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Clinicopathological analysis of small-sized thymoma with podoplanin and Ki 67 expression analysis

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Abstract. Thymoma is the most common tumor of the anterior mediastinum for which surgical resection is currently the primary form of treatment. An increase in the incidence of a small-sized (≤3 cm) thymoma (SST) has recently been noted. Clinicopathological factors and prognosis of SST have not been reported previously. In this study, the clinicopathological data of 21 SST patients were reviewed and podoplanin and Ki67 immunohistochemistry were assessed to determine the biological behavior of SSTs. Pathological diagnosis of SSTs revealed the following types: A (n=1), AB (n=8), B1 (n=5), B2 (n=6) and B3 (n=1). The Masaoka-Koga stages of 21 thymoma patients were I (n=16), II (n=3), III (n=1) and IVb (n=1). In the case of the stage IVb thymoma, phrenic nerve, mediastinal pleura invasion and anterior mediastinal lymph node metastasis were observed. The Ki67 labeling index of this stage IVb was found to be 3.2. This case was also positive for podoplanin and was one of the only 2 cases that were positive for podoplanin. This patient succumbed to thymoma. Advanced stage thymomas are possibly included in SSTs although the majority of SSTs are classified into early stages of disease. Findings of this study suggest that podoplanin analyzed by immunohistochemistry may be useful to determine the malignant behavior of SSTs.

Introduction

Thymoma is the most common tumor of the anterior mediastinum. Surgical resection has been advocated as the principal treatment and completeness of resection has been considered to be the most important determinant of long-term survival in thymomas (1-3). Recently, an increase in the incidence of a small-sized thymoma (SST) has been noted. SST appears to usually be classified into early stages. However, SSTs are rare and clinical reports are not currently available.

To identify the individual biological behavior of thymomas, various factors including p53, bcl-2, matrix metalloproteinases, proliferating cell nuclear counts, Ki67 index and podoplanin have been analyzed (4-10). In a previous study, podoplanin, a 40-kDa mucin-type transmembrane sialoglycoprotein was correlated with tumor lymphangiogenesis, tumor invasion, lymph node metastasis of thymoma and poor clinical outcome of thymoma patients (11).

In the present study, we evaluated the clinicopathological data of SST patients and assessed the podoplanin and Ki67 immunohistochemistry of SSTs to determine the biological behavior of SSTs.

Patients and methods

Clinical data. A retrospective review was conducted of clinical and pathological data in all patients with thymomas undergoing surgery at the Nagoya City University Hospital, Japan, between January 1989 and December 2010. During this period of time, 21 tumors were diagnosed as SST within 30 mm as a maximal diameter (MD). The present study was approved by the Institutional Review Board (IRB) of Nagoya City University Hospital.

In the present study, the macroscopical measurements of resected specimens were utilized as the MD of a tumor, and the Masaoka-Koga staging system (12) was used for staging of thymoma. The Masaoka-Koga staging system, modified with the Masaoka staging system (1), has been recommended by the International Thymic Malignancy Interest Group (ITMIG) (13,14).

Histopathological analysis. To identify the intensity of malignant behavior the expression of podoplanin and Ki67 was evaluated.

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Immunohistochemistry. MIB-1, a mouse monoclonal anti-human Ki67 antibody (MIB-1, Dako, Glostrup, Denmark) was used. The Dako Envision system (Dako EnVision labeled polymer, peroxidase) was used according to the manufacturer's instructions. The Ki67 labeling index was assessed as the percentage of cells showing definite nuclear staining among 500 randomly selected tumor cells, with high-power (magnification x400) fields (7).

D2-40 monoclonal antibodies (Nichirei Bioscience, Tokyo, Japan) were used for the staining of podoplanin. The immunohistochemical staining was performed using a Benchmark LT automated immunostainer (Ventana Japan K.K., Kanagawa, Japan) by the avidin-biotin-peroxidase method with diaminobenzidine visualization and hematoxylin counterstaining, according to the manufacturer's instructions. The immunoreactivity of the tumor cells was assessed semiquantitatively as negative (no evidence of staining), weakly positive (1-10% of tumor cells were positive), or positive (>10% of the tumor cells were positive).

Statistical analysis. Survival analysis was performed by the Kaplan-Meier method. An outcome measure was utilized with overall and tumor-specific survival. Ki67 labeling indices were presented as the mean ± standard deviation and analyzed using the unpaired t-test using Scheffe's method according to the Masaoka-Koga stage or the WHO histological subtype. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical and pathological data. The study included 10 males and 11 females, with a mean age of 59 years, ranging between 35 and 77 years. Eleven of the 21 thymoma patients had myasthenia gravis (MG) symptoms. The mean of MD of the tumor was 2.4 cm (1.5-3.0). The distribution of MD was $1.5 < MD \leq 2.0$ cm in 7 tumors, $2.0 < MD \leq 2.5$ cm in 5 tumors and $2.5 < MD \leq 3.0$ cm in 9 tumors. Pathological diagnosis of thymoma revealed the following types: A (n=1), AB (n=8), B1 (n=5), B2 (n=6) and B3 (n=1). Surgical procedures including extended thymothymomectomy with sternotomy was performed in 15 patients. These 15 patients included all MG patients (n=11). Tumor resection (resection of thymoma with margin, but some thymic tissue may remain) with VATS (video-assisted thoracic surgery) technique was performed in 6 patients with stage I (n=5) or II (n=1) disease. Masaoka-Koga stages of 21 thymoma patients were stage I (n=16), II (n=3), III (n=1) and IVb (n=1) (Table I).

In the case of stage III thymoma, pericardial and left brachiocephalic vein invasions were observed. This patient underwent thymothymomectomy with sternotomy and partial resection of the pericardium and left brachiocephalic vein. In the case of stage IVb thymoma, phrenic nerve and mediastinal pleura invasion and anterior mediastinal lymph node metastasis were observed. Preoperative phrenic nerve paralysis was not observed. This patient underwent thymothymomectomy with sternotomy and partial resection of the phrenic nerve. All 21 patients, including the 2 patients at advanced stage (stage III and IVb), underwent surgery with complete resection both macroscopically and microscopically. Postoperative

Table I. Clinicopathological factors in 21 thymoma cases.

Factors	Thymoma cases (n=21)
Age	59±11 (35-77)
Gender	
Male	n=10
Female	n=11
MD (cm)	2.4±0.5 cm (1.5-3.0)
$1.5 < MD \leq 2.0$	n=7
$2.0 < MD \leq 2.5$	n=5
$2.5 < MD \leq 3.0$	n=9
Operation procedure 1	
Extended thymothymomectomy	n=15
Tumor resection only	n=6
Operation procedure 2	
Sternotomy	n=16
VATS	n=5
Masaoka stage	
I	n=16
II	n=3
III	n=1
IVb	n=1
WHO histology	
A	n=1
AB	n=8
B1	n=5
B2	n=6
B3	n=1
Ki67 labeling index	2.7±0.8% (1.4-3.8) (n=17)
WHO histology and Ki67 labeling index	
A + AB	2.1±0.7% (n=7)
B1	3.1±0.6% (n=5)
B2 + B3	3.3±0.4% (n=5)
Masaoka stage and Ki67 labeling index	
I	2.5±0.8% (n=12)
II	3.1±0.8% (n=3)
III	3.8 (n=1)
IVb	3.2 (n=1)
Podoplanin immunohistochemistry	
Positive	n=2
Focally positive	n=3
Negative	n=12
WHO histology	
Positive	B2 + B3 (n=2)
Focally positive	B1 (n=2), B2 + B3 (n=1)
Negative	A + AB (n=7), B1 (n=3) B2 + B3 (n=2)
Masaoka stage	
Positive	I (n=1), III + IVb (n=1)
Focally positive	I (n=3)
Negative	I (n=8), II (n=3) III + IVb (n=1)

MD, maximal diameter; VATS, video-assisted thoracic surgery.

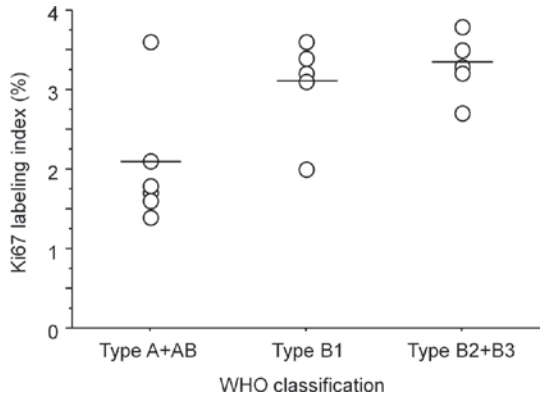

Figure 1. Comparison of Ki67 labeling index according to the WHO classification.

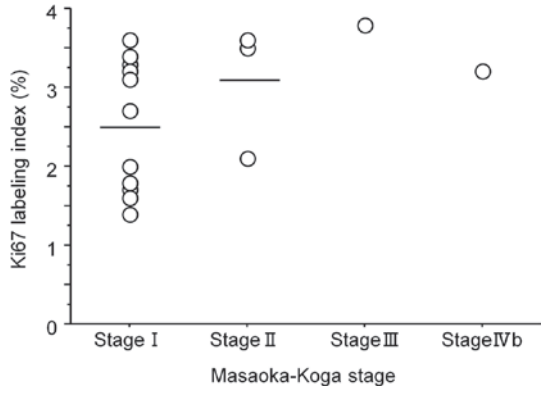

Figure 2. Comparison of Ki67 labeling index according to the Masaoka-Koga stage.

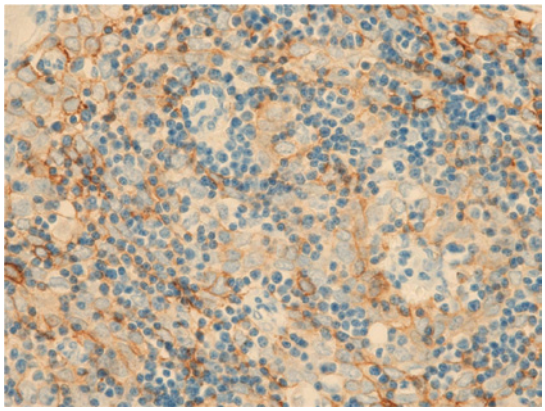

Figure 3. Immunohistochemical staining of D2-40 in an SST. Podoplanin was expressed on the cell membrane of the tumor cells.

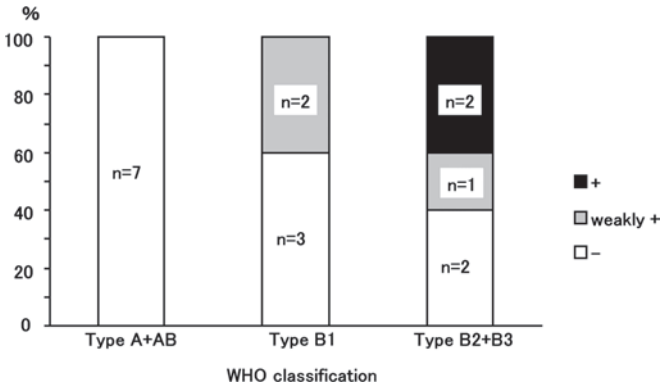

Figure 4. Immunohistochemical analysis for D2-40 in SSTs. Comparison of D2-40 immunohistochemistry according to the WHO classification.

adjuvant radiation was performed in 2 thymoma patients with stage III and IVb disease. No postoperative complication occurred with the exception of one case with phrenic nerve resection (Table I).

Immunohistochemical analysis. Paraffin blocks from 17 of the 21 thymoma patients were analyzed using immunohistochemistry. The mean Ki67 labeling index was 2.7±0.8% (1.4-3.8). The mean Ki67 labeling index of type A and AB tumors combined was 2.1±0.7%, that of type B1 was 3.1±0.6% and that of type B2 and B3 tumors combined was 3.3±0.4% (Fig. 1, Table I). No significant differences were found among the groups. The mean Ki67 labeling index of stage I was 2.5±0.8; of stage II, 3.1±0.8, of stage III, 3.8 and of stage IVb, 3.2% (Fig. 2, Table I). No significant differences were observed among the groups.

To assess the lymphangiogenesis and ability of lymphatic invasion, an immunohistchemical analysis was performed using a D2-40 monoclonal antibody that recognizes human podoplanin. Fig. 3 shows an example of positive podoplanin immunohistochemical staining with D2-40. This case was a WHO-type B2 thymoma. Two cases were positive for D2-40, 3 were weakly positive and 12 were negative. Regarding the WHO classification, the two D2-40-positive cases were type B2, and 2 cases of type B1 as well as 1 case of type B2 were weakly positive (Fig. 4). Regarding the Masaoka-Koga stage, 1 positive case was stage IVb disease and another was

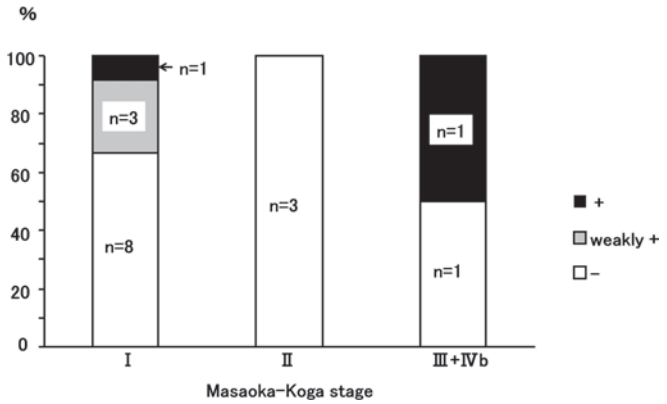

Figure 5. Immunohistochemical analysis for D2-40 in SSTs. Comparison of D2-40 immunohistochemistry according to the Masaoka-Koga stage.

stage I disease. The weakly positive cases were stage I disease (Fig. 5).

The median observation period was 42.1 months (2.1-274.4 months after the primary operation). Pleural disseminated lesions appeared and were diagnosed as a recurrence in only 1 case of stage IVb thymoma, 46 months after the primary operation. Resection of the recurrent lesions and chemotherapy were performed. This patient succumbed to

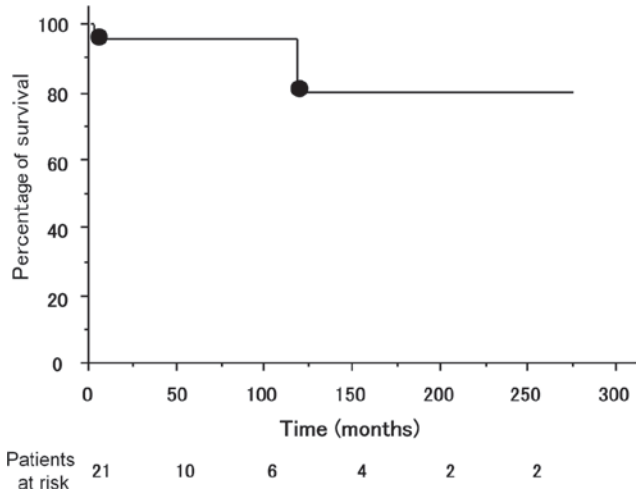

Figure 6. Overall survival of 21 SST patients. Patients at risk 50 months after the primary operation were 10; 100 months, 6; 150 months, 4; 200 months, 2; 250 months, 2; 300 months, 0 patients.

thymoma 118 months after the primary operation. One patient succumbed to crisis of MG 2 months after the operation. The overall survival was 95.2% at 5 years and 79.4% at 10 years after the operation (Fig. 6). Tumor-specific survival (only deaths from thymoma were considered as events) was 100% at 5 years and 83.3% at 10 years after the operation.

Discussion

Investigators in this study experienced a case of SST in which the patient succumbed to the disease. The MD of the tumor was only 2.0 cm but with pericardial invasion and anterior mediastinal lymph node metastasis. Recurrence appeared 46 months after the primary operation. This patient succumbed to thymoma 118 months after the primary operation. To analyze this case, we evaluated 21 SST cases in the present study. Pathological type of WHO classification of the case that succumbed was type B2, which is regarded as one of the most common types of SSTs. To analyze the malignant potential of SSTs, podoplanin and Ki67 immunohistochemistry were utilized. Results showed 2 tumors of positive signals of podoplanin in SSTs. The tumor of the deceased case was positive and the Ki67 labeling index was 3.2, higher than the average index.

The tumor size of thymoma has been identified as a prognostic factor (2). However, 2 cases with advanced-staged SSTs were observed in this study. The 2 tumors invaded the pericardium, identified using intraoperative findings. Intraoperative findings are crucial and this macroscopical diagnosis was included in the Masaoka stage classification. Usually SSTs are classified into early stages. As the prognosis of the patients with early-staged thymoma has been extremely good, limited thymectomy may be utilized. Onuki *et al* reported the efficacy of limited thymectomy for stage I or II thymomas (15). If the tumor invasion to the surrounding organs is apparent intraoperatively, we should convert the operative procedures from limited thymectomy to extended or total thymectomy.

Masaoka stage (1-3) and WHO histological classifications have been regarded as prognostic factors (16). However, the identification of more powerful prognostic factors would be beneficial for the treatment of thymoma. A number of factors, including p53, bcl-2, matrix metalloproteinases and proliferating cell nuclear counts have been assessed thus far. In the present study, we assessed podoplanin and Ki67 by immunohistochemistry to determine their role as prognostic indicators.

The Ki67 labeling indices of small-sized thymomas were less than 4% in all 21 cases in the present study. These values were relatively low compared to other malignancies (17,18). In thymic malignancies, Ghazi *et al* recently reported that Ki67 labeling indices changed 5% in a thymic typical carcinoid at the first surgery to 30% in the invasive recurrent lesions at the second surgery (19). Since Ki67 is a marker of cell proliferation, the result seems to be reasonable in thymomas with slow growth. Even in the low index of Ki67, it was of note that the indices showed an increase concomitant to the progress of staging and histological classification. These results suggest that the Ki67 labeling index may not be an optimal biological marker as a prognostic factor of SSTs.

In a previous study, we showed that podoplanin correlated with tumor lymphangiogenesis, tumor invasion, lymph node metastasis of thymoma and poor clinical outcome of thymoma patients (11). In the present study, a positive expression of podoplanin was demonstrated only in 2 of 17 SSTs. One thymoma was clinically diagnosed as stage IVb disease as stated above. The expression of podoplanin in the remaining 15 cases was negative or weakly positive. Podoplanin immunohistochemistry using a D2-40 antibody may be efficacious to predict lymphatic metastasis and poor clinical outcome. Although another thymoma patient with a positive expression of podoplanin is alive without recurrence, successive follow-up may be necessary.

While the results of this study are encouraging, it is acknowledged that any conclusions should be tempered with some reservations. The small number of patients limited the statistical analysis of the present study. A larger scale study may reveal the usefulness of podoplanin immunohistochemistry more clearly and may demonstrate statistical significance in the analysis of the Ki67 labeling index in SSTs.

In conclusion, we evaluate a deceased case of SST. Advanced-stage thymomas are possibly included in SSTs although the majority of SSTs are classified into early stage disease. In addition, podoplanin analyzed by immunohistochemistry may be useful in determining the malignant behavior of SSTs.

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