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Immunohistochemical Detection of P53, bcl-2 and Retinoblastoma Proteins in Primary Mediastinal Large B Cell Lymphoma

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ABSTRACT

***Background**: Primary mediastinal large B cell lymphoma (PMBL) is an aggressive non-Hodgkin's lymphoma (NHL) and has distinct clinico-pathologic as well as molecular characteristics. Mutation of P53 has been reported in PMBL in 13-16% of cases in the previous studies. The role of Retinoblastoma (Rb) gene, another tumor suppressor gene, in this type of lymphoma genesis has not been established yet. Previous studies showed the absence of bcl-2 gene expression in PMBL.*

***Materials and Methods**: To determine the pathogenic roles of these two tumor suppressor genes and bcl-2, an anti - apoptotic factor, we analyzed formalin fixed paraffin embedded blocks of ten cases of PMBL by Immunohistochemical staining (IHC).*

***Results**: Six cases were P53 positive, and 8 cases were strongly positive for Rb. There were also strong cytoplasmic positivity for bcl-2 antibody in 6 cases. Two P53 positive cases were negative for bcl-2, and nuclear staining for P53 and positive reaction for bcl-2 were detected concurrently in 4 cases.*

***Conclusion**: Our study showed high Rb protein expression (80%) in PMBL, and it implies cell cycle derangement. Rb protein does not seem to be involved in pathogenesis of PMBL.*

Extensive P53 expression by tumor cells correlates with clinico-pathologic features of PMBL. We observed bcl-2 over expression in 60% of the cases; thus, we suggest the role of this factor in pathogenesis of PMBL. The discrepancy between P53 mutation and bcl-2 over expression in previous studies and the study at hand can be explained by different methods of detection, technical factors, and various fixation process.

High frequency of P53 mutation and bcl-2 over expression in our study may be also explained by ethnic and geographic variations.

In summery, our findings indicate severe cell cycle derangement, high P53 mutation, and bcl-2 over expression in PMBL in our population. Our results need more precise molecular studies for confirmation, which may lead to improved clinical outcome for these patients. ***(Tanaffos 2002; 1(4): 27-35)***

***Key words**: Mediastinal lymphoma, P53, Retinoblastoma (Rb), bcl-2*

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INTRODUCTION

Primary mediastinal large B cell lymphoma (PMBL) is a distinct type of non-Hodgkin's lymphoma (NHL). These tumors have been completely separated from other diffuse large B cell lymphomas (DLBLs) in WHO and "Revised European- American classification of Lymphoid neoplasms" (REAL classification), because of their distinct clinico- pathologic and molecular features (1). This tumor is rare and approximately makes up 2.4% of non-Hodgkin's lymphomas (2). Based on present definitions, although it is mostly a focal disease in mediastinum, it involves extramediastinal organs in advanced stages, its size is usually more than 5cm(3).

These tumors were seen in women twice as many as in men, and mean age of the patients is 33-39. Clinical symptoms are commonly due to compression effects or local mass invasion into the anterior mediastinum in these patients. The most common symptom is Superior Vena Cava (SVC) syndrome (4).

Peripheral lymph nodes, pericardium, pleura, Lungs, and sternum are involved in local invasion of this lymphoma; in addition, its distant metastatic involvement in brain, liver, ovary, kidney, and adrenal gland is rarely reported.

Pathologically, it contains characteristics of malignant large lymphoid B cell with clear cytoplasm which are separated by collagen bands. Immunohistochemically, tumor cells reveal common surface B lymphocyte markers.

Reports about molecular features of this specific lymphoma showed differences with the other types of extra mediastinal diffuse large B cell lymphomas. They showed absence of bcl-2 expression versus presence bcl-6 expression in PMBL. These findings were entirely different from those which were in the other extra mediastinal lymphomas.

Evaluation showed that there were bcl-6 rearrangement in 4-6% of cases, C-myc alterations in 19-25% cases, and P53 mutation in 13-19% of cases in these tumors (1,5).

In this study, PMBL cases have been examined regarding to expression of bcl-2, Rb proteins, and P53 with Immunohistochemical method between the years 1999 through 2001.

MATERIALS AND METHODS

During period of 1999-2001, 10 cases of PMBL were detected. These cases were also clinically approved and the clinical data were obtained from hospital records. The slides were reviewed and confirmed for immunohistochemical and pathological results by three pathologists. Pathologically, in all cases, there was proliferation of malignant large lymphoid cells commonly with spreading sclerosis. These cells showed strong and diffuse positivity for CD45 and CD20.

In each case, paraffin sections of 4-6 microns from the samples were prepared on TES-smeared slides. Antigen retrieval process was performed on slides after xylol process, alcohol rehydration, and peroxidase activity blocking. Then, the sections were put in Tris buffer with pH=9, heated by autoclave at 120 ^{0}C and 1.1 Atmosphere for 10 min (about P53 and Rb) or by microwave at 100 ^{0}C for 10 min (about bcl-2).

Primary used antibodies were as follows: Do-7 anti P53 (DAKO, Dilution 1/50), Rb1 anti Rb (DAKO Dilution 1/50), and 124 anti bcl-2 (DAKO, dilution 1/50). After incubating of sections with the above antibodies for 45 minutes at room temperature, staining with LSAB2 Kit (DAKO) and using of DAB were followed. Positive and negative controls were applied in each series and showed favorable results. Staining was repeated in all 10 samples for two times in two different series, but in equal conditions, and the results were similar.

The slides were examined blindly for intensity of staining and positivity rate of tumoral cells by three pathologists.

Nuclear staining for P53 and Rb protein as well as cytoplasmic staining of tumoral cells for bcl-2 were reported as follows:

P53, Rb:

0-10%=Neg/ 10-30%=1+/, 30-60%=2+/, 60-100%=3+

bcl-2:

<25%=Neg/, 25-50%=1+/, 25-75%=2+/, 75-100%=3+

RESULTS

Table 1 showed that most of the patients were women, and female: male ratio was 2: 3 and mean age was 28.8. All of them had a large mass in superior, anterior mediastinum and the mean onset of the symptoms was about 3.7 months.

Table 1. The cases of primary malignant diffuse B cell lymphoma of mediastinum.

Case	Age	Sex	Duration of symptoms	Clinical findings	Radiological findings	Clinical Course
1	47	F	2 months	Cough, chest pain, dyspnea, SVCS weight loss, fever, chills	Anterior mediastinal mass	4 months follow up
2	24	F	4 months	Dyspnea, chest pain	Anterior, superior mediastinal mass	5 months follow up
3	34	F	2 months	Dyspenea, weight loss, SVCS, Cervical Lymphadenopathy,Hepatomegaly, bilateral pleural effusion	Large anterior, mediastinal mass with extension to posterior mediastinum and pressure effect on trachea and bronchi	NA
4	32	M	2 months	Productive cough, SVC, fever, chills	Anterior mediastinal mass, bilateral pleural effusion, elevation hemidiaphragm, abdominal and of inguinal lymphadenopathy	5 months follow up
5	18	M	10 months	SVCS ,chest pain, Hemoptysis abdominal pain , axillary lymphadenopathy Hepatomegaly	Mediastinal mass, bilateral pleural effusion, infiltrate of right lung	Death after 10 months
6	25	F	2 months	Chest pain, dyspnea, left upper extremity pain, cough, weight loss, dysphagia, hemoptysis ,night sweating, neck pain	Anterior mediastinal mass with extensive shift of mediastinal organs to the left	8 months follow up
7	41	M	2months	Dyspnea,mediastinal mass	Mediastinal mass, pericardial effusion	NA
8	28	F	6 months	Cough, dyspnea	Anterior mediastinal mass, bilateral pleural effusion	NA
9	19	F	2 weeks	Cough, dyspnea	Anterior mediastinal mass	17 months follow up
10	20	M	NA	SVC syndrome	Anterior mediastinal mass	NA

SVCS= Superior vena cava syndrome

NA = Not available

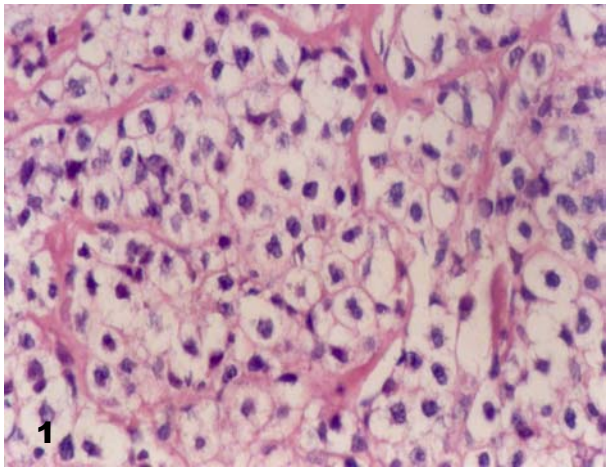
Immunohistochemical findings of the patients are listed in table 2.

Table 2. Immunohistochemical findings of cases with primary mediastinal B cell lymphoma.

Case	Staining characteristic		
	P53	Rb	Bcl-2
1	2+	3+	Neg
2	3+	3+	2+
3	Neg	3+	3+
4	2+	2+	Neg
5	3+	3+	3+
6	2+	3+	1+
7	Neg	2+	2+
8	Neg	Neg	Neg
9	2+	3+	3+
10	Neg	Neg	Neg

In 6 cases (60%), large tumor cells revealed strong nuclear staining for P53 and the positivity varied from 2+ to 3+. In 8 out of 10 cases (80%), tumor cells presented nuclear staining with Rb antibody and the remaining 2 cases were completely negative. Of the 10 cases, 6 showed strong cytoplasmic staining with bcl-2 antibody in tumor cells. In 8 cases, there was nuclear staining for P53 and Rb concurrently and two cases were negative for P53 but positive for Rb in nucleus of cells. Nuclear staining for P53 and positive reaction for bcl-2 were detected concurrently in 4 cases. In 2 cases, tumor cells were negative for both of them. Two cases were P53 positive and bcl-2 negative, but the other 2 cases were on the contrary. Because of severe sclerosis, some of the specimens revealed weak background in immunohistochemical staining, but it didn't prevent assessing of the tumor cells.

In a number of tumors, small lymphocytes became positive for bcl-2 or P53.



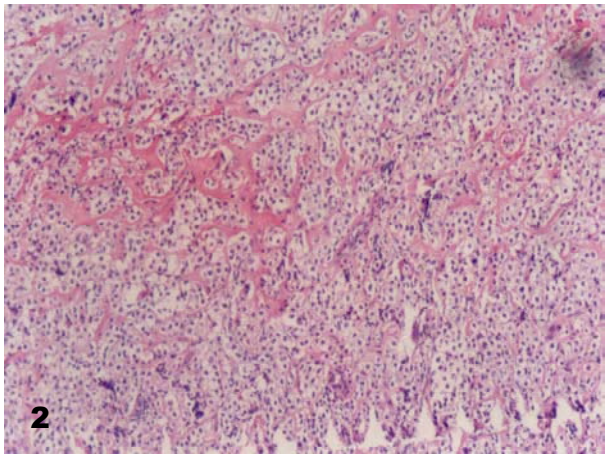


Fig 1,2. Characteristic pathologic features of PMBL: malignant large lymphoid B cell with clear cytoplasms (H&E Staining)

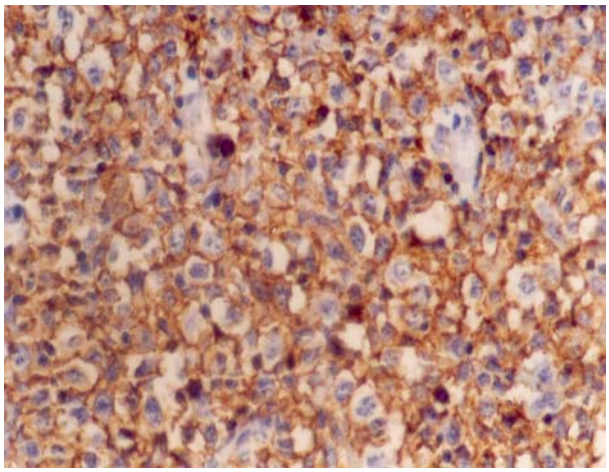


Fig 3. Diffuse CD20 positivity of tumor cell

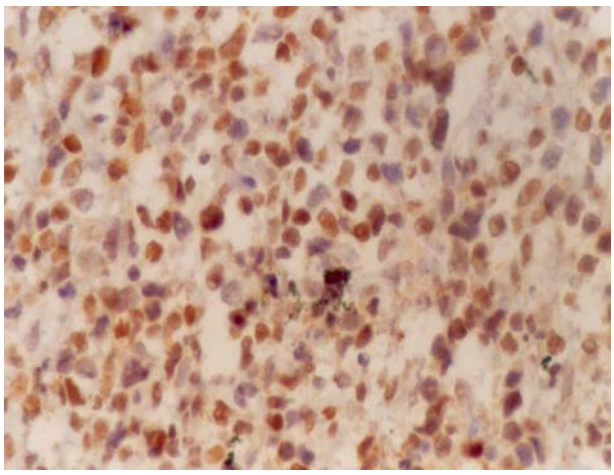


Fig 4. Strong nuclear positivity for P53 in malignant lymphocytes

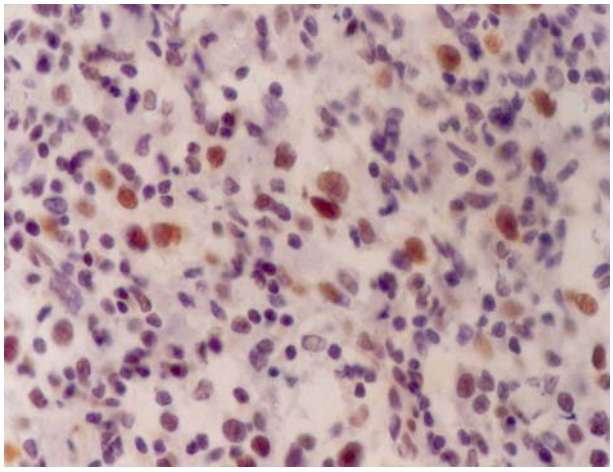


Fig 5. Malignant lymphocytes show presence of Rb protein

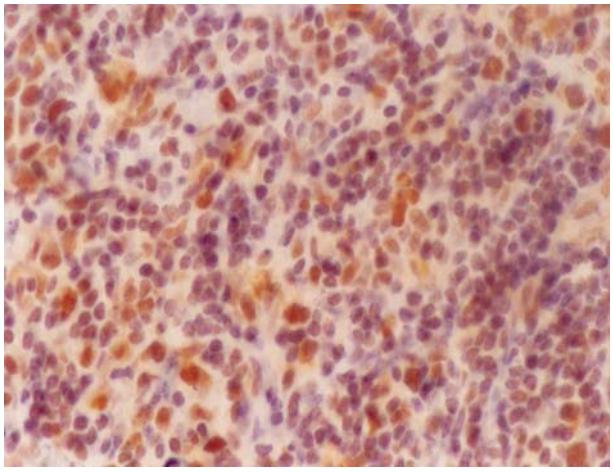


Fig 6. Cytopalmic positivity for bcl-2 in most of the malignant large lymphocytes as well as small background lymphocytes

DISCUSSION

Nowadays, it is clear that losing control of natural cell cycle is a key phenomenon to produce malignant changes. At least, one of the controlling cell cycle genes, like Rb protein, mutates at the most of human malignancies (6). Inactivation of this gene has been detected in pathogenesis of the majority of lymphomas especially high grade malignant types (7,8). We expect that absence and nonfunction of this gene, as a tumor suppressor gene, in nucleus of tumor cells will be equal with no reactivity in immunohistochemical staining. Nevertheless, researchers believe that presence of Rb protein in nucleus is a process due to cell cycle. Although its amount is low at G1 phase, it is very much at S phase.

It must be noticed that most Rb expression in a normal lymph node is in lymphoblasts.

Regarding to these results, the increasing of nuclear staining in immunuhistochemical technique, which was in 80% of cases, may indicate the cell cycle abnormality in tumor cells. On the other hand, we consider that the cause of Rb protein expression could be arising from P53 cell cycle derangement. Thus, the cells, which have P53 mutation, could present secondarily a large accumulation of Rb protein in nucleus (9). This theory can explain concurrent nuclear staining of Rb protein and P53 at the most cases of the present study. Therefore, the positivity of Rb protein in 80% of cases in present study indicates the extensive cell cycle derangement of tumor cells in PMBL at this center because of either P53 mutation or other pathologic molecular mechanisms.

P53 is a tumor suppressor gene which is located on short arm of chromosome 17. It is responsible to produce a nuclear phospholipid controlling cell entrance to S phase in cell cycle division and in normal condition; it also causes stopping growth and apoptosis in injured cells (6).

There was not a similar agreement between authors for degree of P53 mutation in different kinds of tumors, because methods of detection of P53 gene is different, which includes immunohistochemical staining (IHC), loss of heterozygosity, single strand conformational polymorphism analysis, and direct sequencing. Although the easiest way is IHC, it is recognized that P53 over expression might occur in absence of mutation (10).

The mutation of this gene has been observed at the most malignancies such as high grade lymphomas (11,12).

In pervious studies performed on P53 gene mutation in PMBL (9), evaluating method was commonly PCR- SSCP and sequencing analysis. Frequency of this gene mutation reported accurately 13-19% in western population by this method (1,5). The present study, showed the expression of P53 protein with strong nuclear staining in 60% of cases.

Based on the differences in methods, we can not compare, carefully and quantitatively, achieved numbers of this study with pervious studies about P53 gene mutation. Esrig et al., showed that nuclear P53 protein accumulation in IHC by using of 1801 and B P53-12 monoclonal antibodies in bladder cell carcinoma, if cut off point be 10%, has a direct relation and correspondence with obtained results in assessing of these tumors with SSCP methods (13).

It must be considered that the level and mutation rate of P53 may be different in various tumors for different causes; for example, in breast cancer, the percentages vary from 13% to 82% (13). These variations could be due to discrepancy in histological conditions (frozen or paraffin section), inappropriate fixation, incorrect processing methods, type of antibody, and kind of used materials (13).

Thus, we can say that significant differences in frequency percentage of P53 mutation in the present study with previous studies could be due to the difference in methods. Therefore, our study suggests extensive mutation of P53 gene in 6 of 10 PMBL cases at this center.

bcl-2 is one of the anti-apoptosis factors in cell which its high expression has been detected at the most of non-Hodgkin's lymphomas, especially follicular lymphomas (9). The best method for bcl-2 assessment is gene rearrangement on DNA-tumor cells. Performed studies on PMBL with immunohistochemical methods indicate absence of bcl-2 in these tumors.

Positivity threshold of this antigen was considered 25% in our study; thus, overestimation possibility is less established. Therefore, the present study may indicate the role of this gene in the pathogenesis of examined lymphomas. The studies performed in relation of P53 mutation with bcl-2 expression; for example, in breast carcinoma and also follicular lymphomas converting to diffuse large cell lymphomas (9), suggest reverse relationship of these two tumors.

In the present study, 6 cases were concurrently positive for P53 and bcl-2 in tumor cells. Coincidence of these two genes is not a unique finding in biology of lymphomas. Yin H et al. (7) showed that mutation of P53 was accompanied with bcl-2 over expression in a number of lymphomas. As it is mentioned, frequency rate of P53 mutation and bcl-2 expression showed nearly much differences with present study of in the previous reports in Western populations.

Technically all of the specimens had been fixed in formalin, maximum for 24 hours, and all of the examinations with each three antibodies repeated again in two different series and the results didn't show significant differences. According to positivity threshold both in P53 and bcl-2, the possibility of overestimation is refused. After refusing technical causes, the most common explanation for the above findings is environmental, geographical, genetic, and

ethnic variations which are in tumor lesions and lymphomas of various regions of the world.

It is has for a long time been recognized that the rate of various types of lymphoma in Asians is different from other populations, the newest of which has been recently in Malaysia (14).

Researchers believe that the frequency of subtypes of non-Hodgkin's lymphoma and its EBV association rate in various Malaysian populations was deferent from that of Western. It has also been observed that bcl-2 association in follicular lymphomas was less in Eastern than Western countries. Thus we can suggest that frequency rate and the type of molecular alterations of these lymphomas could be different in our country from that of Western populations.

In diffuse large B cell lymphoma (DLBL), over expression of P53 and bcl-2, accompanied with progressive clinical courses, possibly indicates poor prognosis and suppression of chemotherapy induced apoptosis (16). In other words, in our country, more extensive P53 gene mutation and bcl-2 expression in PMBL are more than Western cases. It means that these tumor cells have extensive DNA damage, P53 mutation, and increased proliferation as well as B lymphocyte survival (bcl-2 over expression). These findings could indicate rapid and short clinical course at progression clinical stages and inappropriate response to common therapeutic regimens among the patients living in our country. Other clinico-pathologic studies should be performed on much more numbers of samples with more precise molecular methods in the patients in order to confirm present study results.

Importance of these studies in any certain population reveals specific molecular features regarding to pathogenesis of disease in an area. Furthermore, regarding to appearance of new therapies in oncology, using of drugs which act directly against oncogenes, like what performed in breast cancer and HER2/ neu, more recognition of molecular factors can influence on chemotherapy regimens of these patients in future.

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