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# Detection of Thrombomodulin in Human Lung Cancer Cells

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***Thrombomodulin (TM), which usually exists in vascular endothelial cells and exerts an anticoagulant activity, was detected by Western blot analyses and immunocytochemical staining using three anti-TM monoclonal antibodies in cultured cell lines derived from a squamous cell carcinoma and an adenocarcinoma of the lung, but was not detected in a cell line derived from a small cell carcinoma. Functional assays indicated that TM detected in these cells was functionally active. The presence of TM in 22 specimens of surgically removed lung cancer tissue was also examined by an immunohistochemical method. TM was present along the cell membranes in 4 (36%) of 11 squamous cell carcinomas examined, but was not detected in 10 adenocarcinomas and 1 large cell carcinoma examined. Because TM is identical to fetomodulin, which modulates embryogenesis, the authors have concluded that TM is an oncodevelopmental antigen. The authors have also suggested that functionally active TM on lung cancer cells may modulate cancer cell behaviors in such ways as exhibiting anticoagulant activity. (Am J Pathol 1993, 142:79–85)***

Thrombomodulin (TM), which is a receptor for thrombin on the surface of the vascular endothelial cells, neutralizes thrombin-clotting activity by forming a complex with thrombin and the formed thrombin-TM complex activates protein C. Activated protein C in turn inactivates factor Va and VIIIa, retarding the process of the coagulation cascades. Thus, TM converts procoagulant thrombin to an anticoagulant and acts as an important modulator of intravascular coagulation.[1–3] Recently, the structure of TM has been determined,[4] and TM has been found distributed in vascular endothelial cells,[5] syncytiotrophoblasts,[5] platelets,[6] and nonvascular body cavity surfaces.[7]

In an independent study, Imada et al have found a type of cell surface protein called fetomodulin (FM),[8] and have reported that it is closely related to cell differentiation and that it is controlled by cyclic-adenosine monophosphate for its expression. Further, FM has recently been shown to be identical to TM, based on protein analysis, DNA sequencing, and functional assays.[9] During embryonic development, TM has been found to be present in nonvascular tissues such as atrium, lung bud epithelium, and neuroepithelium.[9] Because an amino-terminal region of FM/TM is homologous in sequence with the animal lectins[10] and the cell-to-cell adhesive interaction in embryogenesis is thought to be associated with a lectin-like activity,[11] FM/TM may be an additional member of cellular adhesive molecules.[9]

In addition, TM has been found present in some kinds of tumor cells, such as angiosarcoma cells[12] transformed from endothelial cells and choriocarcinoma cells[13] transformed from syncytiotrophoblasts.

Cancer cells frequently express embryonic antigens when undergoing a "retrodifferentiation" process during the course of malignant transformation. Human lung cancer cells have been found to express varying degrees of several kinds of oncodevelopmental antigens, such as carcinoembryonic antigen[14] and stage-specific embryonic antigen-1-related antigens,[15–17] which are found expressed in stage-specific lung buds of human embryos and may play some role in the cell-to-cell interactions.[18] Therefore, if TM is not only a thrombin receptor but also an oncodevelopmental antigen, then TM should be found expressed in lung cancer cells because TM is expressed in the lung bud epithelium.[9]

In this study, we first examined lung cancer cells in culture and in tissue for expression of TM and then discussed the characteristics of TM expressed in the lung cancer cells.

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## Materials and Methods

### Cultured Cells of Lung Cancer

Three types of lung cancer cell lines were selected: A549, derived from an adenocarcinoma; A431, derived from a squamous cell carcinoma; and SBC3, derived from a small cell carcinoma. These cell lines were provided by the Japanese Cancer Research Resource Bank.

### Monoclonal Antibodies

Monoclonal anti-human TM antibodies (KA2, KA3, and KA4) have been described elsewhere in detail.[19] Informatively, each epitope for KA2, KA3, and KA4 differs: the epitope for KA2 is most likely located in the *O*-glycosylation-site rich domain, that for KA3 somewhere other than the thrombin-binding region in the epidermal growth factor homology domain, and that for KA4 in the thrombin-binding region (the fifth EGF-like repeat[20]).

### Western Blot Analyses of Cultured Cells

Confluent cancer cells in 100-mm culture dishes were rinsed several times with phosphate-buffered saline, pH 7.4, then suspended in 1 ml of 10 mM TRIS-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100, and incubated for 15 minutes at 4 C. Clear lysates were then obtained by centrifugation for 5 minutes at 7,000 × *g*. These samples were subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli,[21] under nonreducing conditions.

After separation by SDS-PAGE, these samples were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, MA), as essentially described by Towbin et al.[22] The transferred membranes were then blocked by incubation in 50 mM TRIS-HCl buffer (pH 7.6) containing 5% low fat milk at 25 C for 1 hour, washed in the washing buffer (50 mM TRIS-HCl containing 0.5 M NaCl, 0.05% Tween 80, and 5 mM $CaCl_2$; pH 7.6) and separately incubated with each one of the monoclonal antibodies (1 μg/ml), which had been dissolved in the washing buffer, at 25 C for 3 hours. After washing, the polyvinylidene difluoride membranes were incubated with peroxidase-conjugated anti-mouse IgG (Bio-Rad, CA), 1 μg/ml, at 37 C for 1 hour, followed by an addition of 3–3′-diaminobenzidine hydrochloride (50 mg/dl) as the substrate.

### TM Cofactor Activity for Protein C Activation in Cultured Cells

TM activity on the surface of the cancer cells was determined by using confluent cancer cells (A549, A431, and SBC3) in 48-well tissue culture plates in the same way as previously described for cultured endothelial cells.[23] Cultured cells were washed three times with 20 mM TRIS-HCl (pH 7.4) buffer containing 150 mM NaCl, 2.5 mM $CaCl_2$, and 5 mg/ml bovine serum albumin. After the washings, the cells were incubated for 30 minutes at 37 C in 75 μl of the same buffer containing 3.3 U/ml human thrombin and 10 μg/ml human protein C. Activated protein C generated in 50 μl of the reaction mixture was determined by measuring the cleavage of the chromogenic substrate, S-2366, in the presence of 6 U/ml antithrombin III and 15 U/ml heparin.[24] A $V_{max}$ Kinetic Microplate Reader interfaced to the Softmax software (Molecular Devices, CA) was used to quantitate cleavage of S-2366 at 405 nm. We also measured TM activity on the surface of cultured endothelial cells[23] in the same way for the sake of comparison.

### Immunocytochemistry for Cultured Cells

A549, A431, and SBC3 cells were cultured on culture plates (LAB-TEK, Miles Scientific, IN), washed three times with phosphate-buffered saline, and fixed in alcohol using Rapid Spray (Mutoh Chemical, Japan).

Immunocytochemical staining for TM was performed by the ABC immunoperoxidase method[25] using ABC kit (Vector Laboratories, CA) and the monoclonal antibodies. Because the binding of KA4 to TM depends on $Ca^{2+}$ ion,[19] 50 mM TRIS-HCl (pH 7.6) buffer containing 5 mM $CaCl_2$ was used for the dilution of antibodies and for the washing of the specimens. Peroxidase activity was visualized by incubating the specimens in the same buffer containing 3–3′-diaminobenzidine hydrochloride (20 mg/dl) and 0.005% hydrogen peroxidase, and counterstaining the specimens with methyl green.

The specificity of the immunostainings was confirmed by a negative control, using nonimmune mouse IgG in place of the primary antibody.

### Tissues and Immunohistochemistry

Twenty-two lung cancer tissues were taken from surgically removed specimens. The clinical profiles of these cancer cases are shown in Table 1.

Five-μm-thick, fresh frozen sections (fixed with acetone) and paraffin-embedded sections (fixed

Table 1 *Patient Characteristics*

| Histologic type | No. of patients | Sex (m/f) | Age (yr) (mean ± SD) | Stage* (recurrence†) I | II | III |
|---|---|---|---|---|---|---|
| Squamous cell carcinoma | 11 | 10/1 | 65.8 ± 8.6 | 4 (0) | 0 (0) | 7 (5) |
| Adenocarcinoma | 10 | 8/2 | 62.8 ± 6.4 | 4 (0) | 3 (2) | 3 (3) |
| Large cell carcinoma | 1 | 0/1 | 64 | 0 (0) | 0 (0) | 1 (1) |

* According to the classification of the international staging for lung cancer.
† After operation recurrence within 1 year.

with each one of the following solutions: methanol-Carnoy, Carnoy, Bouin, Zamboni, PLP, and formalin) were prepared from these tissue specimens. Immunohistochemical staining of these sections was performed in the same way as for the immunocytochemical staining previously mentioned.

## Results

### Western Blot Analyses

All three monoclonal antibodies against human TM reacted in Western blot analyses with A549 cells and with A431 cells, but not with SBC3 cells (Figure 1). The estimated molecular size of TM detected in A549 cells was 76 kd, which is similar to the size of a major, native TM form,[26] whereas TM detected in A431 cells exhibited several bands that distributed over a region from 71 to 79 kd approximately. This may have been caused by the presence of TM in a variety of forms with a different quality and degree of glycosylation.[27] Further, a faint band of 52 kd was observed with the antibody KA2 on samples from all three cell lines, including SBC3 cells (Figure 1A). Because the faint band was not detected with the other antibodies and SBC3 cells did not exhibit a major component that reacts with the antibodies, this

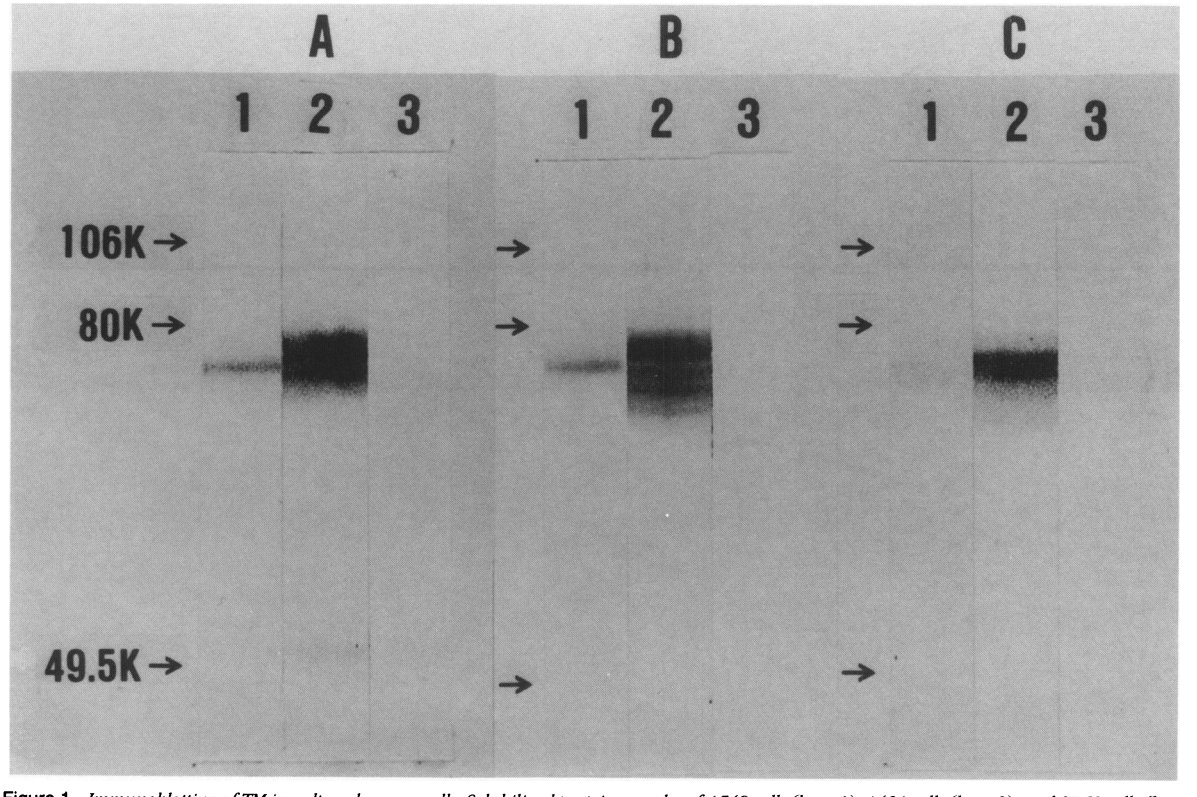


Figure 1. *Immunoblotting of TM in cultured cancer cells. Solubilized protein samples of A549 cells (lane 1), A431 cells (lane 2), and SBC3 cells (lane 3) were analyzed on a 10% SDS-polyacrylamide gel using monoclonal antibodies KA2* (A), *KA3* (B), *and KA4* (C). *The molecular size markers used were rabbit muscle phosphorylase β (106K), bovine serum albumin (80K), ovalbumin (49.5K), and carbonic anhydrase (32.5K). Positions of the markers are indicated by arrows.*

faint band may represent a different antigen containing an epitope structurally common to the *O*-glycosylation-site rich domain recognized by KA2.

## *Immunocytochemistry*

All three monoclonal antibodies also reacted with A549 cells and A431 cells on immunostaining, but not with SBC3 cells (Figure 2, A-E). The staining pattern indicating TM positivity in A549 cells differed from that of A431 cells. The cytoplasm of A549 cells was stained by anti-TM antibodies, whereas the cell membrane surfaces stained in A431 cells. The staining of A549 cells is quite variable, and some cells stained strongly and some cells stained very weakly.

## *Functional Assays*

TM antigens detected on the cultured cells by the antibodies were tested for their activities for protein C activation. The mean activities were determined ($n$ = 4) as follows: 16.0 ± 0.28 mOD/minute/well in A549, 10.4 ± 0.68 mOD/minute/well in A431, 0.48 ± 0.047 mOD/minute/well in SBC3, 22.6 ± 0.92 mOD/minute/well in endothelial cells, and 0.82 ± 0.072 mOD/minute/well in negative controls (no cells). These results indicate that TM antigens detected on A549 and A431 cells are active in protein C

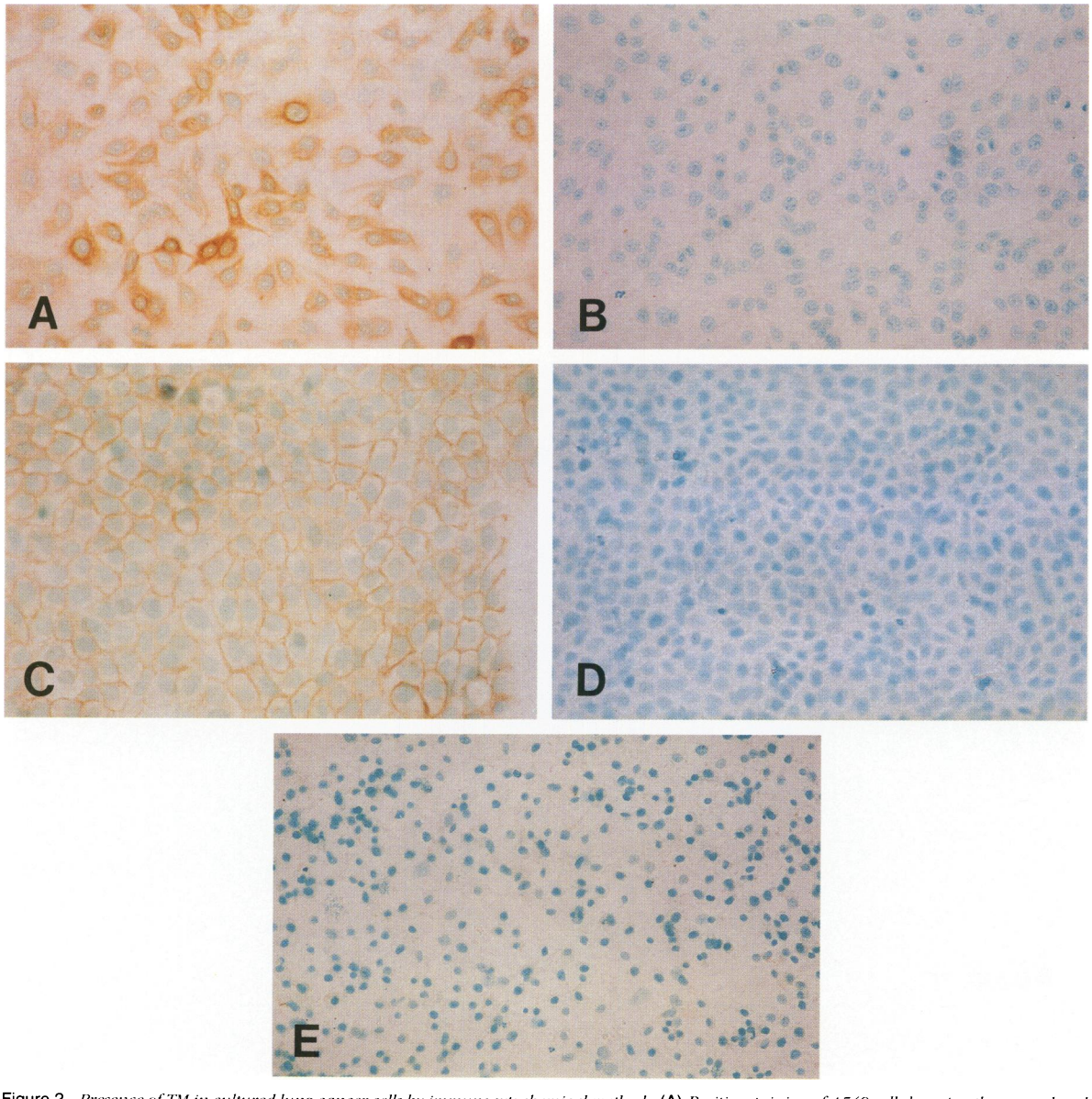


Figure 2. *Presence of TM in cultured lung cancer cells by immunocytochemical methods.* (A) *Positive staining of A549 cells by using the monoclonal antibody KA3. The staining pattern of TM is cytoplasmic and the staining intensity is heterogeneous.* (B) *Negative control of A549 cells.* (C) *Positive staining of A431 cells by KA3. The staining pattern of TM is discretely punctate along cell membranes and the staining intensity is homogeneous.* (D) *Negative control of A431 cells.* (E) *Negative staining of SBC3 cells by KA3 (original magnification, ×100).*

activation and that SBC3 cells did not contain active component for protein C activation. The results also indicate that TM activity detected on the cancer cells is comparable to that on the endothelial cells.

## *Immunohistochemistry*

Lung cancer tissues from 22 patients were examined. In 4 of 11 patients (36%) with a squamous cell carcinoma, all antibodies showed positive staining on the surface of frozen sections of the lung cancer cells. Also, in 1 of 4 positive cases, TM-positive cells were seen on the paraffin-embedded sections (Figure 3, A, B). The cell membrane surfaces of these squamous cell carcinoma cells both *in vivo* and *in vitro* were stained by anti-TM antibodies.

In contrast to squamous cell carcinoma specimens, adenocarcinoma specimens from 10 patients and a large cell carcinoma specimen from a single ts patient were not found to be TM-positive.

On each specimen, the TM positivity among the squamous cell carcinoma was apparent in the lesser differentiated areas close to the stroma, not the mature, central keratinized areas. However, as a whole, the TM positivity did not seem to correlate with the grade of differentiation, the pathological stages, and after operation recurrences of the tumors (Table 2).

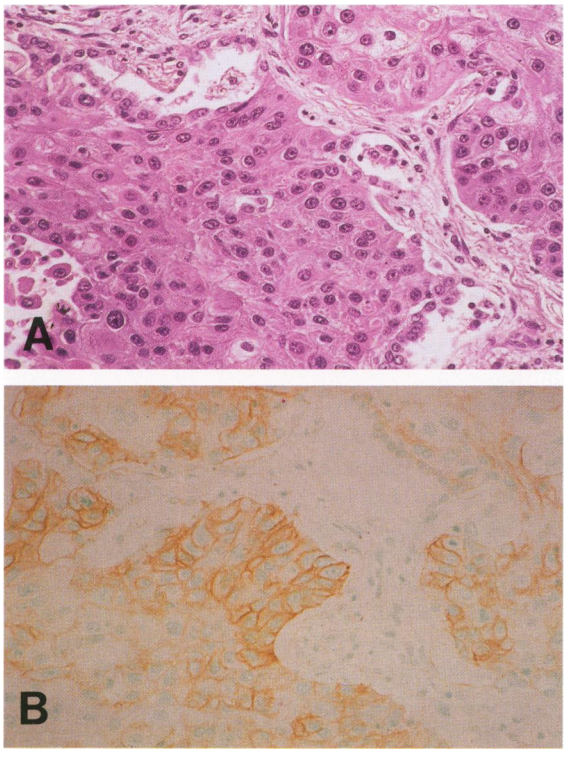


**Figure 3.** *Presence of TM in tissue specimens of lung cancers by immunohistochemical methods.* **(A)** *H & E-stained sections.* **(B)** *Positive staining of squamous cell carcinoma cells by using KA3, applied on a paraffin-embedded section fixed with methanol-Carnoy solution. Staining pattern of TM is discretely punctate along cell membranes, and the positivity was apparent only in the lesser differentiated areas close to the stroma (original magnification, ×100).*

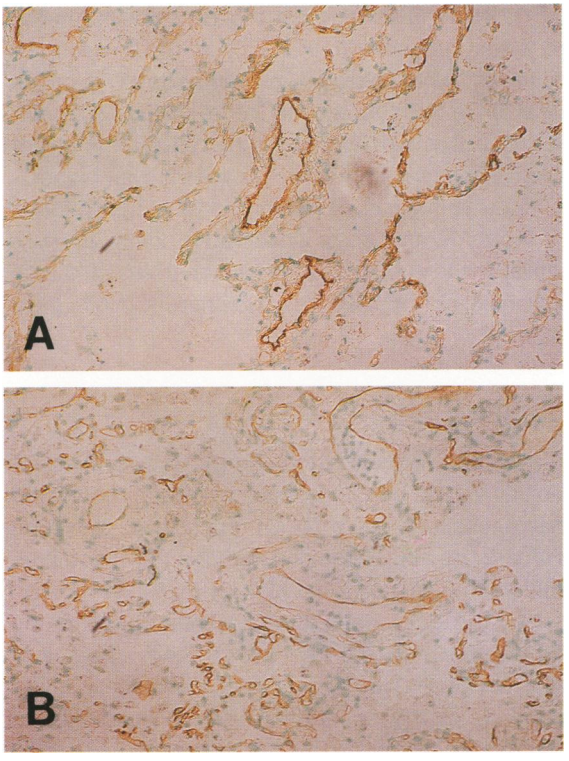


**Figure 4.** *Presence of TM in tissue specimens of normal lungs by immunohistochemical methods.* **(A)** *Positive staining of vascular endothelial cells by KA4, applied on a frozen section.* **(B)** *Positive staining of capillary endothelial cells by KA2, applied on a paraffin-embedded section fixed with methanol-Carnoy solution (original magnification, ×100).*

The pulmonary vascular endothelial cells showed positive staining to all antibodies tested in both the paraffin-embedded sections and the frozen sections from all the patients (Figure 4, A, B).

**Table 2** *Clinical Profiles and Immunoreactivity of Anti-TM Antibodies in Cases of Squamous Cell Carcinoma (Applied on Frozen Sections)*

| Case no. | Age | Sex | Grade of differentiation | Stage* | Recurrence† | Staining for TM |
|---|---|---|---|---|---|---|
| 1‡ | 76 | M | Well | I | – | + |
| 2 | 60 | M | Moderate | I | – | + |
| 3 | 81 | M | Well | $III_A$ | – | + |
| 4 | 63 | M | Well | $III_A$ | + | + |
| 5 | 76 | M | Well | I | – | – |
| 6 | 62 | M | Moderate | I | – | – |
| 7 | 71 | M | Poor | $III_A$ | – | – |
| 8 | 61 | M | Well | $III_A$ | + | – |
| 9 | 60 | M | Well | $III_A$ | + | – |
| 10 | 57 | M | Well | $III_B$ | + | – |
| 11 | 57 | F | Well | $III_B$ | + | – |

* According to the classification of the international staging for lung cancer.
† After operation recurrence within 1 year.
‡ A case showing positive staining to anti-TM antibodies on paraffin-embedded sections.

The staining intensity of the paraffin-embedded sections was poorer than that of the frozen sections. However, when the paraffin-embedded sections were fixed with methanol-Carnoy, Carnoy, and/or PLP, a recommended procedure to retain the glycosylated antigens, the staining intensity was higher.

The staining intensity of immunostaining exhibited by the three antibodies observed in this study was similar to the one of Western blot analyses of human recombinant TM[27]: KA3 always showing the highest intensity.

## Discussion

In this study, immunocytochemical studies, Western blot analyses, and functional studies all indicated that functionally active TM is present in some of lung cancer cells and TM belongs among the oncodevelopmental antigens.

Like the other oncodevelopmental antigens, the TM expression in the lung cancer cells appears to vary depending on the cellular conditions: about one-third of the squamous cell carcinoma tissue specimens expressed TM, but this was not seen in the adenocarcinoma and large cell carcinoma tissue specimens.

Yesner[28] believed that various histological types of common lung neoplasms develop from a small cell carcinoma to a large cell carcinoma, which may then develop in turn into an adenocarcinoma and/or a squamous cell carcinoma. Thus, the TM expression seen in this study may have been TM expressed at a certain stage during the differentiation pathway leading to a lung cancer, probably a late stage toward the development of a squamous cell carcinoma.

Focal findings on each specimen revealed that the TM expression of our squamous cell carcinoma cells took the form of a punctate pattern along the cell membranes in the lesser differentiated areas close to the stroma. This characteristic staining pattern may indicate that TM on squamous cell carcinoma cells plays some roles in cell-to-cell interaction as a cellular adhesive molecule, because the same pattern is also seen in the staining of a squamous cell carcinoma for desmoplakin, one of the cellular adhesive molecules.[29]

In contrast to squamous cell carcinoma cells, adenocarcinoma cells have been found to express TM in their cytoplasm in culture but not in tissue specimens. The heterogeneous expression of an embryonic antigen in cultured tumor cells is influenced by the variability of the membrane fractions associated with cell growth.[30] Further, immunohistochemical studies to determine the presence of embryonic antigens in lung adenocarcinoma cells have demonstrated local variability of staining within an individual tumor, indicating the heterogeneity for the production of the antigens.[14,15] Similarly, this immunocytochemical study may also represent the variable production of TM in A549 cells associated with the heterogeneity of the tumor cell population.

It appeared that there was no distinct correlation between the presence of TM in tissue specimens and the patient characteristics. However, there have been many *in vitro* investigations on the association between cancer and the coagulation system: fibrin deposits,[31] platelet aggregation directly induced by the tumor cells,[32] and secondary aggregation caused by thrombin.[33] Because TM plays an important role in the regulation of intravascular coagulation by exerting the inhibitory activity on thrombin-induced platelet aggregation[34] and/or the anticoagulant activity on fibrin formation, it can be roughly speculated that functionally active TM on lung cancer cells may modulate the biological behaviors of these cells, such as invasiveness and metastatic potential.

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