It was examined by PCR for human β globin (HBG) gene whether each DNA solution that was gotten by means of one of the above-mentioned methods. A pair of PC03 and 04 primers can amplified 110bp DNA of HBG gene. A pair of GH20 and 21 primers can amplify 408bp

Figure 4. A case of Chinese primary gastric B-cell lymphoma (Case 9, diffuse large B-cell lymphoma)
a) Diffuse proliferation of lymphoma cells is noted in the ulcer base. b) Atypical giant cells resembling Hodgkin cells are seen. c) Lymphoma cells are various-sized cells. Intermingling small lymphocytes are seen.



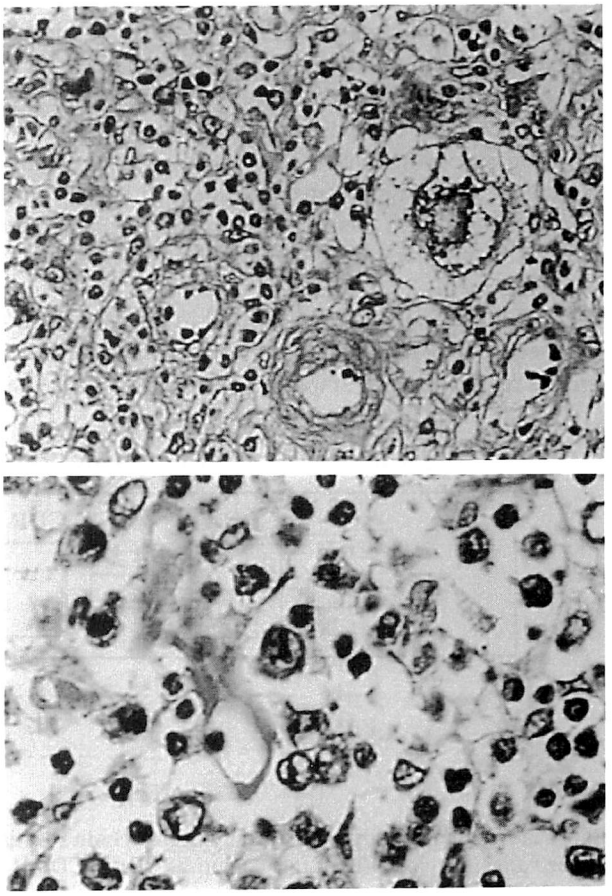


Figure 5. Nature of DNA extracted from paraffin sections of Chinese primary gastric B-cell lymphomas

The length of the DNA extracted from paraffin sections of cases 6 and 8 of diffuse large B-cell lymphoma was examined by means of the PCR for human β globin (HBG) gene. In the first PCR and in the second PCR employing 1,000 times diluted solution of the first PCR product, the PCR of a pair of primers GH20 and 21 yielded no amplification of 408bp long DNA. But the PCR of a pair of PC03 and 04 yielded amplification of 110bp long DNA.

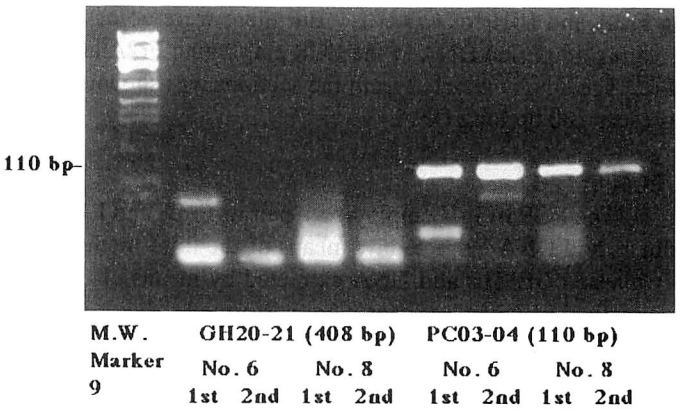


Figure 6. The PCR of immunoglobulin heavy chain)IgH0 gene variable region in Chinese primary gastric B-cell lymphomas

Only in a lane of case 5 the PCR of a pair of primers Fr3A and LJH showed a band of amplified DNA at 100bp length. The lane Cad is of a control case with gastric poorly differentiated adenocarcinoma.

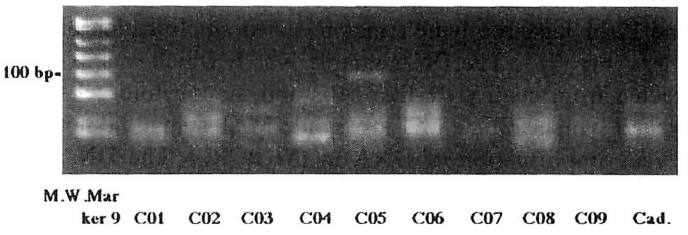
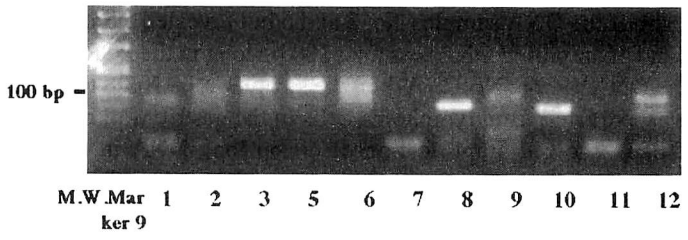


Figure 7. The PCR of immunoglobulin heavy chain (IgH) gene variable region in Japanese primary gastric and extranodal B-cell lymphomas

The number of lane corresponds to the DNA sample number. The relation between the case number and the DNA sample number should be refer the text.



or 204bp DNA of HBG gene. The PCR was two times PCR that amplified DNA in the second PCR employing 5 μl of 100 to 1,000 times diluted solution of the first PCR product.

PCR of immunoglobulin heavy chain (IgH) gene variable region

As shown in Fig. 2, PCR employing a combination of Fr2A and LJH amplifies 240 to 260bp long DNA including CDRII and CDRIII. In this PCR non-specific 100bp long DNA amplification can be seen sometimes[11]. By sequencing DNA arrangement in the amplified DNA, somatic mutation in IgH gene can be evaluated[12]. PCR employing Fr2A and LJH amplified about 100 bp long DNA that includes 30 to 60bp long rearranged CDRIII region[13]. The PCR employing pairs of primers; Fr2A and LJH, Fr3A and LJH, was two times PCR that amplified DNA in the second PCR employing 5μl of 100 to 1,000 times diluted solution of the first PCR product.

Analysis of DNA sequence in the PCR product

The PCR product was loaded on 4% agar gel electrophoresis. The band of amplified DNA on the gel was removed under ultraviolet light to TAKARA SIPREC™01 microtube with membrane filter and was extracted by means of centrifugation. By dye terminator method employing one of Fr3A and VLJH primers, bi-directional reading of DNA sequence was performed.

Result

1, Histopathology of Chinese and Japanese gastric B-cell malignant lymphomas

Chinese pGBMLs comprises three case of low grade MALTomas including one case of immunocytoma, two cases of high grade MALTomas with low-grade component or revealing lymphoepithelial lesions (LEL) and four cases of diffuse large B-cell lymphomas (DLBLs). In one case (Case 1, Fig. 3) of low grade MALToma, diffuse infiltrative proliferation of centrocyte-like cells and monocytoid cells in the submucosal layer, and colonization of these lymphoma cells in the mucosal lymph follicle germinal centers. In a few foveolar glands, there was H. pylori-like microorganism. One of DLBLs showed atypical giant cells resembling Hodgkin cells (Fig. 4).

Japanese cases of pGBML comprises three low grade MALTomas and three cases of DLBLs.

2. Nature of DNA extracted from paraffin sections

PCR of HBG genes, employing PC03-04 primes and DNA extracted by means of the second and third methods, showed 110 bp long DNA amplified in each case of Chinese pGBMLs (Fig. 5), whereas the PCR employing GH20-21 primers did not amplified DNA. The PCR of HBG genes in Japanese cases did the same result of the agar-gel electrophoresis as that of Chinese pGBMLs. At least, less than about 110bp long DNA was extracted from the paraffin sections of Chinese and Japanese pGBMLs. There were some cases of Chines and Japanese pGBMLs revealing amplified 100bp long DNA in the PCR employ-

ing Fr3A and LJH for CDRIII. But there were no cases revealing amplified DNA in the PCR employing Fr2A and LJH. The DNA extracted from the sections was shown to have less 260 bp long DNA.

3. PCR of IgH

The PCR of IgH employing a pair of Fr3A and LJH primers and DNA extracted by means of the third method in Chinese pGBMLs and DNA extracted by means of the first method in Japanese cases were performed.

There was only one case (high grade MALToma) of Chinese pGBMLs, which revealed amplification of about 100bp long DNA (Fig. 6).

On the other hand, DNA samples No. 3, 5, 8, 9, 10 and 12 of Japanese lymphomas revealed amplification of DNA (Fig. 7). The DNA sample No. 7, which was extracted from centrocyte-like cells and monocytoid cells around lymph follicle in a low grade MALToma, did not reveal amplification of DNA in the PCR. The DNA samples No. 2. 3 and 5, which were extracted from lymphoma cells in different areas of the mucosa in a low grade MALToma (case 2), showed amplified DNA in the PCR of the DNA samples No. 3 and 5 but revealed a broad band of amplified DNA less than 100 bp long in the PCR of the DNA sample No. 2. When the sample DNA No. 8 that was extracted from lymphoma cells in the gastric wall in a DLBL, showed amplification of 60 bp long DNA in the PCR, the sample No. 9 that was extracted from lymphoma cells in the regional lymph node of the same case of DLBL indicated amplification of 80 bp long DNA. The difference in the length of the amplified DNA indicated that different clones of lymphoma cells proliferated in the gastric wall and in the regional lymph node. The DNA sample No. 11 that was extracted from myeloma cells (Case 7) did not show amplification of DNA. The DNA sample No. 12, which was extracted from lymphoma cells of immunocytoma (case 8), revealed two bands of amplified DNA at 60 bp and 80 bp long.

4. DNA sequence of the amplified DNA in the PCR

The DNA band in the agar gel, on which the PCR products of Chinese case 5, Japanese case 2 (DNA samples No 3 and 5), case 5 (DNA samples No. 8 and 9) and case 6 (DNA sample No. 10) and case 8 (DNA sample No. 12) were loaded, was extracted. DNA sequence of the DNA was analyzed. But only in two DNA samples (DNA sample No. 8 of Japanese case 5 and DNA sample No. 10 of case 6) the DNA sequence could be read.

The DNA sequence of the DNA sample No. 10 of Japanese case 10 indicated 72.7% matching% with the DNA sequence at the 11300 position of human myotonic dystrophy kinase (DM kinase) gene (HUMDKIN) in the homology analysis (DYNASIS).

The DNA sequence of the DNA sample No. 8 of Japanese case 5 indicated 50.0% matching% with H. sapience (D138260) DNA segment containing (CA) repeat (HA177XF4) and 51.9% matching% with Human ornithin decarboxylase (ODC) gene, 5' flanking region (HUMORNDECA).

Discussion

This study was performed, employing DNA extracted from paraffin sections of lymphomas. It was examined which of the three methods of DNA extraction was the best for PCR analysis. The length of the DNA extracted was less than expected. In fixation of the tissue and in storage of the paraffin block DNA fragmentation would develope beyound expected. On the other hand, radical oxygen that H. pylori produced and peroxynitrite induced in the tissue with MALToma would effect on DNA even after embedded in paraffin block. Because of the shortness of the extracted DNA, the IgH gene variable region was examined by the PCR of Fr3A and LJH primers, which amplifies less than 100bp long DNA.

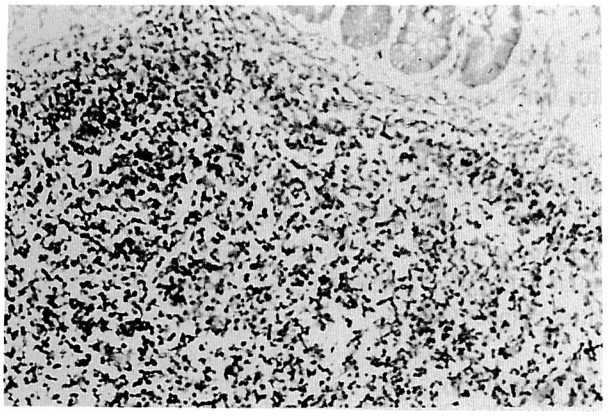
Ratio of cases, of which the PCR product showed a band of amplified DNA in the agar-gel electrophoresis, was different between in Chinese cases and in Japanese cases, although the number of cases examined was small. It might be explained why the ratio of cases was low in Chinese cases, as the concentration of free radicals differ between Chinese and Japanese cases. It suggested the other reasons inducing high concentration of free radicals in the gastric tissue with pGBML other than H. pylori that only one case of Chinese pGBMLs indicated infestation of H. pylori.

On the other hand, there were Japanese cases of low grade MALTomas, of which the extracted DNA showed no amplification of DNA or a broad band of the amplified DNA in the PCR. Even in a case the fashion of the DNA amplification in the PCR varied area to area. These result could be understood as many preneoplastic cells in such low-grade MALTomas. Some of such cases might be of H. pylori-associated gastritis[1] rather than low-grade MALToma. There is possibility that the DNA or rearrangement of variable region of IgH gene of low-grade MALTomas is unstable in the microenvironment rich in NO-related substances including peroxynitrite. Further examination of NO-related substances in the tissue with pGBMLs must be performed[14]. Such unstable rearrangement of IgH variable region should be understood to reflect ongoing somatic mutation, because IgH gene variable region of Hodgkin cells is known to be in ongoing somatic mutation and not to be evaluated by the usual molecular method such as PCR. One case of Chinese pGBML showed atypical giant cells resembling Hodgkin cells (Fig. 4). The reason why the number of Chinese cases that showed amplification of DNA in the PCR was quite small might be explained as the frequent ongoing somatic mutation in IgH gene variable region. On the other hand, as suggested in myeloma[11], neoplastic change with activation of immunoglobulin light chain gene and oncogenes must be examined in Chinese cases.

Previous studies reported that lymphoma cells of low grade MALTomas originate in autoantibody-associated lymphocytes, whereas those of high grade MALToma come from B-cells sensitized against foreign antigens[5,6]. But this study suggested that IgH gene variable region CDRIII of lymphoma cells in Japanese DLBLs oriented against DM kinase and DNA segment containing (CA) repeat that are endogenous. Then, expression of MHC on follicular dendritic cells in the germinal center with coloni-

Figure 8. A EBER-1-positive primary gastric B-cell lymphoma

A Chinese case of primary gastric B-cell lymphoma (diffuse large B-cell lymphoma) was positive for EBER-1 in-situ hybrization. This case was not an immunocompromised host.



zation of MALToma cells must be studied, because an endogenous antigen is expressed on antigen-presenting cells by means of MHC class 1 antigen.

Wotherspoon[8] reported that a small number of low grade MALTomas even in the immunocompromised hosts with HIV-1 infection showed a relation to H. pylori infestation, although most of pGBMLs in such immunocompromised hosts were Epstein-Barr virus (EBV)-related large B-cell lymphomas. We found EBV infection that was recognized by EBER-1 in-situ hybridization in one pGBML case without evidence of an immunocompromised host (Fig. 8). It is well known that there are many EBV-related nasopharyngeal carcinomas in China[15] so that fashion of EBV infection and sensitivity to EBV infection must be considered to effect on the occurrence of pGBML in China. There might be de nevo occurrence of gastric diffuse large B-cell lymphoma[16], whereas low grade MALToma transforms in the germinal center colonization to high grade MALToma. Metachronous occurrence of gastric T-cell rich B-cell lymphoma and nodal T-cell lymphoma in a HTLV-1 carrier suggested effects of HTLV-1 infection on the microenvironment of pGBML occurrence[7]. The effects of EBV and HTLV-1 infection might be seen in the progression of low grade to high grade MALToma and in the de nevo occurrence of high grade MALToma and gastric diffuse large B-cell lymphoma.

Therefore, a comparative study of Chinese and Japanese pGBMLs needs the analysis of H. pylori infestation, NO-related substances in the tissue with pGBML, expression of MHC in follicular dendritic cells and antigen-presenting cells in H. pylori-associated gastritis, somatic mutation and ongoing somatic mutation in IgH gene variable region of lymphoma cells, EBV infection in pGBMLs and HTLV-1 infection in the tissue with pGBMLs.

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