ORIGINAL RESEARCH ARTICLE

Tanaffos (2003) 2(5), 7-14

Expression of Apoptosis Genes Related Protein in Thymoma and Invasive Thymoma

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ABSTRACT

***Background**: The involvement of apoptotic genes in tumor genesis is well recognized. It was reported that P53 to be expressed more frequent in invasive thymoma than thymoma and expression of bcl-2 is positively correlate with aggressiveness of thymic epithelial neoplasm. We aimed to assess the expression of P53, bcl-2, and Rb genes related protein in thymoma and invasive thymoma.*

***Materials and Methods**: We analyzed formalin-fixed paraffin embedded tissue sections of thymic epithelial tumor during 1999 to 2001 for P53, bcl-2, and Rb protein by Immunohistochemistry.*

***Results**: Fifteen cases of thymic epithelial tumor including nine thymoma and six invasive thymoma were collected. P53 nuclear staining were found in epithelial cells of 14 cases (8 thymoma and 6 invasive thymoma). Rb protein was present in 10 cases (5 thymoma and 5 invasive thymoma). Cytoplasmic staining for bcl-2 was positive in six cases, of these, two were invasive.*

Conclusion**: Between Products of these three genes, P53 protein accumulation is more pronounced than other two, and there is inverse relationship between P53 and bcl-2 expression. The accumulation of P53, bcl-2, and Rb does not correlate with invasiveness of tumor. We may suggest that difference in expression of P53, in our study, may occur in the absence of mutation due to some ethnic or environmental factors which needs more investigation. We also suggest that organotypical differentiation present at the molecular level in Thymoma. **(Tanaffos 2003; 2(5): 7-14)

***Key words**: Apoptosis, P53, bcl-2, Rb gene, Thymoma, Invasive thymoma*

INTRODUCTION

It is now appreciated that genes which either inhibit or promote apoptosis (programmed cell death) are also important variables in tumorogenic equation.

In this regard, a large family of genes that regulate apoptosis has been identified. Among these, some act as anti-apoptosis gene like bcl-2, and some enhance apoptosis such as p53. Although Rb gene is not recognized as apoptotic gene, there is a report that

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Rb-negative mice which died in uterus have shown evidence of apoptosis in their nervous system. (1)

P53, bcl-2, and Rb genes are cell cycle regulatory agents that act through their protein expression. As a rule for neoplastic growth to occur, an imbalance between these proliferation regulatory genes must be established. (1)

Thymic epithelial neoplasm belongs to a single family of closely related lesions displaying a wide spectrum of differentiation. Today, in routine surgical pathology with regards to clinical outcome, thymic epithelial neoplasm is classified as thymoma, a slow growing tumor, and thymic carcinoma, a malignant epithelial tumor of thymus. Thymoma itself is also categorized according to the clinical findings or morphology to benign thymoma and aggressive (invasive) thymoma. The latter shows different behavior and sometimes acts very similar to malignant counterpart (1,3).

The investigators have suggested that assessing the gene expression determined the biological nature of neoplasm. There are some reports that show the expression of P53 protein was more frequent in invasive thymoma than thymoma (2,3,4). In the present study, we are going to show whether there is any clue, in addition to morphology, to differentiate the biological nature of thymoma from invasive thymoma. We are not dealing with thymic carcinoma. Assessment of P53, Rb, and bcl-2 gene proteins in thymoma and invasive thymoma is the aim of this study.

P53, bcl-2, and Rb status could be determined by either immunostaining, single strand conformational polymorphism analysis, or direct sequencing; however, immunostaining is the simplest and the most reliable way. Thus, we have evaluated the expression of P53, Rb, and bcl-2 genes related protein in thymoma, and aggressive thymoma by immunohistochemistry. To our knowledge, the combination of these three protein expression has not been reported yet.

MATERIALS AND METHODS

Case Selection

Representative sections of formalin-fixed paraffin-embedded tissue samples were obtained from fifteen patients with thymic epithelial neoplasm.

The samples were retrieved from histopathology laboratory of National Research Institute of Tuberculosis and Lung Disease (NRITLD) between 1999 and 2001. The specimens were either surgical biopsy or complete excision of neoplasm. All sections were hematoxylin-eosine stained at 3-4 micron thickness and reviewed by three pathologists to confirm the histological diagnosis, regarding the standard morphologic criteria for invasive and non-invasive thymic tumors.

The patients with thymoma include 8 males and 7 females whose age ranged from 16 to 76 years old (table 1).

Table 1. The age and gender of patients with thymoma

Case	Gender	Age (Years Old)
1	M	16
2	M	76
3	F	69
4	M	19
5	F	36
6	M	48
7	M	38
8	F	22
9	F	35
10	M	43
11	M	22
12	F	62
13	F	34
14	M	70
15	F	30

Immunohistochemical Method

Immunohistochemical staining was performed on formalin-fixed paraffin embedded 5 microns tissue sections (3-5 blocks for each case). Subsequently, they were deparaffinized by xylene and rehydrated through graded alcohol. Endogenous peroxidase was inhibited with hydrogen peroxide. For retrieval, tissue sections were placed in Tris buffer (pH=9) for P53, and citrate buffer (pH=6) for PRb, then heated with autoclave at 120°C for 10 minutes at 101 atmospheric pressure. For bcl-2, all slides were placed in Tris buffer (pH=9) and microwaved at 100°C for 10 minutes. Sections were incubated for 45 minutes at room temperature with anti P53 antibody (Do7, DAKO), dilution 1/50. For anti-Rb antibody clone Rb1 (DAKO), dilution 1/50 and for anti-bcl-2 clone 124 (DAKO) dilution 1/50 were used (Table 2).

Table 2.

Antibody	Clone	AR buffer	AR method
P53	Do7 (DAKO)	Tris pH9	Autoclave
Rb	Rb 1(DAKO)	Citrate pH6	Autoclave
bcl-2	124 (DAKO)	Tris pH9	Microwave

Finally, they were stained with LSAB Kit (DAKO) using DAB. After washing, staining with hemotoxylin was performed.

For double checking, the process of staining was performed in two separate running for all cases. Moreover, all specimens were placed in citrate buffer and then stained for P53 antibody, the way as above. For all cases, positive and negative controls were used.

All cases were reviewed by three pathologists, and the field with the highest percentage of tumor cells with positive staining was considered to be representative of IHC of tumor cells. An immunostaining positive scoring with the following criteria have been used.

0-1%= Negative

1-10%= +

11-50%= ++

> 50%= +++

RESULTS

Histological Findings

Of 15 cases of thymic neoplasm, 7 were thymoma of lymphocytic predominance, two were invasive, five were epithelial type of these, and one was regarded as invasive thymoma. Three cases were mixed lymphocytic and epithelial, all of them were invasive. Totally, six cases were considered to have invasive thymoma.

Immunohistochemical Findings

The staining reaction of P53, pRb, and bcl-2 are presented in table 3.

P53 Protein Expression: P53 nuclear staining was found in epithelial cell of 14 thymoma (including six invasive thymomas). The results of second checking run of process for P53 and results of the run with citrate buffer were similar to the first run staining.

The thymocytes (lymphocytes) of 3 cases also revealed positive nuclear staining. The thymocytes around epithelial cells showed more intense staining (Table 3).

Rb Protein Expression: pRb nuclear staining was present in the epithelial cells of 10 thymomas (of these, five were invasive). The lymphocytes also revealed positive staining in 11 thymomas (Table 3).

bcl-2 Expression: Cytoplasmic staining for bcl-2 was positive in epithelial cells in 6 cases (of these, 2 were invasive), but lymphocytes revealed positive staining in 12 cases (Table 3)

Table 3. The staining reaction of P53, PRb and bcl-2.

Case No	Type of Tumor	P53		PRb		Bcl -2	
		E	L	E	L	E	L
1	Lymphocytic Predominance	++	+	++	+++	-	++
2	Epithelial *	+++	+	+	++	-	+
3	Epithelial	++	-	-	-	+	++
4	Mixed *	++	-	+++	-	+/++	++
5	Lymphocytic Predominance *	++	+	+	+++	-	++
6	Epithelial	+	-	+++	+	-	+++
7	Lymphocytic Predominance *	++	-	+	+++	-	+++
8	Lymphocytic Predominance	+++	-	++	+++	-	+++
9	Lymphocytic Predominance	++	-	-	+++	+	++
10	Mixed *	+++	-	-	+++	-	++
11	Lymphocytic Predominance	++	-	+	+++	-	+++
12	Mixed *	+	-	+++	-	++	-
13	Lymphocytic Predominance	+++	-	-	+++	+	-
14	Epithelial	-	0	-	0	++	0
15	Epithelial	+++	-	++	+++	-	++

* Invasive tumor, 0: Tumor devoid of lymphocyte, E: Epithelial, L: Lymphocyte

\- = 1< % positive cell

\+ = 1 –10% positive cell

++ = 11 –50 % Positive cell

+++= > 50% Positive cell

DISCUSSION

Thymic neoplasms are divided into thymoma and thymic carcinoma. The thymic carcinoma is very similar to other carcinomas and usually shows cytologic and morphologic criteria of malignancy. Thymoma which is a benign-looking tumor also divided into non-invasive and invasive types. The latter occasionally shows pleural implantation and rarely metastasis, and it sometimes behaves as thymic carcinoma. Cytologically, no distinction exists between invasive and non-invasive thymoma. Virtually, all thymomas are made up of mixture of epithelial cells and a variable infiltration of non-neoplastic lymphocytes (thymocytes) (1,4). Although the relative proportion of the epithelial cells and lymphocyte components are supposed to be less significance (Pan CC et al 1994), tymomas are divided into lymphocytic predominance, epithelial and mixed according to cytologic pictures (1,4). In this context, we have studied the procedure of IHC staining methods for three most important proteins in thymoma and invasive thymoma.

P53: Prior investigators have reported that P53 protein expression was more frequent in thymic carcinoma and invasive thymoma than thymoma (2,4,5,6). Fukiwake N (1999) reported that two of 13 (15%) noninvasive thymomas were positive for P53 (2). In similar study, Oyama et al. found that 18% of thymomas were positive for P53 (4).

There are other reports indicating that positive P53 exists, but it is low for non-invasive thymoma (3,6). Our results show positivity of P53 is much higher, eight of nine non-invasive thymomas were positive for P53, and all six cases of invasive types were also positive for this protein. In other words, we had 14 P53 positive cases in our series (93% altogether). This finding is like the study of Chen et al. (5) in

which there was no correlation between P53 positivity and aggressiveness of tumor.

The level of P53 positivity varies greatly among different neoplasms; for example, P53 expression in ductal carcinoma of breast varies between 13% to 82% (7). The variation may be caused by condition of tissues(frozen vs. paraffin). Inequality in fixation or processing, the quality of the antibodies which are used and the criteria were employed for interpretation of the results (3,7,16). It was reported that some fixation methods weakened the antigenicity of P53 and antigen retrieval by microwave which could increase positivity of P53 (6,7). In the present study, a step of autoclave heating was performed prior to incubation with P53 antibody. Hino et al. (1997), by using polyclonal antibody (CM-1), have reported that the sensitivity of anti-P53 antibody may make the differences in P53 expression. They have found that only one of 17 thymomas was stained positive for P53 (6). In contrast, Tateyama et al. (1995), used anti-P53 antibody Do 7 and showed that 57% of thymomas revealed P53 expression (8). We have used clone Do7 antibody with higher percentage of positivity. Therefore, the differences in results may be partly due to the sensitivity of the anti-P53 protein antibody and partly due to technical diversity. According to Hino et al. study, anti-P53 antibody Do7 is more sensitive than CM-1 for the analysis of P53 (6). To rule out any technical problems in our study, all specimens stained repeatedly with anti P53 anti-body, but the results were similar.

M. Etebary et al. on evaluation of P53 expression on breast cancer of Iranian patients found that 40.3% of their cases were positive for P53 (15), which was higher than some of other related reports (14), so one possibility is that P53 expression may be due to ethnic or environmental factors. In this regard, it has been postulated that P53 over expression can occur in the absence of mutation (1,18).

Rb: pRb, the product of Rb gene, is a nuclear phosphoprotein that plays a key role in regulating the cell cycle. Mutation of Rb gene is expected to result in loss of protein expression (1,12,16). In this study, ten thymomas including five invasive thymoma revealed positive nuclear staining of epithelial cells. Anwar et al. examined different thyroid lesions and reported that non-neoplastic thyroid tissue and benign thyroid tumors including follicular adenoma showed positive staining for pRb (16). In contrast, follicular carcinomas were negative with Rb antibody. According to these investigators, mesenchymal and lymphoid cells stain positive which serve as internal quality control. Eleven thymomas in our study revealed positive staining for lymphoytes which used as internal positive control. Thus, the significance of pRb positivity for differentiation of invasive from non-invasive thymoma remains in question.

bcl-2: Cytoplasmic staining for bcl-2 was present in epithelial cells of 6 thymoma examined in this study, and 9 were negative for bcl-2. In contrast to our findings, all thymomas investigated by Engle et al. (1998) were positive for bcl-2. They concluded that bcl-2 was positively correlated with aggressiveness of thymic epithelial neoplasm (3). In our study only 2 out of 6 positive bcl-2 cases were invasive thymoma.

There are in vitro findings suggest that both mutant and wide type of P53 may have suppression effect on bcl-2 expression in epithelial cells. Stefenaki et al. (1997) reported that all eighteen P53 positive cases of their thymomas were negative for bcl-2 (10). Inverse relationship between P53 and bcl-2 was seen in 9 cases of our thymomas. This pattern of expression for these two proteins has been

demonstrated in adenoma of colon, non-Hodgkin's lymphoma, and breast cancer (3).

Our interesting finding is that lymphocytes in thymoma were stained by P53 in 3 cases, pRb in 11 cases, and bcl-2 in 12 cases, while no report of P53 and Rb staining of lymphocytes in thymoma exists, but staining of lymphocytes by bcl-2 has been reported by Stefenaki et al. (10). Gilhus NE et al. reported that bcl-2 was not detected in the neoplastic cells of thymomas but was present in the intermingling thymocytes which was also observed in most of our thymomas. They concluded that bcl-2 staining pattern in lymphocytes illustrated the broad spectrum of maturational stages in thymocytes (11).

During emberyogenesis, the interaction between lymphocytes (thymocytes) and thymic epithelial cells occur. At this stage, genes and surface receptor molecules of lymphocytes are activated by various inductive signals from epithelial cells. Thymoma is defined as thymic epithelial neoplasm displaying features with thymic organotypical differentiation (13,14,19). According to our above findings, we may suggest that this organotypical differentiation also presents at the molecular level in thymoma, and there is a molecular interaction between lymphocytes and epithelial cells of thymoma. This is supported by the fact that even in metastatic deposits of invasive thymoma, lymphocytes was also present which exhibit immature T-cell phenotype (17). In support of this theory, there is intense staining of lymphocytes adjacent to epithelial cells of three lymphocytic predominance thymoma for P53 antibody.

CONCLUSION

P53 is a tumor suppressor gene which induced apoptosis, and bcl-2 is a proto-oncogen inhibiting apoptosis. Rb gene is also a tumor suppressor gene which may be involved in apoptosis. Between protein products of these three genes, P53 protein accumulation is more pronounced than other proteins investigated (Rb, bcl-2). This accumulation does not correlate with invasiveness of thymoma. We suggest that the expression of P53 protein may differ in the same tumor due to some ethnic differences or environmental factors, which, of course, requires further investigations.

In most cases in our study, there was inverse relationship between P53 and bcl-2, but there was no correlation between invasiveness of tumor and positive expression of bcl-2. Also, for pRb, there was no correlation between invasiveness of thymoma and positive or negative expression of pRb.

Finally, we may suggest that there are interactions between epithelial cells and thymocytes of thymoma, and organotypical differentiation is also present at the molecular level of thymoma.

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