Human Tissue Distribution of TA02, Which is Homologous with a New Type of Aspartic Proteinase, Napsin A

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The N-terminal amino acid sequence of TA02 (molecular weight 35.0 kDa, isoelectric point 5.29), which is associated with primary lung adenocarcinoma, was determined and a fragment peptide was used to generate mouse monoclonal antibodies (mAbs) against TA02. The amino acid sequence suggested that TA02 might be homologous with napsin A, a new type of aspartic proteinase. In this context, we confirmed the expression of napsin A in primary lung adenocarcinoma using reverse-transcription polymerase chain reaction (RT-PCR) and showed that the TA02 mAbs reacted with glutathione-S-transferase (GST)-napsin A fusion protein. We concluded that TA02 is the same molecule as napsin A, and showed immunohistochemically that it is distributed mainly in type II pneumocytes, alveolar macrophages, renal tubules and exocrine glands and ducts in the pancreas. In particular, type II pneumocytes and alveolar macrophages showed high expression of TA02 among human normal tissues. In primary lung adenocarcinoma, 47 out of 58 (81.0%) primary lesions were positive. All well-differentiated adenocarcinomas except those of goblet cell type showed high expression of TA02. In addition, two out of seven (28.6%) large cell carcinomas showed low expression of TA02. The other histopathological types of primary lung cancer did not express TA02 at all. A few cases of renal cell cancer, pancreatic cancer, breast cancer, thyroid cancer, colon cancer and ovarian cancer showed low expression, but the staining patterns were completely different from that of primary lung adenocarcinoma, which showed a granular staining pattern. Our novel mAbs should be valuable for immunohistochemical detection of TA02/napsin A.

Key words: TA02 — napsin A — monoclonal antibody against TA02 — human aspartic proteinase — primary lung adenocarcinoma

Primary lung carcinoma incidence and mortality are still increasing. Even though primary lung carcinoma originates in bronchial epithelium, it shows a wide range of histopathological patterns. Adenocarcinoma accounts for approximately 40% of primary lung carcinoma in Japan. Most adenocarcinomas appear in peripheral bronchi. It is thought that this type of tumor originates from type II pneumocytes of the alveolar sac and Clara cells of the bronchiole. Other types of adenocarcinoma, which occur in relatively large bronchi, seem to derive from mucus cells, duct epithelial cells, goblet cells or bronchial glands. Based on the suspected cells of origin, primary lung adenocarcinoma can be subdivided into four types: type II pneumocyte type, Clara cell type, goblet cell type and bronchial gland type.1,2

We have already reported the specific two-dimensional polyacrylamide gel electrophoresis patterns of each histological type of primary lung carcinoma.2,3 In that investigation we discovered TA02 polypeptide (TA02; molecular weight 35.0 kDa, isoelectric point 5.29), which is associated with primary lung adenocarcinoma.3,4 The intensity of TA02 expression reflected the degree of histopathological differentiation of primary lung adenocarcinoma, except for the goblet cell type. Metastatic lung adenocarcinoma from colon or breast cancer did not express TA02. In summary, we have produced a novel monoclonal antibody (mAb) against TA02 and used it to investigate the TA02 molecule itself and to evaluate the possibility that TA02 may be a tumor marker for the clinical diagnosis of primary lung adenocarcinoma. The TA02 molecule seemed to be homologous with napsin A, which is a human aspartic proteinase.3,4 Consequently, we also examined whether TA02 and napsin A are identical.

MATERIALS AND METHODS

Non-enzymatic sample preparation from surgically resected specimens of primary lung carcinoma and two-dimensional polyacrylamide gel electrophoresis for the collection of TA02. Surgically resected well-differentiated adenocarcinomas, which expressed TA02, were sub-
jected to a non-enzymatic sample preparation technique employing two-dimensional electrophoresis, as reported previously. A sample corresponding to 100 µg of protein was subjected to isoelectric focusing (IEF) for 14.5 h at 800 V and for 1 h at 1000 V using a Protein II cell (Bio-Rad Laboratories, Hercules, CA) and a Model 1000/500 Power Supply (Bio-Rad). The IEF gels were placed on top of a linear gradient of 10–13% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoresed overnight at 10 mA per gel at 10°C.

Collection of TA02 polypeptide and analysis of amino acid sequence

The TA02 polypeptide was transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using the western blotting method and visualized using Coomassie Brilliant Blue staining. The TA02 spots were collected from the dried membrane. TA02 polypeptide was applied to a gas-phase protein sequencer (HPG1005A Protein Sequencing System; Hewlett-Packard Co., Palo Alto, CA) to determine the N-terminal amino acid sequence.

Production and purification of monoclonal antibodies against TA02 polypeptide

The synthetic peptide used for immunization was KPIFVPLSNYRDQFYGEIC, which corresponds to the K1 to I18 amino acid sequence of TA02. This peptide was coupled with bovine thyroglobulin and mixed with incomplete adjuvant, then injected subcutaneously into mice (BALB/c).

After immunization, spleen cells were collected and polyethylene glycol-mediated cell fusion between spleen cells and X63-Ag8-653 cells was induced. After five rounds of fusion, a hybridoma cell line cells were screened using an antigen-coated enzyme-linked immunosorbent assay (ELISA). Positive clones were cultured for production of antibodies in RPMI 1640 medium. TA02 polypeptide was applied to a gas-phase protein sequencer (HPG1005A Protein Sequencing System; Hewlett-Packard Co., Palo Alto, CA) to determine the N-terminal amino acid sequence.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of napsin A/B transcript expression and distinction between A and B transcripts

From the partial amino acid sequence of TA02, the TA02 molecule was suspected to be homologous with napsin, which is a type of human aspartic proteinase. In order to distinguish between napsin A and B transcripts, PCR products were digested with MspA1, which has a specific restriction site only in napsin B cDNA. After digestion, fragments were analyzed by 2% agarose gel electrophoresis.

Cloning of human napsin A cDNA and expression in Escherichia coli

The full-length cDNA of napsin A was amplified from the first-strand cDNA obtained from human primary lung adenocarcinomas by using forward primer (5′-GGCAATTTCATGTCCTCCACCCCGCTG-3′; NAP-1) and reverse primer (5′-CGCTCGAGCGTACCCGGGGAACTG-3′; NAP-6) synthesized based on the published human pro-napsin A cDNA sequence. The amplified products were digested with EcoR1 and Xho1, ligated into pGEX-4T-1 vector (Amershams Pharmacia Biotech) and used to transform E. coli JM109 cells. Napsin A cDNA cloned by PCR was completely sequenced.

Western blot and immunodetection with monoclonal antibodies against TA02

Surgically resected human primary lung carcinomas or the glutathione-S-transferase (GST)-pro-napsin A fusion protein expressing E. coli were subjected to 12.5% SDS-polyacrylamide gel electrophoresis as described previously, and proteins were transferred electrophoretically to Immobilon PVDF membrane (Millipore). The membrane was immunoblotted with anti-TA02 mAb and detected with an enhanced chemiluminescence (ECL) western detection reagents kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Furthermore, after sample preparation from well-differentiated adenocarcinoma in which a high level of TA02 had already been confirmed, we performed western blotting and immunodetection using anti-TA02 mAbs.

Immunohistochemistry

Four-micrometer-thick tissue sections were prepared from formalin-fixed, paraffin-embedded specimens and collected on glass slides. After deparaffinization, the specimens were stained immunohistochemically by the ABC method using mouse monoclonal antibodies against TA02. Meyer’s hematoxylin was used for counterstaining.

Evaluation of immunohistochemical staining

The staining patterns were evaluated as positive staining with a granular pattern, or positive staining with a diffuse pattern, or negative. In primary lung cancer, granular staining was classified into four groups: high expression (+), intermediate expression (++), low expression (±) or negative (−). When more than 80% of cancerous cells were positive, we evaluated the case as (+). Cases with 40–80% positive cells were evaluated as (±). Those with 10–40% positive granular staining were (−). Even if some cancerous cells showed a diffuse staining pattern, we evaluated lung cancer cases with less than 10% positive granular staining cells as negative (−).

RESULTS

N-Terminal amino acid sequence

A thirty-amino-acid N-terminal sequence of the TA02 molecule was deter-
mined, except for the twenty-seventh amino acid. The sequence was KP1FVPLSNYRDQYFGEIGLGTPPQ- (F)FYV.

**Monoclonal antibodies against TA02** We succeeded in producing four kinds of clones and designated them as mAb-4B2, 34D1, 22A and 6A1. Based on the reactivity on western blot analysis we chose clone 4B2, and utilized this clone in further experiments.

We also confirmed that clone 4B2 recognized the TA02 molecule in western blot analysis of a sample from well-differentiated lung adenocarcinoma (Fig. 1). Other minor spots including TA01 (molecular weight 35.0 kDa, isoelectric point 5.45) were also visualized. The spots other than TA01 and TA02 showed weak intensity.

**RT-PCR analysis of napsin A/B transcript expression and distinction between napsin A and B transcripts** We detected napsin A/B transcript expression in surgically resected primary lung adenocarcinoma in which high expression of TA02 had been confirmed and also in Raji cells. The RT-PCR product (bp 465–648 region) in lung adenocarcinoma expressing TA02 was not digested with MspA1, but that from Raji cells was digested (Fig. 2a). This analysis reveals that primary lung adenocarcinoma expressing TA02 at the protein level also expressed napsin A mRNA, and that Raji cells expressed napsin B mRNA, because a specific restriction site for MspA1 exists only in napsin B cDNA.

**Cloning of human napsin A cDNA, expression in E. coli and western blot analysis with anti-TA02 mAbs** From human primary lung adenocarcinoma, we cloned full-length cDNA which corresponded to the full-length napsin A cDNA, and amplified it using RT-PCR. We then prepared glutathione-S-transferase (GST)-napsin A fusion protein from transformed E. coli JM109 cells.

In the western blot analysis, we confirmed that anti-TA02 mAbs recognized the GST-napsin A fusion protein as a molecule with a molecular weight of approximately 67 kDa (Fig. 2b).

**Immunohistochemical staining**

**Normal tissues** (Table I): The cytoplasm of type II pneumocytes and alveolar macrophages showed strong staining with a granular expression pattern (Fig. 3a). Also, renal tubules (Fig. 3c) and pancreatic exocrine glands and ducts

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![Fig. 1. A: Two-dimensional electrophoretic analysis of well-differentiated adenocarcinoma sample. After western blotting, the residual protein on the gel was visualized by silver staining. B: Western blot analysis of well-differentiated adenocarcinoma sample. Western blot analysis and immunodetection using anti-TA02 mAb (clone 4B2) and a chemiluminescence detection technique were performed after two-dimensional electrophoresis. Spot A: TA01; spot B: TA02; spots C–D: precursor of TA02 (pro-napsin A). C: The X-ray film in western blot analysis was placed on top of the silver-stained gel film to reveal how spots A and B corresponded to TA01 and TA02.]

![Fig. 2. A: RT-PCR analysis of napsin A/B transcript expression and distinction between napsin A and B transcripts. Lane 1: Surgical specimens of well-differentiated adenocarcinoma of the lung. Lane 2: Surgical specimens of well-differentiated adenocarcinoma after digestion with MspA1. Lane 3: Raji cells (a B-cell line derived from human Burkitt’s lymphoma), which express the napsin B gene. Lane 4: Raji cells after digestion with MspA1. Well-differentiated adenocarcinoma expressed napsin A gene, because digestion with MspA1 was not seen. B: Western blot analysis using anti-TA02 mAb (clone 4B2). Lane 1 and 2: Surgically resected specimens of well-differentiated adenocarcinoma. Lane 3: GST-napsin A fusion protein. Lane 4: GST. Clone 4B2 recognized both TA02 derived from well-differentiated adenocarcinoma of the lung and GST-napsin A fusion protein.]

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Fig. 3. Immunohistochemical staining. A, Alveolar sac in the peripheral lung. Type II pneumocytes are positive. Alveolar macrophages (black arrowhead) also showed positive immunoreactivity. B, Bronchus. Bronchial epithelium is negative. C, Kidney. Some renal tubules are weakly positive. However, glomerulus (white arrowhead) is negative. D, Pancreas. Pancreatic duct is weakly positive. E, Well-differentiated adenocarcinoma of the lung, showing positive staining with a granular pattern. This case was evaluated as (2+). All cases with well-differentiated adenocarcinoma were positive. F, Poorly differentiated adenocarcinoma. A heterogeneous staining pattern was observed. However, a few adenocarcinoma cells showed high expression with a granular pattern in most poorly differentiated adenocarcinomas. This case was evaluated as (±). G, Well-differentiated squamous cell carcinoma of the lung. All cases with squamous cell carcinoma showed negative staining. H, Breast carcinoma. A few cases of breast cancer were weakly positive. Scale bar: 10 μm.
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(Fig. 3d) showed weak cytoplasmic staining. In some of these tissues a granular expression pattern could be observed, even though the immunoreactivity was weak. In other organs it was not possible to observe such a pattern. A part of the follicular epithelium in the thyroid, duct in the mammary gland and parietal cells in the stomach showed low expression with a diffuse pattern. Ciliated epithelium in the bronchus was completely negative (Fig. 3b).

Cancerous tissues (Table I and Table II): Cancerous tissues except lung carcinoma (Table I): A few cases of pancreatic cancer, renal cell cancer, breast cancer, thyroid cancer colon cancer and ovarian cancer showed weak staining with a diffuse pattern (Fig. 3h). Although these cases showed positive staining, it was heterogeneous.

Primary lung carcinoma (Table II): Forty-seven out of 58 (81.0%) adenocarcinoma cases showed positive stain-

Table I. Distribution of TA02 (Napsin A) in Normal and Cancerous Tissues

| TA02 expression                     | Normal tissues                        | Cancerous tissues                     |
|-------------------------------------|---------------------------------------|---------------------------------------|
| High expression with a granular pattern | Type II pneumocytes                  | Lung adenocarcinoma                   |
|                                     | Alveolar macrophages                  |                                       |
| Low expression with a granular pattern | Renal tubules                        | Large cell carcinoma of the lung      |
|                                     | Exocrine gland and duct in the pancreas |                                       |
| Low expression with a diffuse pattern | Follicular epithelium in the thyroid | Renal cell cancer                     |
|                                     | Duct in the mammary gland             | Pancreatic cancer                     |
|                                     | Parietal cells in the stomach         | Breast cancer                         |
|                                     |                                       | Thyroid cancer                        |
|                                     | Low expression                        | Colon cancer                          |
|                                     | with a granular pattern               | Ovarian cancer                        |
| Negative                            |                                       |                                       |
|                                     | Type I pneumocytes                    | Squamous cell lung carcinoma          |
|                                     | Ciliated bronchial epithelum          | Small cell lung carcinoma             |
|                                     | Bronchial gland                       | Carcinoid of the lung                 |
|                                     | Glomerulus in the kidney              | Gastric cancer                        |
|                                     | Insula in the pancreas                | Hepatoma                              |
|                                     | Lobule and myoepithelium in the       | Gall bladder cancer                   |
|                                     | mammary gland                         |                                       |
|                                     | Crypta in the stomach                 | Prostatic cancer                      |
|                                     | Colon                                 | Cervical cancer                       |
|                                     | Liver                                 |                                       |
|                                     | Gall bladder                          |                                       |
|                                     | Prostate                              |                                       |
|                                     | Uterus                                |                                       |
|                                     | Bladder                               |                                       |

Table II. Relationship between Expression of TA02 and Histopathological Types of Lung Carcinoma

| Histology                    | Expression levels of TA02 | Positive rate (%) |
|------------------------------|---------------------------|-------------------|
|                              | 2+ | + | +/- | -   |                  |
| Adenocarcinoma               |    | 25 | 11  | 11  | 11  | 81.0             |
| Well-differentiated          |    | 16 | 3   | 0   | 0   | 100.0            |
| Moderately differentiated    |    | 8  | 2   | 4   | 4   | 77.8             |
| Poorly differentiated        |    | 1  | 6   | 7   | 7   | 66.7             |
| Squamous cell carcinoma      |    | 0  | 0   | 14  | 0   |                  |
| Large cell carcinoma         |    | 0  | 0   | 2   | 5   | 28.6             |
| Small cell carcinoma         |    | 0  | 0   | 0   | 9   | 0                |
| Undifferentiated carcinoma   |    | 0  | 0   | 0   | 1   | 0                |
| Carcinoid tumor              |    | 0  | 0   | 0   | 2   | 0                |
ing with a granular pattern (Fig. 3, e and f). Particularly in well-differentiated cases, a strong immunoreactivity with a granular pattern could be seen compared with poorly differentiated cases. Other histopathological types showed completely negative staining except for a few cases of large cell carcinoma which had a weak expression with granular pattern. The positive staining pattern in primary lung carcinoma was completely different from that in other cancerous tissues.

**DISCUSSION**

Primary lung adenocarcinoma is an aggressive, highly malignant type of tumor that frequently exhibits lymph node metastases and distant metastases. Most primary lung adenocarcinomas appear in the peripheral bronchi and alveolar sac. Also, metastatic adenocarcinomas from other organs frequently appear in the periphery of the lung. However, few tumor markers are useful for distinction between primary lung adenocarcinoma and metastatic lesions with adenocarcinoma.

We had already detected TA02 on two-dimensional electrophoresis gels from primary lung adenocarcinoma samples.6 However, in metastatic lung adenocarcinoma these spots were not detected. We also recognized a close relationship between the expression level of TA02 and histopathological differentiation of lung adenocarcinoma. 3) Therefore, we speculated that this molecule might be useful for distinguishing between primary lung adenocarcinoma and metastatic lung adenocarcinoma from other organs, and attempted to produce mAbs against TA02 in order to analyze this molecule in more detail.

Protein database examination suggested that the TA02 molecule might be homologous with napsin A. Therefore, we examined the expression of the napsin A gene in primary lung adenocarcinoma. In well-differentiated adenocarcinoma of the lung, which highly expressed TA02, we succeeded in confirming the expression of the full-length pro-napsin A cDNA, and anti-TA02 mAbs could recognize the GST-napsin A fusion protein. This suggests that napsin A is identical with the TA02 molecule. A report concerning the homology between TA02 and pro-napsin A has appeared. 9) It gave the amino acid sequence of the TA02 molecule and described the distribution of TA02 using northern blot analysis and in-situ hybridization. Our result confirms that findings in other way.

The nucleotide sequence encoding pro-napsin A consists of a 1263-bp open reading frame that predicted a 420-amino acid polypeptide. 4) In the mature form of napsin A, 64 amino acids (64 residues) are lost from the N-terminal of pro-napsin A. The TA02 molecule extracted from primary lung adenocarcinoma corresponds to the mature form of napsin A. Therefore, it is highly likely that anti-TA02 mAb recognizes not only the mature form of napsin A, but also pro-napsin A. We detected some spots of approximately 40 kDa in molecular weight with weak intensity by western blot analysis (Fig. 1). They might be precursors of napsin A. Their amount was relatively low in primary lung adenocarcinoma compared to the mature form of napsin A.

Furthermore, we investigated the immunohistochemical distribution of TA02 (napsin A) at the protein level in normal human tissues and cancerous tissues. Only type II pneumocytes and alveolar macrophages in the peripheral lung showed high expression with a granular staining pattern. It was reported that napsin A is a new type of human aspartic proteinase. 4) Some human aspartic proteinases are secretory enzymes (for instance, pepsin, gastricsin and renin). If napsin A is a secretory enzyme produced by type II pneumocytes, alveolar macrophages could phagocytize napsin A molecules. Therefore, it is doubtful whether alveolar macrophages express the napsin A gene, because macrophages in other organs do not show high expression levels of TA02 (napsin A) immunohistochemically.

Based on the TA02 (napsin A) expression level, normal epithelial tissues could be classified into four groups (high expression: type II pneumocytes; moderate expression: renal tubules, pancreatic exocrine gland and duct; low expression: mammary gland duct, follicular epithelium in the thyroid and parietal cells in the stomach; and negative). Our data are consistent with a previous small-scale analysis of human tissues at the RNA level. 4) Furthermore, among cancerous tissues, only primary lung adenocarcinoma showed high intensity with a granular expression pattern, though a few other kinds of cancerous tissues had low expression with a diffuse pattern. It seems that distinguishing primary lung adenocarcinoma from metastatic adenocarcinoma of the lung originating from other organs is possible in terms of the TA02 immunohistochemical staining pattern. Among primary lung adenocarcinomas, type II pneumocyte type and Clara cell type expressed TA02. All three cases of goblet cell type in this study showed negative staining, even though these cases were histopathologically diagnosed as having moderate differentiation.

It is known that surfactant apoprotein A (SpA) is expressed in type II pneumocytes and primary lung adenocarcinoma. 10) Thus, the distribution of TA02 (pro-napsin A) was compared with that of SpA in each histopathological type of primary lung carcinoma using PE-1 mAb (DAKO, Copenhagen, Denmark). Either TA02 (napsin A) or SpA was expressed only in adenocarcinoma and large cell carcinoma. TA02 (napsin A) showed a higher positive rate in adenocarcinoma than SpA, and there was no case in which SpA was positive and TA02 (napsin A) was negative (data not shown).

The main conclusions of this work were that TA02 is homologous with napsin A, based on anti-TA02 mAbs’
reactivity with napsin A fusion protein and the distribution of TA02 in human tissues. TA02 may be useful as a tumor marker for primary lung adenocarcinoma. It remains important to clarify the functions of the TA02 molecule. The functional regions in napsin A have been identified and are similar to those of other human aspartic proteinases. Therefore, TA02 (napsin A) may play critical roles in the peripheral lung, the renal tubules and pancreatic exocrine gland and duct. We should evaluate the clinical utility of TA02 in the diagnosis of primary lung adenocarcinoma, because this is the only entity that expresses a high level of the molecule.

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