Utility of PAX8 mouse monoclonal antibody in the diagnosis of thyroid, thymic, pleural, and lung tumors: a comparison with polyclonal PAX8 antibody

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Abstract

Aims: The purpose of this study was to compare the immunohistochemical staining profiles of PAX8-polyclonal, PAX8-monoclonal, PAX5-monoclonal, and PAX6-monoclonal antibodies in several histologic types of primary thoracic and thyroid tumors. In addition, we analyzed PAX8 mRNA expression by using in situ hybridization.

Methods and results: We compared polyclonal PAX8 and monoclonal PAX8, PAX5, and PAX6 antibodies in 898 samples (687 cases of lung carcinoma, 40 cases of malignant pleural mesothelioma, 138 cases of thymic tumor, and 33 cases of thyroid tumors) using tissue microarray technique. Among thyroid tumors, the monoclonal and polyclonal PAX8 antibodies showed a high positive rate (97.0%). Of 167 polyclonal PAX8 antibody-positive tumors, except for thyroid tumors, 54 cases tested positive for PAX5 and/or PAX6 (31 lung carcinomas and 23 thymic tumors). No PAX8 mRNA expression was detected using RNAscope (in situ hybridization technique) other than in thyroid tumors. A portion of polyclonal PAX8 antibody-positive tumors showed cross-reactivity for PAX5 or PAX6 protein.

Conclusions: Monoclonal PAX8 antibody showed high specificity to thyroid tumors and was superior to the polyclonal antibody.

Key words: PAX8, thoracic tumors, cross-reactivity, immunohistochemistry
Introduction

The lung is the most common site of metastasis in case of malignant tumors. Distinction of primary lung tumor from metastatic tumor is important because the treatment modalities and prognosis for these 2 lesions are quite different. When lung tumors present with typical morphology, the diagnosis is straightforward, and immunohistochemical staining is not necessary. However, poorly differentiated tumors are sometimes more challenging. Immunostaining of thyroid transcription factor-1 (TTF-1) is a useful positive marker for confirming adenocarcinoma of a unknown primary site as having a pulmonary origin. However, TTF-1, as the name implies, is also positive for thyroid tumors.

The paired box transcription factor PAX8 is a nephric-lineage transcription factor. In human tissues, it is expressed in various normal tissues, such as the thyroid gland, kidney, and müllerian system, as well as in tumors arising in these regions. The usefulness of PAX8 in distinguishing ovarian serous tumors from malignant mesothelioma or renal collecting duct carcinoma from urothelial carcinoma has been reported.\textsuperscript{1,2} In addition, PAX8 expression was reported in thymic neuroendocrine carcinomas,\textsuperscript{3} normal B lymphocytes,\textsuperscript{4,5} and B cell lymphomas.\textsuperscript{4}

However, some recent studies, mostly employing PAX8 polyclonal antibodies, have shown cross-reactivity with the B-cell-specific transcription factor PAX5.\textsuperscript{6,7} As a result, some previously reported PAX8 immunopositive normal tissues and tumors represented by B lymphocytes that did not express PAX8 mRNA could be considered to be due to this cross-reactivity. This cross-reactivity was attributed to the high sequence homology between PAX5 and PAX8 in the N-terminal region.\textsuperscript{6} PAX8 polyclonal antibody was raised against a 212-amino acid acid-long polypeptide encompassing the N-terminal region of PAX8. In addition, a recent study revealed that one of the causes of aberrant polyclonal PAX8 positivity could be cross-reactivity for PAX6, which is crucial for neuronal development.\textsuperscript{8}
The purpose of the present study was to compare the immunohistochemical staining profiles of PAX8-polyclonal, PAX8-monoclonal, PAX5-monoclonal, and PAX6-monoclonal antibodies in several histologic types of primary thoracic and thyroid tumors. In addition, we analyzed PAX8 mRNA expression by using in situ hybridization with the novel, highly sensitive method of RNAscope.

Materials and methods

Case selection and construction of tissue microarrays

The institutional review board approved the study (2010-0077). The materials for the present study were extracted from cases deposited in the pathology files of the National Cancer Center Hospital, Tokyo. A total of 898 cases were used this analysis; 687 cases of lung carcinoma (adenocarcinoma, 253 cases; squamous cell carcinoma, 158 cases; large cell neuroendocrine carcinoma, 106 cases; small cell carcinoma, 67 cases; carcinoid tumor, 51 cases; pleomorphic carcinoma, 41 cases; large cell carcinoma, 11 cases), 40 cases of malignant pleural mesothelioma (epithelioid, 28 cases; biphasic, 12 cases), 138 cases of thymic tumor (thymoma, 102 cases; thymic carcinoma, 36 cases), and 33 cases of thyroid tumors (papillary carcinoma, 21 cases; follicular carcinoma, 5 cases; undifferentiated carcinoma, 4 cases; follicular adenoma, 3 cases).

All diagnoses were based on conventional histopathological features evident in slide preparations stained with hematoxylin and eosin, some specific stains, immunohistochemical, and molecular techniques available at the time. Tissue microarray (TMA) utilized a core sample measuring 2.0 mm in diameter.

Immunohistochemistry

For immunohistochemical staining, 4-μm-thick sections were deparaffinized and treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity, followed by washing in deionized water for 2–3 min. Heat-induced epitope retrieval with Target Retrieval
Solution High pH (DAKO, Carpinteria, CA, USA) was performed. After the slides were allowed to cool at room temperature for 40 min, they were rinsed with deionized water and then washed in phosphate-buffered saline for 5 min. The slides were then incubated with PAX8 polyclonal antibody (1:200; Proteintech, Chicago, IL, USA), PAX8 monoclonal antibody (PAX8R1, 1:50; Abcam, Cambridge, MA, USA), PAX5 monoclonal antibody (clone 24, 1:200; BD Biosciences, Franklin Lakes, NJ, USA), and PAX6 monoclonal antibody (P3U1, 1:500; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 1 h at room temperature. Immunoreactions were detected by the Envision-plus system (DAKO), and visualized with 3,3′-diaminobenzidine, followed by counterstaining with hematoxylin.

Immunohistochemical staining was scored independently by 2 observers (A.T. and K.T.). Nuclear staining was evaluated and its intensity was graded on a scale of 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong), and the extent of staining (estimated percentage of stained cells) was recorded. Tumors were considered positive if >5% of the tumor nuclei were stained.

**In situ hybridization for PAX8 mRNA**

For detection of PAX8 mRNA among polyclonal PAX8-antibody positive cases, an RNAscope FFPE Assay Kit (Advanced Cell Diagnostics Inc., Hayward, CA, USA) and an RNAscope Probe Hs-PAX8 (Advanced Cell Diagnostics Inc.) were used according to the manufacturer’s instructions. Briefly, sections were pretreated with heat and protease. They were then incubated with a probe targeting PAX8 for 2 h at 40°C. The slides were washed thoroughly with Wash Buffer (Advanced Cell Diagnostics Inc.) after each hybridization step at room temperature. Diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin. A probe for POLR2A was used as a positive control. Positive staining was identified as brown, punctate spotting in the nucleus and/or cytoplasm.

The expression was scored using the instructions in the RNAscope FFPE Assay Kit; no
staining (score, 0), 1–3 dots/cell (score, 1), 4–10 dots/cell (score, 2), >10 dots/cell in addition to <10% positive cells have dot clusters (score, 3), and >10 dots/cell in addition to >10% positive cells have dot clusters (score, 4).

Results

Reactivity of monoclonal and polyclonal PAX8 antibodies

Immunoreactivity of polyclonal PAX8 antibody was observed in 32 of 33 (97.0%) thyroid tumors: 21 (100%) cases of papillary carcinoma, 5 (100%) cases of follicular carcinoma, 3 of 4 (75.0%) cases of undifferentiated carcinoma, and 3 (100%) cases of follicular adenoma; as well as 55 of 687 (8.0%) cases of lung carcinoma: 6 of 253 (2.4%) cases of adenocarcinoma, 3 of 158 (1.9%) cases of squamous cell carcinoma, 17 of 106 (16.0%) cases of large cell neuroendocrine carcinoma, 27 of 67 (40.3%) cases of small cell carcinoma, 2 of 11 (18.1%) cases of large cell carcinoma; 91 of 102 (89.2%) cases of thymoma; and 21 of 36 (58.3%) cases of thymic carcinoma (Table 1). No malignant pleural mesotheliomas were positive for polyclonal PAX8 antibody. On the other hand, immunoreactivity of monoclonal PAX8 antibody was observed in 32 of 33 (97.0%) cases of thyroid tumor: 20 of 21 (95.2%) cases of papillary carcinoma, 5 (100%) cases of follicular carcinoma, 4 (100%) cases of undifferentiated carcinoma, and 3 (100%) cases of follicular adenoma, whereas other tumors showed no reactivity.

When thyroid tumors were regarded as the gold standard for calculating the sensitivity of the PAX8 antibodies, the same sensitivity (97.0 %) was observed for both monoclonal and polyclonal PAX8 antibodies. However, compared to the polyclonal antibody, the monoclonal antibody showed lower intensity and percentage of positive cells (Fig.1A, B). Strong intensity staining was observed in 28 (84.8%) cases of thyroid tumor, 1 (0.9%) cases of large cell neuroendocrine carcinoma, and 4 (6.0%) cases of small cell carcinoma for polyclonal PAX8 antibody, whereas most of the others exhibited weak labeling.
Reactivity of PAX5 and PAX6 antibodies

PAX5 immunoreactivity was observed in 24 (3.5%) cases of lung carcinoma: 7 (6.6%) cases of large cell neuroendocrine carcinoma, 16 (23.9%) cases of small cell carcinoma, and 1 (2.0%) cases of carcinoid tumor; and 7 (19.4%) cases of thymic carcinoma (Fig.2). Thyroid tumors, lung carcinomas except for neuroendocrine tumors, malignant pleural mesotheliomas, and thymomas were negative for PAX5.

PAX6 was positive in 2 (50.0%) cases of thyroidal undifferentiated carcinoma; 107 (15.6%) cases of lung carcinoma: 26 (10.3%) cases of adenocarcinoma, 29 (18.4%) cases of squamous cell carcinoma, 2 (4.9%) cases of pleomorphic carcinoma, 20 (18.9%) cases of large cell neuroendocrine carcinoma, 20 (29.9%) cases of small cell carcinoma, 3 (27.3%) cases of large cell carcinoma, and 7 (13.7%) cases of carcinoid tumor, 5 (12.5%) cases of malignant pleural mesothelioma; and 20 (14.5%) cases of thymic tumor.

Cross-reactivity among polyclonal PAX8, PAX5, and PAX6 antibodies

Among 32 polyclonal PAX8 antibody-positive thyroid tumors, PAX5 and/or PAX6 were positive in only 1 (3.1%) case. In contrast, 167 polyclonal PAX8 antibody-positive other than thyroid tumors, PAX5 and/or PAX6 were positive in 54 (32.3%) cases. In detail, PAX 5 was positive in 16 lung carcinomas (4 of 17 cases of large cell neuroendocrine carcinoma and 12 of 27 cases of small cell carcinoma), and 7 of 21 cases of thymic carcinoma (Fig.2A-C).

PAX6 was positive in 20 cases of lung carcinomas (1 of 6 cases of adenocarcinoma, 3 of 3 cases of squamous cell carcinoma, 8 of 17 cases of large cell neuroendocrine carcinoma, 8 of 27 cases of small cell carcinoma), and 18 of 112 cases of thymic tumor (16 of 91 cases of thymoma and 2 of 21 cases of thymic carcinoma). The overlapping cases are depicted in Venn diagrams (Fig.3).

PAX8 mRNA expression

To confirm that aberrant PAX8 detection was induced by cross-reactivity of other protein
expression, we evaluated PAX8 mRNA expression by using an in situ hybridization technique. Among 199 polyclonal PAX8 antibody-positive cases, 15 cases were negative for POLR2A mRNA, which was considered as insufficient material for mRNA evaluation; thus, the final cohort included 184 (92.4%) samples: 29 cases of thyroid tumors and 155 cases of non-thyroid tumors. PAX8 mRNA expression was observed in 28 (96.6%) of 29 analyzed thyroid tumors. On the other hand, the other polyclonal PAX8 antibody-positive non-thyroid tumors were negative for PAX8 mRNA expression (Fig. 1C, D).

Discussion

The current study showed that the monoclonal PAX8 antibody was a highly specific and equally sensitive marker compared to polyclonal PAX8 antibody. In addition, the aberrant detection by polyclonal PAX8 was partially caused by the cross-reactivity with PAX5 and/or PAX6.

When thyroid tumors were regarded as the gold standard for calculating the sensitivity for PAX8, the monoclonal PAX8 antibody showed a high positive detection rate identical to that of the polyclonal antibody. When compared with the findings of the present study, identical sensitivity was observed for polyclonal and monoclonal PAX8 antibodies in ovarian serous carcinoma. On the other hand, monoclonal PAX8 antibody showed higher sensitivity in ovarian endometrioid and renal cell carcinomas, which were regarded as the gold standard in the same study. These conflicting results for the detection of PAX8 expression may be due to differences in target organ, scoring system, or possibly staining techniques with different antigen-retrieval methods. In general, the immunoreactivity of the monoclonal antibody was lower compared with that of the polyclonal one because the monoclonal antibody recognizes a single epitope, whereas the polyclonal antibody can recognize multiple epitopes and is less likely to be affected by changes in protein conformation.

Similar to previous reports, 81.2% of cases of thymic tumor and 25.4% of cases of
pulmonary neuroendocrine carcinoma showed polyclonal PAX8 reactivity, and PAX8 was not detected in cases of pulmonary carcinoid tumor. However, these observations were attributed to the cross-reactivity of the polyclonal PAX8 antibody because detection using monoclonal PAX8 was negative in these tumors. Previous studies suggested a similar reactive pattern (positive for polyclonal PAX8 but negative for monoclonal PAX8) and these reactions observed in B cell lymphomas were considered to be caused by antibody cross-reactivity. 6, 7, 9

As described above, the cross-reactivity of PAX5 or PAX6 protein was one of the known reasons for aberrant detection by polyclonal PAX8 antibody. 6-8 However, a proportion of polyclonal PAX8 antibody-positive lung and thymic tumors showed PAX5 protein expression. In addition, PAX6 expression was limited in a proportion of polyclonal PAX8 antibody-positive lung and thymic tumors. The possibility of cross-reactivity with other PAX families of similar structures needs to be considered because PAX gene family members share similar sequence homology. 13 In fact, cross-reactivity between PAX2 protein and PAX5 antibody has been reported. 14

We observed that PAX8 mRNA expression was not observed among polyclonal PAX8-positive tumors other than those in the thyroid. Although mRNA expression is not always parallel to protein expression, the current data reinforce the hypothesis that polyclonal PAX8-positive tumors, other than those in the thyroid, are not genuine PAX8 protein expressions. 6 To reduce the possibility of false-negative results in the currently used immunohistochemical technique, we used commercially available kits and probes. In addition, the currently used method is highly sensitive for the detection of a small mRNA copy number from the formalin-fixed, paraffin-embedded samples. Finally, we excluded cases that tested negative for the expression of POLR2A mRNA internal control. This result indicated that the difference in the positive results for polyclonal and monoclonal PAX8 antibodies in tumors, other than those in the thyroid, was not induced by reduced sensitivity of PAX8 monoclonal
antibody.

The utility of PAX5 and PAX6 antibodies for solid organ is limited because PAX5 expression was positive in a proportion of pulmonary neuroendocrine tumors and thymic carcinomas. However, PAX5 expression may be useful in distinguishing pulmonary neuroendocrine tumors from poorly differentiated non-neuroendocrine carcinomas. In agreement with previous reports, pulmonary small cell carcinoma showed high PAX5 reactivity compared with other low-grade pulmonary neuroendocrine tumors.\textsuperscript{15-17} Although PAX6 expression was reported in pancreatic neuroendocrine tumors\textsuperscript{8} and the neuroepidermis\textsuperscript{18}, present data indicate that this is not high and is restricted to pulmonary neuroendocrine tumors.

In conclusion, the monoclonal PAX8 antibody is an equally sensitive and highly specific thyroidal cell marker as the polyclonal PAX8 antibody and would be better suited, in daily practice, for identification of PAX8-expressing tumors.
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References


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Figure legends

Figure 1.

Follicular carcinoma immunoreactivity

A, Diffuse, strong (3+) positive staining for polyclonal PAX8 antibody (original magnification, 400×). B, Staining with the monoclonal PAX8 antibody shows more focal and weaker staining than the polyclonal antibody (original magnification, 400×). C, PAX8 mRNA shows dot-like staining and clusters (original magnification, 400×). D, POLR2A shows mRNA dot-like staining (original magnification, 400×).

Figure 2.

Thymic carcinoma immunoreactivity

A, Diffuse, moderate (2+) staining for the polyclonal PAX8 antibody (original magnification, 400×). B, PAX5 shows similar, but weak, positive staining patterns with PAX8 (original magnification, 400×). C, Tumor cells are focally positive for PAX6 (original magnification, 400×).

Figure 3.

Venn diagrams showing overlapping positive cases

The circles depict the number of positive cases for each antibody: monoclonal PAX8 (yellow), polyclonal PAX8 (red), PAX5 (purple), and PAX6 (green).
<table>
<thead>
<tr>
<th>Tumor types</th>
<th>Staining pattern in neoplastic cells, no. (%)</th>
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<tbody>
<tr>
<td></td>
<td>PAX8 polyclonal</td>
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<tr>
<td><strong>Lung tumors</strong></td>
<td></td>
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<tr>
<td>Adenocarcinoma (n = 253)</td>
<td>6 (2.4)</td>
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<td>Squamous cell carcinoma (n = 158)</td>
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<td>Large cell neuroendocrine carcinoma (n=106)</td>
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<td>Carcinoid tumor (n=51)</td>
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<td>Pleomorphic carcinoma (n=41)</td>
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<td>Large cell carcinoma (n=11)</td>
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<td>Malignant pleural mesothelioma (n = 40)</td>
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<td><strong>Thymic tumors</strong></td>
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<td>Thymoma (n=102)</td>
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<td>Thymic carcinoma (n=36)</td>
<td>21 (58.3)</td>
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<tr>
<td><strong>Thyroid tumors</strong></td>
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<td>Papillary carcinoma (n=21)</td>
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<td>Follicular adenoma (n=3)</td>
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| Table 1. Positive cases stained with polyclonal PAX8, monoclonal PAX8, PAX5, and PAX6 antibodies |