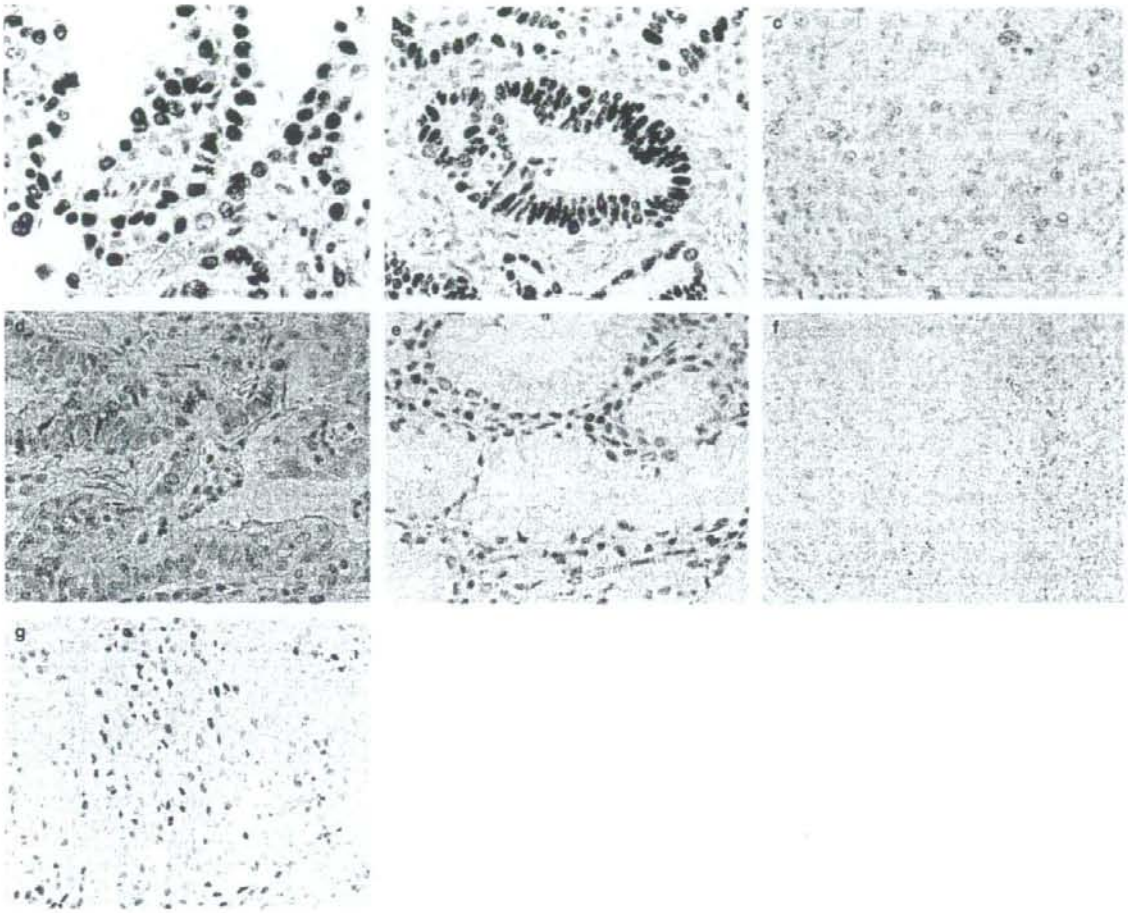

Figure 3 Immunohistochemical thyroid transcription factor-1 expression. Strongly positive in (**a**) Clara and (**b**) bronchial surface epithelial component (BSE), and weakly positive in (**c**) poorly differentiated component (POR) in group A tumor. Weakly positive in (**d**) BSE, and negative in (**e**) GOB and (**f**) POR in group B tumor. (**g**) Positive in group C tumor. (HE).

Table 3 Immunohistochemical TTF-1 expression in adenocarcinomas

Positive degree	0 (0%: negative)	1+ (1–10%)	2+ (11–50%)	3+ (51–90%)	4+ (91–100%)
BSE (no. cases)					
Group A ($n = 5$)	0	1	2	1	1
Group B ($n = 15$)	9	3	2	0	1
POR					
Group A ($n = 44$)	3	6	9	14	12
Group B ($n = 15$)	11	2	1	0	1
Group C ($n = 10$)	2	1	2	3	2

BSE, bronchial surface epithelial component; POR, poorly differentiated component; TTF-1, thyroid transcription factor-1.

metastatic sites even if they had dedifferentiated to poorly differentiated adenocarcinoma.

BSE in group B (lacking Clara/type II or mCB) were frequently TTF-1 negative, in contrast to constant TTF-1 expression of BSE in group A. Although it is impossible to distinguish a BSE component of these two groups solely on the basis of cytological and structural morphology, the natural histories through which they acquire their morphologies may

be different. Group B tumors are suspected to be derived from bronchial epithelium or bronchial metaplastic epithelium, and not from peripheral alveolar epithelium such as group A tumors. Although the possibility remains that group B tumors are derived from peripheral alveolar epithelium and lose their TTF-1 expression completely during progression, this seems unlikely when considering the difference in cytological constituents between these two groups, as well as the conserved expression of TTF-1 in group A. A similar hypothesis can be suggested for POR. Although POR in group A and POR in group B show similar morphology, they are suggested to have different characters (history and biological nature), which reflect the presumed natural history of each group.

All GOB lesions were TTF-1 negative, both in group A and in group B. Although most goblet cell adenocarcinomas are of the pure form, small numbers of mixed goblet cell and non-goblet cell adenocarcinoma also exist.[27,33] Lau *et al.* reviewed seven cases of mixed non-mucinous and mucinous BAC, and reported that 6/7 of the mucinous component (almost identical to BAC comprising only the goblet component) were TTF-1 negative, whereas 5/7 of the non-mucinous component were TTF-1 positive.[27] Also in the present study, the non-goblet cell component in mixed goblet cell and non-goblet cell adenocarcinomas in both groups A and B expressed TTF-1 in some cases. On the basis of previous reports and the present data, we hypothesize that TTF-1 expression is completely lost when adenocarcinomas differentiate to a goblet cell morphology in mixed goblet cell and non-goblet cell adenocarcinoma. However, in pure goblet cell adenocarcinoma it is difficult to explain the lack of TTF-1 expression, and therefore the mechanism of controlling TTF-1 expression should be clarified from the viewpoint of the physiological function of TTF-1 and mucin production.

Eight of 10 group C tumors were TTF-1 positive and some of them expressed TTF-1 strongly. Although their number was small, it is suggested that the majority of group C tumors were derived from group A tumors.

In summary, group A and group B tumors are suggested to have different natural histories, from the viewpoint of immunohistochemical expression of TTF-1. Group A tumors might be derived from peripheral alveolar epithelial cells and retain their TTF-1 expression even if they differentiate to BSE or dedifferentiate to POR. Conversely, the majority of BSE and POR in group B tumors were TTF-1 negative. Although BSE and POR in both groups had a similar morphology, their TTF-1 expression patterns were different, thus reflecting their different natural histories. From the viewpoint of TTF-1 expression, the majority of group C tumors are suggested to be derived from group A tumors.

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Analysis of Expression Patterns of Breast Cancer–Specific Markers (Mammaglobin and Gross Cystic Disease Fluid Protein 15) in Lung and Pleural Tumors

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• *Context.*—The lung is the most common site of metastasis during the natural history of malignant tumors. Breast carcinoma has a propensity for distant metastasis, and the lung and pleura are among the most common metastatic sites. Although it is often difficult to make a clear-cut differential diagnosis between the two, distinguishing primary lung carcinoma from breast carcinoma metastatic to the lung is important because the treatment modalities are different.

Objective.—To elucidate the utility of mammaglobin and gross cystic disease fluid protein 15 (GCDFP-15), which are known to be breast-specific antigens, in distinguishing various primary lung and pleural tumors from breast carcinoma metastasizing to the lung.

Design.—A total of 20 cases of breast carcinoma metastatic to the lung and 263 tumors of nonbreast origin located in the lung and pleura were analyzed.

Results.—Of the 20 cases of breast carcinoma metastatic to the lung, 10 (50.0%) were immunoreactive for mammaglobin and 9 (45.0%) for GCDFP-15, the frequency of positivity being slightly higher for the former than for the latter. The area immunopositive for mammaglobin showed more diffuse staining than the area immunopositive for GCDFP-15. Furthermore, the specificity of mammaglobin for breast carcinoma metastatic to the lung was superior (98.9%) to that of GCDFP-15 (91.8%).

Conclusion.—The sensitivity of mammaglobin is equal or superior to that of GCDFP-15 for investigation of breast carcinoma. Immunopositivity for mammaglobin is more diffuse than that for GCDFP-15. In terms of practical diagnosis, mammaglobin immunohistochemistry can serve as a differential marker of breast carcinoma and should be added to the immunohistochemical panel.

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The lung is the most common site of metastasis during the natural history of malignant tumors.[1] In particular, breast carcinoma has a propensity for distant metastasis, and the lung and pleura are the second most common metastatic sites following bone.[2] It is also well known that breast carcinoma metastatic to the lung may be found even after a postoperative disease-free interval up to 20 years after resection of the primary lesion. Distinction of primary lung carcinoma from breast carcinoma metastatic to the lung is important under certain clinical situations, such as when a solitary solid nodule is found in the peripheral lung of a patient with a history of resected breast carcinoma, because the treatment modalities for these 2 lesions are different. Furthermore, histologic information regarding hormone receptor status and HER2 status provides a target for therapy in breast carcinoma.

Differentiating between primary lung carcinoma and breast carcinoma metastatic to the lung is often problematic when only a small amount of material is available, as the histologic features may not be sufficient to permit unequivocal distinction. Therefore, reliable immunohistochemical markers are required to facilitate the differentiation of these malignancies.

In breast cancer, the estrogen receptor (ER)/progesterone receptor (PgR) status of the tumor is useful for both prognosis and therapy, with more chemotherapeutic options being available for patients with hormone receptor–positive tumors.[3,4] Breast adenocarcinoma has been shown to be positive for ER in 24% to 63% of cases and positive for PgR in 9% to 37% of cases.[5,6] Breast adenocarcinoma may demonstrate immunophenotypic variability in its expression of ER and PgR, with differences that are dependent on the histologic grade, histologic subtype, antibody clone applied, and immunohistochemical techniques used. These factors limit the sensitivity of these markers for excluding metastatic breast adenocarcinoma in cases of unknown primary site.

When primary unknown metastatic tumor is suspected

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Table 1. Immunoreactivity of Mammaglobin

	No. of Cases Examined	% Positive	Staining Area			
			0	1+	2+	3+
Breast carcinoma, metastasis to lung	20	50	10	4	6	0
Primary lung carcinoma	250	1.2	247	3	0	0
Adenocarcinoma	100	0	100	0	0	0
Squamous cell carcinoma	60	1.6	59	1	0	0
Pleomorphic carcinoma	20	0	20	0	0	0
Carcinoid tumor	19	5.2	18	1	0	0
Large-cell neuroendocrine carcinoma	20	0	20	0	0	0
Small-cell carcinoma	15	0	15	0	0	0
Adenoid cystic carcinoma	11	9.1	10	1	0	0
Mucoepidermoid carcinoma	5	0	5	0	0	0
Malignant mesothelioma	13	0	20	0	0	0

to have originated from the breast, ER, PgR, and gross cystic disease fluid protein 15 (GCDFP-15) have been shown to be useful immunohistochemical markers.[7–9] However, ER and PgR have also been documented in many neoplasms from various organs. In lung tumors, ER and PgR expressions are reported to range from 0% to 96.7% and from 0% to 46.5%, respectively.[10–12] A panel consisting of anticytokeratin 7 and anticytokeratin 20 (CK7/CK20) antibodies is useful for determining the origin of an unknown primary tumor. However, numerous tumors exhibit an identical $CK7^+/CK20^-$ immunophenotype, including nearly all breast carcinomas and adenocarcinomas of the ovary, lung, endometrial, thyroid, and salivary gland.[13]

Additionally, GCDFP-15 has also been documented in many neoplasms from various locations. In lung tumors, GCDFP-15 expression has been reported to range from 0% to 3.3%[14,15]

The mammaglobin gene sequence fragments were first isolated in 1994 by Watson and Fleming.[16] The mammaglobin gene encodes a 10-kDa molecule, which is related to a family of secretory proteins, including rat prostatic steroid-binding protein subunit C3, human Clara cell 10-kDa protein, and rabbit uteroglobin. Mammaglobin is expressed specifically in breast tissue.[16–18] Recently, an anti-mammaglobin antibody that can be applied to formalin-fixed, paraffin-embedded sections has become commercially available.

In the present study, we elucidated the expression of mammaglobin and GCDFP-15 in order to distinguish various primary lung and pleural tumors from breast carcinoma metastatic to the lung.

MATERIALS AND METHODS

Histologic Analysis

Materials for the present study were extracted from the pathology files of the National Cancer Center Hospital (Tokyo, Japan). The specimens comprised 20 cases of breast carcinoma metastatic to the lung and 263 lung and pleural tumors other than metastatic breast carcinomas: 100 adenocarcinomas, 60 squamous cell carcinomas, 20 pleomorphic carcinomas, 20 large-cell neuroendocrine carcinomas, 15 small-cell carcinomas, 19 carcinoids (14 cases typical and 5 cases atypical), 16 salivary gland–type tumors of the bronchus and/or trachea (11 cases of adenoid cystic carcinoma and 5 cases of mucoepidermoid carcinoma), and 13 malignant pleural mesotheliomas.

The 100 cases of adenocarcinoma were divided into 2 subtypes according to the growth pattern: 60 cases showing lepidic growth and 40 cases without lepidic growth. The 60 cases showing lepidic growth were further divided into 20 cases of the nonmucinous type (tumor cells resembling Clara cells or type II pneumocytes) and 40 cases of the mucinous type (tumor cells resembling goblet cells and/or bronchial surface epithelial cells). Furthermore, the 40 cases without lepidic growth were divided into 20 cases of the acinar-cribriform type (tumor showing an acinar and/or cribriform growth pattern with some degree of cytoplasmic mucin) and 20 cases of the solid type (tumor showing solid growth with some degree of cytoplasmic mucin formation, such as intracytoplasmic lumina).

The 60 cases of squamous cell carcinoma were divided into the well-differentiated type (tumor cells showing a stratified pattern and abundant keratinization), moderately differentiated type (cells showing a lower degree of stratification than that of the well-differentiated type), and poorly differentiated type (the tumor composed of more atypical cells that show only focal squamous cell differentiation).

Immunohistochemistry

For immunohistochemical staining of mammaglobin (clone 304-1A5, 1:200; DAKO, Carpinteria, Calif) and GCDFP-15 (clone D6, 1:200; Signet, Dedham, Mass), 5-μm-thick formalin-fixed sections from each paraffin block were routinely deparaffinized. The sections were exposed to 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, and then washed in deionized water for 2 to 3 minutes. Then, for heat-induced epitope retrieval, the sections stained for mammaglobin were subjected to a 0.02M concentration of citrate buffer (pH 6.0) in a steamer at 120°C for 20 minutes. The sections were allowed to cool at room temperature for 40 minutes, and after rinsing with deionized water and washing in phosphate-buffered saline for 5 minutes, the slides were incubated with primary antibody for 1 hour at room temperature. Then the slides were washed in phosphate-buffered saline 3 times for 5 minutes each time. Subsequently, the slides were labeled with EnVision+/HRP system (DAKO). Diaminobenzidine was used as the chromogen, and Meyer hematoxylin as the counterstain.

Grading the intensity of immunostaining was performed using a sliding scale of 0 to 3+ according to the percentage of reactive cells (0 = <1%; 1+ = 1%–10%; 2+ = 26%–50%; 3+ = 51%–100%).

RESULTS

The results of the immunostains of mammaglobin and GCDFP-15 on each tumor are listed in Tables 1 and 2, respectively.

Breast Carcinoma Metastatic to the Lung

Of the 20 cases of breast carcinoma metastatic to the lung, mammaglobin (Figure 1, A and B) and GCDFP-15 (Figure 1, C and D) stained 10 cases (50.0%) and 9 cases (45.0%), respectively, with mammaglobin showing a

Table 2. Immunoreactivity of Gross Cystic Disease Fluid Protein 15						
	No. of Cases Examined	% Positive	Staining Area			
			0	1+	2+	3+
Breast carcinoma, metastasis to lung	20	45	11	5	4	0
Primary lung carcinoma	250	8.2	231	19		
Adenocarcinoma	100	15	85	15	0	0
Squamous cell carcinoma	60	0	60	0	0	0
Pleomorphic carcinoma	20	5	19	1	0	0
Carcinoid tumor	19	5.2	18	1	0	0
Large-cell neuroendocrine carcinoma	20	0	20	0	0	0
Small-cell carcinoma	15	0	15	0	0	0
Adenoid cystic carcinoma	11	0	11	0	0	0
Mucoepidermoid carcinoma	5	40	3	2	0	0
Malignant mesothelioma	13	0	13	0	0	0

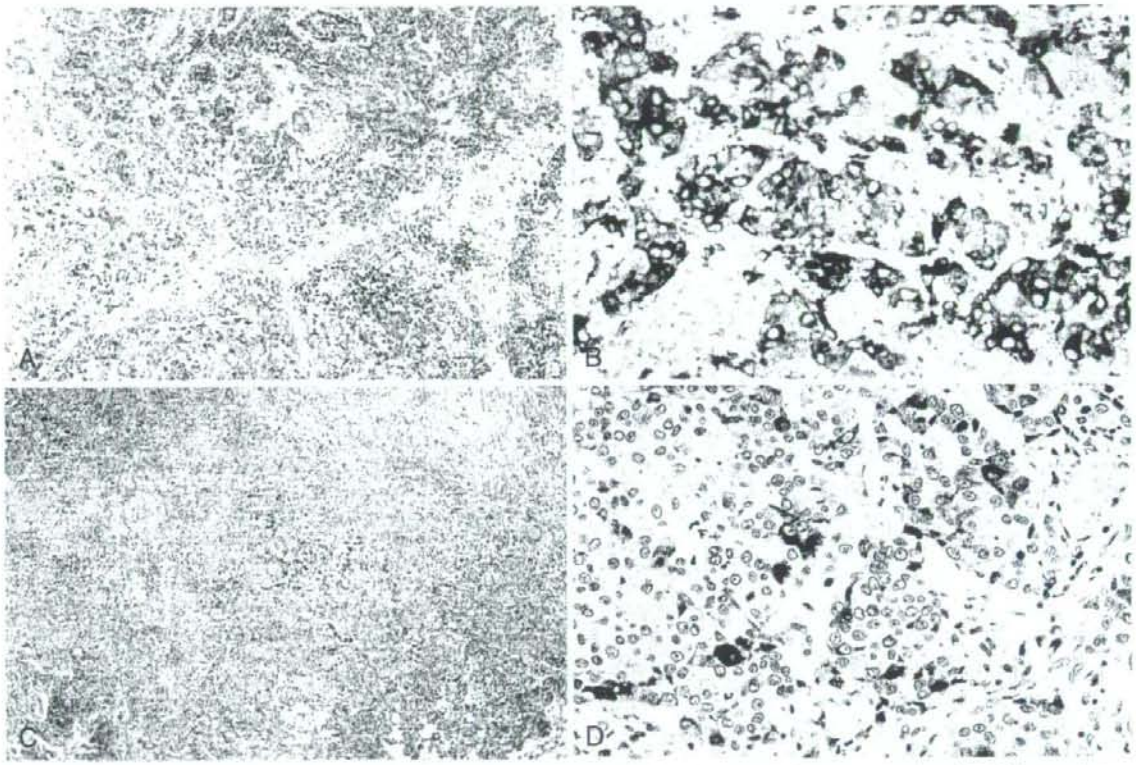

Figure 1. *Breast carcinoma metastatic to the lung, demonstrating solid growth. A to D represent the same case. A and B, Diffuse and strong staining for mammaglobin (original magnifications ×2 [A] and ×20 [B]). C and D, Focal but strong staining for gross cystic disease fluid protein 15 (original magnifications ×2 [C] and ×20 [D]).*

slightly higher frequency of immunostaining than GCDFP-15. Furthermore, mammaglobin showed diffuse immunoreactivity in 60% of immunopositive cases, whereas diffuse immunoreactivity of GCDFP-15 was observed in 44% of all immunopositive cases of breast carcinoma metastatic to the lung. As positivity for either marker was identified in 13 cases, a combination of mammaglobin and GCDFP-15 as a panel raised the sensitivity for identifying breast carcinoma metastatic to the lung to 65%.

The specificity of mammaglobin for detecting breast carcinoma metastatic to the lung (3/263 cases; 98.9%) was higher than that of GCDFP-15 (19/263 cases; 92.8%).

Primary Lung and Pleural Tumors

Primary Lung Adenocarcinomas.—All primary lung adenocarcinomas were immunonegative for mammaglobin. However, GCDFP-15 expression was observed in 15 (15%) of 100 cases of primary lung adenocarcinoma. The lepidic growth pattern was positive in 12 (20%) of 60 cases, and the nonlepidic growth type was positive in 3 (7.5%) of 40 cases. In the lepidic growth type, the non-

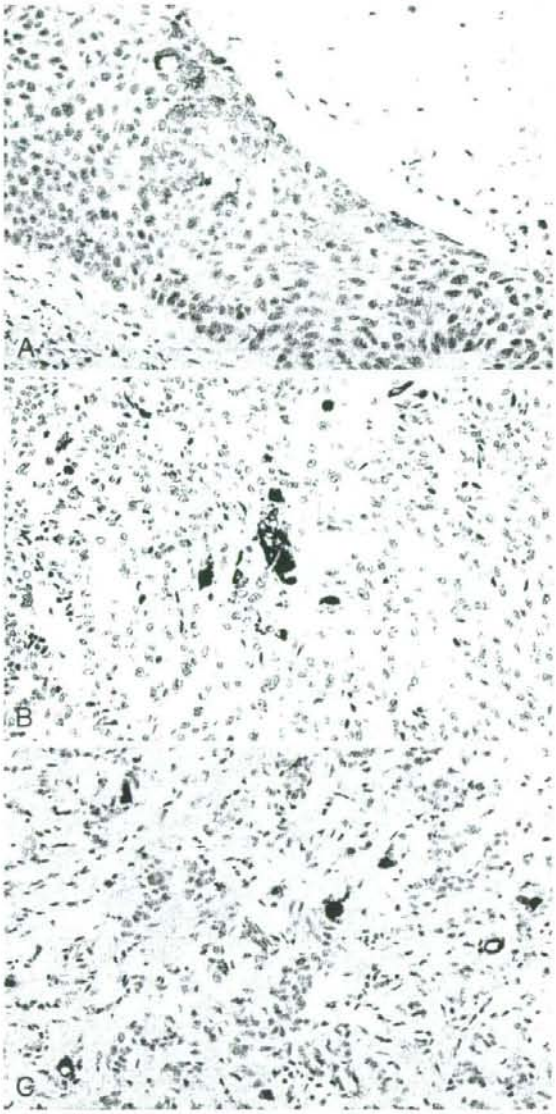

Figure 2. *A, Focal but strong staining for mammaglobin was evident in moderately differentiated squamous cell carcinoma of the lung (original magnification ×20). B, Focal but strong staining for mammaglobin was evident in carcinoid of the lung (original magnification ×20). C, Focal but strong staining for mammaglobin was evident in adenoid cystic carcinoma. Positivity was observed on the luminal side of the cytoplasm of true ductal cells (original magnification ×20).*

mucinous type was positive in 2 (10.0%) of 20 cases, and the mucinous type was positive in 10 (25%) of 40 cases. In the nonlepidic growth type, the acinar-cribriform type was positive in 3 (15.0%) of 20 cases, and the solid type was negative in all cases.

Squamous Cell Carcinomas.—Mammaglobin expression was observed in only one case of moderately differentiated squamous cell carcinoma, but this positive area was restricted to a small part of the tumor (Figure 2, A). All other squamous cell carcinomas were negative for mammaglobin. All squamous cell carcinomas were immunonegative for GCDFP-15.

Pleomorphic Carcinoma.—In pleomorphic carcinomas, expression of GCDFP-15 was observed in 1 (5.0%) of 20 cases, whereas all cases were immunonegative for mammaglobin.

Neuroendocrine Tumors.—Expression of mammaglobin was observed in 1 (5.2%) of the 19 carcinoid tumors, but positivity was limited to a small area (Figure 2, B). Furthermore, expression of GCDFP-15 was observed in 1 different case (5.2%), but the positivity again was limited to a small area (Figure 2, C).

All high-grade neuroendocrine tumors (large-cell neuroendocrine carcinoma and small-cell carcinoma) were immunonegative for both mammaglobin and GCDFP-15.

Salivary Gland–Type Tumors (Mucoepidermoid Carcinoma and Adenoid Cystic Carcinoma) of the Bronchus and/or Trachea

In mucoepidermoid carcinomas, expression of GCDFP-15 was observed in 2 (40.0%) of 5 cases, whereas all cases were immunonegative for mammaglobin. In adenoid cystic carcinoma, 1 (9.1%) of 11 cases was immunopositive for mammaglobin, whereas all cases were immunonegative for GCDFP-15. Mammaglobin (Figure 2, C) and GCDFP-15 showed focal staining.

Malignant Mesotheliomas

All malignant mesotheliomas were immunonegative for both mammaglobin and GCDFP-15.

COMMENT

In the present study, we demonstrated 3 advantages of using mammaglobin over GCDFP-15 to identify breast carcinoma metastatic to the lung. The first advantage is that the sensitivity of mammaglobin is slightly higher than that of GCDFP-15. The mammaglobin positivity rate for primary breast cancer is reported to be between 47.9%[19] and 71%.[20] Although we focused on analyzing breast carcinoma metastatic to the lung, the overall prevalence of mammaglobin expression in our series (50%) is almost accordant with the findings of previous reports[19,20] on primary breast cancer. The expression status of some molecules may be altered between primary and metastatic lesions. For example, the expression of surfactant apoprotein in lung cancer is frequently reduced in metastatic sites.[21] However, a previous report has demonstrated that the concordance rate of mammaglobin expression between the primary site and lymph node metastases was 93%.[20] These findings indicate that mammaglobin expression is not altered in the metastatic lesion.

Several studies have analyzed the mammaglobin expression pattern in breast carcinoma using immunohistochemical methods. Two of three studies used a noncommercial antibody[15] or a cocktail of antibodies[20] to identify mammaglobin, and the positivity rate in primary breast carcinoma was around 70%. However, Sasaki et al[19] reported that the positivity rate for mammaglobin in primary breast carcinoma analyzed by commercially available monoclonal antibody (clone 304-1A5) was lower than that using a noncommercial antibody or a cocktail of antibodies. These findings suggest that commercially available monoclonal antibody has a lower sensitivity and that there might be differences in the patient population. According to analyses by histologic type, mammaglobin ex-

pression was reported to be higher in lobular carcinoma than in ductal carcinoma.[19,20] Another study has suggested that mammaglobin expression is evident mainly in well-differentiated hormone receptor–positive breast carcinomas.

The second advantage of using mammaglobin is that the immunopositive area shows more diffuse staining than that of GCDFP-15. Similar findings have also been reported by Bhargava et al.[20] These findings indicate that examination of mammaglobin expression would be advantageous when the diagnosis is based on a limited sample, such as biopsy material. However, as cases that are immunopositive for mammaglobin and GCDFP-15 are partly exclusive, the combined use of both markers is important.

The third advantage of using mammaglobin is that its expression was found in only 1.1% of nonbreast tumors of the lung and pleura. One carcinoid tumor, 1 squamous cell carcinoma, 1 adenoid cystic carcinoma of the trachea, and none of the primary lung adenocarcinomas were positive. In primary lung adenocarcinoma, about 30% of cases demonstrated a nonlepidic growth pattern.[22] In particular, the cribriform and/or acinar and solid type might be confused with a lung metastasis from breast cancer. In the present study, we demonstrated that mammaglobin was negative in the cribriform and/or acinar and solid types of primary lung adenocarcinoma. The mammaglobin expression rate in lung tumors is reported to range from 0% to 16.7%.[13,19,20,23] Mammaglobin expression was reported to have been found in 20% of salivary gland tumors.[15,20] Therefore, the finding that adenoid cystic carcinoma of the trachea showed immunoreactivity for mammaglobin is not surprising but does require attention.

The differential diagnosis of malignant effusion involving the serosal membrane may be difficult. In the present study, all malignant mesotheliomas were immunonegative for mammaglobin. These findings indicated that mammaglobin should be added as one of the mesothelioma-negative markers, especially in female patients and/or cases of peritoneal mesothelioma.

Thyroid transcription factor 1 (TTF-1) has been shown to play a crucial role in the morphogenesis and function of the lung by regulating gene expression of surfactant proteins.[24] Most studies have reported finding TTF-1 expression in more than 70% of primary adenocarcinomas of the lung.[25] Therefore, TTF-1 has been considered a reliable marker to distinguish between primary lung adenocarcinoma and metastatic adenocarcinoma. It is reasonable that TTF-1 should be added to the antibody panel as a negative marker for metastatic tumors.

In conclusion, we demonstrated that the sensitivity of mammaglobin is equal or superior to that of GCDFP-15 for investigation of breast carcinoma metastatic to the lung. Immunopositivity for mammaglobin is more diffuse than that for GCDFP-15. In terms of practical diagnosis, mammaglobin immunopositivity can serve as a differential marker of breast carcinoma and should be added to immunohistochemical panels.

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SHORT COMMUNICATION

Mucoepidermoid carcinoma of the lung: High-resolution CT and histopathologic findings in five cases

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Summary
Objective: The purpose of this study was to characterize the high-resolution computed tomography (HRCT) findings of mucoepidermoid carcinoma of the lung and correlate them with the histopathological features.
Methods: The study included five patients with pathologically proven mucoepidermoid carcinoma who underwent HRCT before treatment. The HRCT findings were then compared with the histopathological features in all patients.
Results: The HRCT images showed lesions in the central lung in four patients and in the peripheral lung in one. All the lesions were well defined nodules or masses with a smooth margin. The contour of the tumours was oval ($n=3$), round ($n=1$) or lobulated ($n=1$). The contrast-enhanced CT images showed marked heterogeneous enhancement with foci of relatively low attenuation in four of the five lesions and mild heterogeneous enhancement in the other lesion. There was an admixed distribution of areas that are heterogeneous in the densities of blood vessels, as highlighted by immunohistochemical staining of CD31. Most mucin-secreting areas of the tumours showed more densely distributed blood vessels, mostly capillaries, in between tumour cell nests, whereas other areas did less. All five patients in our series underwent lobectomy plus lymph node dissection or sampling. All the patients are alive without evidence of disease an average of 50.4 months after surgery (range, 15–82 months; median, 57 months).

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Conclusion: Mucoepidermoid carcinoma of the bronchus is often visualized as marked heterogeneous contrast enhancement on HRCT images. The results of this study suggest that the presence of abundant microvessels, detected immunohistochemically by microscopic examination, affects the enhancement pattern on HRCT.

1. Introduction

Mucoepidermoid carcinoma of the lung is an extremely rare tumour, comprising less than 5% of primary bronchial tumours and 0.1–0.2% of all lung cancers [1–4]. The largest series (56 cases over 26 years) has been published by Yousem and Hochholzer [3]. These tumours are thought to originate from bronchial gland of minor salivary gland-type lining the bronchi, and are classified into low grade and high grade on the basis of histological criteria [1,3,5]. The most important factors in the prognosis include the histological grade and whether complete surgical resection is possible. Completely resectable low-grade tumours generally have an excellent prognosis [3,6].

The radiological appearance of mucoepidermoid carcinoma of the lung depends on tumour location, size and whether obstructive pneumonia is present. The reported computed tomographic (CT) appearance of mucoepidermoid carcinoma of the lung is a well circumscribed oval or lobulated mass arising within the bronchus [7]. Although some investigators have reported the CT features of this tumour [7–10], few reports have included detailed findings of high-resolution CT (HRCT) or correlated them with histopathologic features. The purpose of this study was to characterize the HRCT findings of mucoepidermoid carcinoma of the lung and correlate them with the histopathologic features.

2. Materials and methods

The patients investigated in this study presented at the National Cancer Center, Tokyo, Japan, for diagnosis and treatment during the period from January 1999 through December 2005. Only patients with primary mucoepidermoid carcinoma of the lung were included; patients with pulmonary metastasis from remote sites were excluded. Five patients underwent HRCT and were treated for primary mucoepidermoid carcinoma. The diagnosis was confirmed by histopathologic examination of the surgical specimen in all five patients. All clinical records, including the follow-up information, HRCT findings, endoscopic images and gross and microscopic specimens, were reviewed retrospectively.

2.1. HRCT protocols

HRCT was performed with either a 4-row or 16-row multi-detector CT (MDCT) scanner (Aquilion V-detector, Toshiba Medical Systems Corp., Tokyo Japan). The patients were evaluated with the MDCT scanner by using axial 2.0 mm × 4 mm or 16 modes, 120 kVp, 200–250 mA, and thin-section CT images were obtained using 1.0 mm sections reconstructed at 2.0 mm intervals with a high-spatial-frequency algorithm and retrospectively retargeted to each lung with a 20 cm field of view (FOV). All patients were intravenously injected with 80–150 ml of non-ionic contrast medium at a rate of 2.0–3.0 ml/s with an autoinjector (Autoenhance A-250, Nemoto Kyorindo, Tokyo, Japan), and scanning was started after a 40 s delay. Hard-copy images were photographed at window settings for the lung (center, −600 HU; width, 2000 HU) and the mediastinum (center, 35 HU; width, 400 HU). The intervals between the CT examinations and surgery ranged from 2 days to 4 weeks. All patients were followed up regularly in our institute. Follow-up CT images were obtained in all patients.

The HRCT images were assessed by two independent observers without reference to the clinical findings. The location of the pulmonary nodule was classified as peripheral or central. Nodules present within the peripheral two-thirds of the lung were arbitrarily classified as peripheral type and those within the central one-third or in contact with lobar or segmental bronchi were classified as central. The CT analysis included determination of the attenuation coefficient of the pulmonary lesion. CT attenuation coefficient was evaluated before and after administration of contrast media. The contrast enhancement of the tumour was compared with that of the chest wall musculature. Whether intratumoral calcification was present was also noted. After making independent initial evaluations, the two observers reviewed all cases in which their interpretations differed and reached a final consensus.

2.2. Histopathologic examination

Surgical specimens were inflated and fixed by transpleural and transbronchial infusion with formalin. The specimens were sectioned transversely in the same planes as the HRCT images, stained with hematoxylin-eosin and immunostained for the endothelial marker CD31. One of the authors, an experienced pulmonary pathologist, reviewed the histopathologic findings. The characteristics of the tumours on the HRCT images were compared with the histopathologic findings.

3. Results

3.1. Clinical features

The clinical data are summarized in Table 1. The five patients (two males and three females) ranged in age from 22 to 58 years, and their average age was 41.6 years. Only two of them were smokers. Four of the patients complained of chronic symptoms, including cough, increased sputum production and episodic fevers. These symptoms were related to bronchial irritation, partial or complete bronchial obstruction and distal pneumonia. The remain-

Table 1 Clinical data of patients with mucoepidermoid carcinoma of the bronchus

Case	Age (year)	Sex	Symptom	Tumour location	Tumour site	Preoperative diagnosis
1	22	M	Cough, sputum	Central	Lt. LLB (B6)	Mucoepidermoid Ca.
2	40	W	Fever, chest pain	Central	Rt. MLB	Mucoepidermoid Ca.
3	58	W	Cough, sputum, fever	Central	Rt. BB (B9)	Non-typed malignant tumour
4	51	M	None	Peripheral	Rt. MLB (B4a)	No malignancy
5	37	W	Cough, sputum, fever	Central	Lt. UDB	No biopsy

Lt. LLB, Left lower lobe bronchus; Rt. BB, right basal bronchus; Rt. MLB, right middle lobe bronchus; Lt. UDB, left upper division bronchus.

ing patient was asymptomatic, and the lesion was detected during routine health examination.

The serum sialyl Lewis X-i antigen (SLX) values were high in all five cases. The serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) values were high in three cases. The serum cytokeratin fragment 19 (CYFRA 21-1), squamous cell carcinoma antigen (SCC), neuron specific enolase (NSE), progastrin-releasing peptide (pro-GRP) values were all within the normal range.

3.2. HRCT findings

On the CT images, the tumours ranged in diameter from 18 to 38 mm (mean, 28.4 mm) (Table 2). The lesions were located in the central lung in four cases and in the peripheral lung in one. All the lesions were well defined nodules or masses with a smooth margin (Fig. 1). The contour of the tumours was round ($n = 1$), oval ($n = 3$) or lobulated ($n = 1$). Non-enhanced CT scans revealed intratumoral punctate calcification in one of the five lesions (Case 1). CT findings suggestive of bronchial stenosis or obstruction were seen in all cases (distal obstructive pneumonia in four cases, distal bronchial dilation in four and atelectasis in three). Atelectasis with recurrent or non-resolving pneumonia was observed distal to the site of obstruction.

CT attenuation coefficients were evaluated before and after administration of contrast medium. Thus, the change of CT attenuation or the degree of contrast enhancement was described. CT images enhanced by intravenous contrast medium showed marked heterogeneous enhancement with foci of relatively low attenuation in four of the five lesions and mild heterogeneous enhancement in the other lesion. Measurement of Hounsfield unit (HU) data was possible in every patient. The attenuation coefficients of the four markedly enhanced tumours (range, 95–139 HU; mean, 118.5 HU) were much higher than those of the chest wall musculature (range, 48–68; mean, 61.3 HU), whereas that of the one mildly enhanced tumour was slightly higher than that of the chest wall musculature. The ratio of the attenuation coefficient of the tumour to that of the musculature in the mildly enhanced case was 1.5, whereas those of the markedly enhanced cases were much higher (range, 2.0–2.2) (Table 2). None of the patients had lymphadenopathy in the mediastinum, pulmonary hilum or around the bronchi, on the basis of the CT findings.

3.3. Bronchoscopic findings

Bronchoscopy was performed in all five cases and the tumours were easily visualized except the peripheral lesion. The tumours were located in the lobar or segmental bronchi and had filled the bronchial lumen. They were soft, polypoid with a sessile base and pink like the bronchial mucosa. Three of the tumours were covered by a highly vascular mucosa. Although bronchoscopic brushing or biopsy was performed in four cases, a preoperative diagnosis of mucoepidermoid carcinoma was made in only two of them. Bronchoscopy in the other two cases revealed a non-typed malignant tumour or non-diagnostic inflammatory cells.

3.4. Treatment

The treatment chosen for all patients was surgical resection, and the procedure consisted of routine lobectomy including right middle and lower lobectomy (Table 2). The surgical procedures resulted in tumour-free margins. Lymph node dissection or sampling of pulmonary hilar and mediastinal lymph nodes was performed in all cases.

3.5. Histopathologic findings

The histologic diagnosis was low-grade mucoepidermoid carcinoma in all five cases (Table 3). The central tumours

Table 2 HRCT findings of mucoepidermoid carcinoma of the bronchus in four patients

Case	Tumour size (mm)	Tumour margin	Tumour contour	Pattern of enhancement	Ratio of attenuation coefficient
1	38 × 35	Well defined (smooth)	Oval	Heterogeneous	2.1
2	26 × 18	Well defined (smooth)	Lobulated	Heterogeneous	1.5
3	34 × 22	Well defined (smooth)	Oval	Heterogeneous	2.1
4	24 × 24	Well defined (smooth)	Round	Heterogeneous	2
5	33 × 29	Well defined (smooth)	Oval	Heterogeneous	2.2

Ratio of attenuation coefficient: Ratio of the attenuation coefficient of the tumour to attenuation coefficient of the musculature

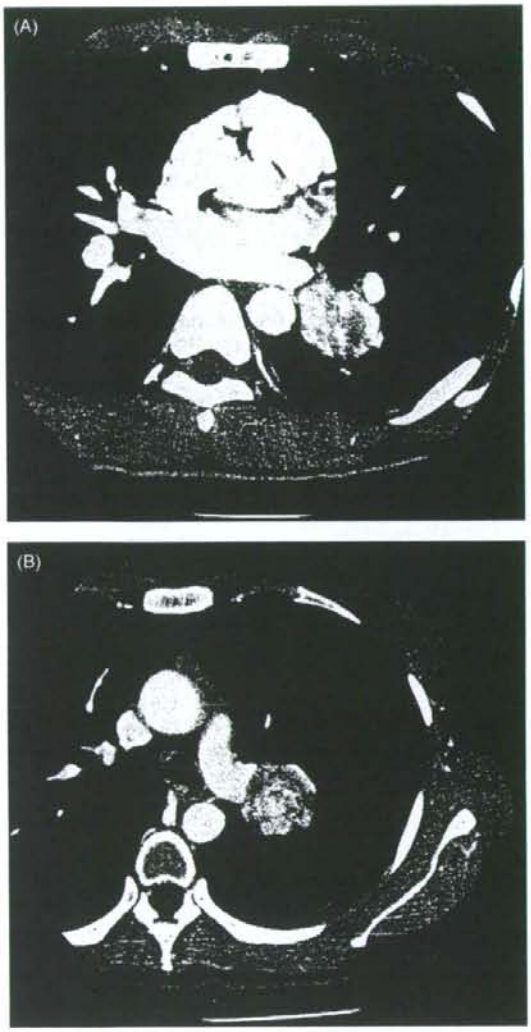

Fig. 1 Mucoepidermoid carcinomas of the bronchus were well defined mass and had a smooth margin. Enhanced CT images shows marked heterogeneous enhancement with foci of relatively low attenuation (A, Case 1; B, Case 5).

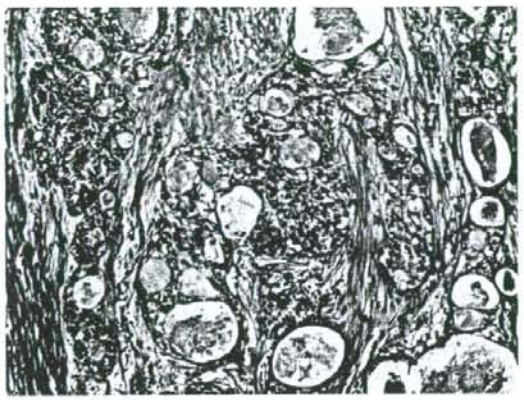

Fig. 2 High-magnification photomicrograph showed the epithelial component of the tumours consisted of mucin-secreting cells, squamoid cells and intermediate-type cells that displayed no specific differentiation.

protruded into the lumen of the bronchus and almost totally occluded it. On cut sections, the tumours were light yellow or tan polypoid masses. The margins and contours of the tumours were smooth, and they were well circumscribed and oval or round, consistent with their CT appearance.

Microscopically, the tumours were seen to arise from bronchial glands and to have infiltrated the bronchial wall. The epithelial component of the tumours consisted of mucin-secreting cells, squamoid cells and intermediate-type cells that displayed no specific differentiation (Fig. 2). Cystic change predominated in some areas, and the solid areas comprised mucin-secreting columnar epithelium that had formed small glands, tubules and cysts. There were no prominent nucleoli, and mitotic figures and necrosis were absent or minimal (less than five mitoses per 50 high-power fields). Keratinization was rare or absent in the epidermoid areas. These pathologic findings are characteristic of low-grade mucoepidermoid carcinoma. There was an admixed distribution of areas that are heterogeneous in the densities of blood vessels, as highlighted by immunohistochemical staining of CD31. Most mucin-secreting areas of the tumours showed more densely distributed blood vessels, mostly capillaries, in between tumour cell nests, whereas other areas did less (Fig. 3). Stromal calcification and ossification with a granulomatous reaction was observed in Case 1. The histologic specimens in Case 1, in which intratumoral punctate calcifications were observed on non-enhanced HRCT scans, showed microscopic calcification. Distal obstructive pneu-

Table 3 Histopathologic findings and outcome

Case	Treatment	p-Stage (TNM)	Grade	CD31	Outcome
1	Left lower lobectomy	T2N0M0 IB	Low-grade	(++)	NED
2	Right middle and lower lobectomy	T1N0M0 IA	Low-grade	(++)	NED
3	Right lower lobectomy	T2N0M0 IB	Low-grade	(+)	NED
4	Right middle lobectomy	T1N0M0 IA	Low-grade	(++)	NED
5	Light upper lobectomy	T2N0M0 IB	Low-grade	(++)	NED

NED: No evidence of disease.

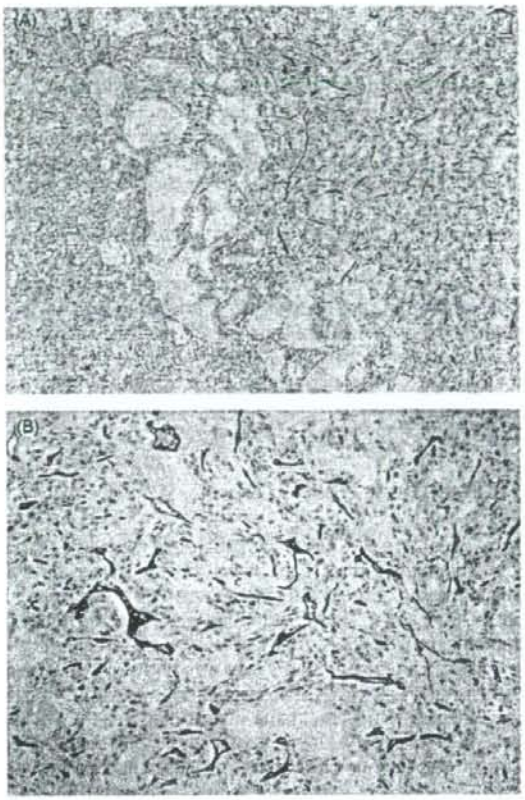

Fig. 3 (A) Immunohistochemical findings showing a border of areas with and without dense blood vessels as highlighted by anti-CD31 antibody. Note the abundant glands with mucus production in the right half, where there are more vessels labeled by anti-CD31 antibody. (B) Higher magnification of the area with plenty of mucin-secreting glands. Immunoreactive CD31 label the surface of endothelial cells, but not mucin of the tumour.

monia was observed in four patients with lesions of the central type, and distal bronchial dilation and mucoid impaction was seen in all five cases. The secondary findings associated with bronchial stenosis or obstruction on the HRCT scans largely reflected these pathologic findings. No lymph node metastasis was found in any of the surgical specimens.

4. Discussion

Mucoepidermoid carcinoma of the lung was first reported by Smetana et al. [11], and accounts for only a very small proportion of primary lung cancers. The tumours are classified as low grade or high grade based on their histologic appearance, and grading is based on cellular atypia, mitotic activity, local extension and tumour necrosis. Low-grade mucoepidermoid carcinoma is the most common type, and all five of the tumours in our series were low-grade. Although there is good clinicopathological evidence for the existence of the low-grade type, it has been questioned whether the high-grade type is a separate entity, mainly because of its histological similarity to mixed pulmonary carcinomas [5]. The high-grade variant is occasionally difficult to differentiate from adenosquamous carcinoma [12–14].

The most common symptoms are related to intraluminal growth, and these include persistent cough and sputum, wheezing, dyspnea, recurrent pneumonia, and, less frequently, hemoptysis [15]. Since the symptoms do not differ from those of other forms of lung tumour, they do not contribute to the differential diagnosis. Most patients with mucoepidermoid carcinoma are misdiagnosed as having bronchitis or lung carcinomas of other types. In our series, one patient was asymptomatic and the others had such similar symptoms that they were initially misdiagnosed as having chronic obstructive airway disease or other airway tumours.

Although the central-type tumours in our series were readily visible, only two of them were diagnosed preoperatively as mucoepidermoid carcinoma. Since mucoepidermoid carcinomas of the bronchi are usually covered by normal respiratory mucosa, bronchial brushing and lavage are seldom diagnostic, and it is better to perform a biopsy with forceps. Despite the theoretical risk of severe hemorrhage by performing a biopsy on a vascular mass, hemorrhage has never been reported as a complication of biopsy for mucoepidermoid carcinoma of the bronchus. Nevertheless, care is required because of the highly vascular nature of many of the tumours.

CT scan is non-invasive and useful for evaluating suspected endobronchial lesions, and fine morphological features have been revealed since the introduction of HRCT. In this series, HRCT images were essential for identifying the more detailed characteristics of the tumours, such as the margin, shape, density and pattern of enhancement. For the most part, the HRCT images reflected the pathologic features of the tumours well. There have been a few case reports of the HRCT appearance of mucoepidermoid carcinoma of the lung [7,10]. The HRCT features of the tumours in our series, such as a smooth margin and a well defined oval or round shape, were similar to those reported by Kim et al., who also found intratumoral punctate calcification on non-enhanced CT scans. Secondary findings associated with bronchial stenosis or obstruction, such as distal obstructive pneumonia, bronchial dilatation and atelectasis, were also seen. Although in their series Kim et al. reported mucoepidermoid carcinoma of the bronchus as showing mild contrast enhancement on CT scans, the three lesions of the central type and one lesion of the peripheral type in our series demonstrated marked contrast enhancement on HRCT images. The attenuation coefficients of the markedly enhanced tumours were much higher than those of the chest wall musculature.

Immunohistochemical staining for CD31 highlighted the heterogeneous distribution of blood vessels from mucin-secreting areas to non-secreting areas in a single tumour. In other words, these lesions may have characteristics of both hypervascular and hypovascular components, and the presence of both was probably the explanation for the features we observed on HRCT. The results of this study suggested that the presence of abundant microvessels, detected immunohistochemically by microscopic examination, affected the enhancement pattern on HRCT. These histopathologic findings correlated with the HRCT findings in all patients.

Bronchogenic carcinomas with more common histologic features, including adenocarcinoma, squamous cell carcinoma and small cell carcinoma have a variety of radiologic manifestations. Adenocarcinoma is often distinct from the other histologic subtypes of lung cancer. Non-solid nodules (ground glass opacities) and partly solid nodules (mixed solid/ground glass opacities) are recognized patterns of adenocarcinoma. Henschke et al. reported that the malignancies in subsolid nodules were typically bronchioloalveolar carcinomas or adenocarcinomas with bronchioloalveolar features, whereas in solid ones the malignancies were typically other subtypes of adenocarcinoma [16]. The proportion occupied by the non-solid component based on volumetric analysis by CT scan is a reliable predictor of tumours without vessel invasion in patients with adenocarcinoma of the lung [17]. Central squamous cell carcinoma is characterized by two major patterns of spread: intraepithelial spread with or without subepithelial invasion, and endobronchial polypoid growth. Polypoid tumours often occlude the bronchial lumen, resulting in atelectasis and obstructive pneumonia. Peripheral squamous cell carcinomas are seen as solid nodules, occasionally with cavitation and irregular margins. Approximately 90–95% of all small cell lung cancers are located centrally and show mediastinal or hilar lymphadenopathy with displacement or narrowing of the tracheobronchial tree or major vessels [18]. These common histologic types of lung cancer usually show mild or less contrast enhancement on CT images. Since these CT findings in common forms of lung carcinoma differ from those of mucoepidermoid carcinoma, which are relatively characteristic, contrast-enhanced CT may be helpful for lesion characterization and tumour classification in affected patients. If a marked heterogeneous contrast enhancement pattern is observed in well circumscribed oval or round masses of the bronchus, mucoepidermoid carcinoma can be considered in the differential diagnosis.

Large cell neuroendocrine carcinoma shows non-specific CT findings similar to those of other non-small cell lung cancer. On contrast-enhanced CT scans, tumour attenuation varies from slightly less to more than that of the chest wall muscle, with a homogeneous or heterogeneous pattern [18,19]. However, large cell neuroendocrine carcinoma is more likely to appear in the peripheral lung. Adenosquamous carcinoma of the peripheral type also usually shows heterogeneous soft-tissue attenuation [20]. Histopathologically, adenosquamous carcinoma is occasionally difficult to differentiate from high-grade mucoepidermoid carcinoma, which invades the pulmonary parenchyma in nearly 46% of the cases [3,12–14].

Pulmonary carcinoid tumours, which are low-grade malignancies accounting for 2–3% of all lung neoplasms [21], show CT findings similar to those of mucoepidermoid carcinoma. Pulmonary carcinoid tumours are also known to be vascular, and often show marked contrast enhancement on CT images [18,22]. Therefore, it is difficult to differentiate pulmonary carcinoid tumour from mucoepidermoid carcinoma on the basis of the CT contrast enhancement pattern alone.

Follow-up information was available for all five of the present cases. The clinical course of the patients was correlated with the histologic grade of their tumours. Low-grade mucoepidermoid carcinoma generally grows locally and is amenable to complete surgical resection. Low-grade tumours spread to regional lymph nodes by local growth in less than 5% of cases, and distant spread is rare [3,15]. The prognosis of low-grade tumours is usually excellent, with no evidence of local recurrence or metastasis. However, Barsky et al. [23] reported cases that were diagnosed as well differentiated and low-grade malignancy histologically but were rated as high-grade malignancy clinically. It can therefore be concluded that the histologic malignancy level of the tumour is not always the same as its clinical malignancy level. This suggests that complete surgical resection plus lymph node dissection should be performed for low-grade mucoepidermoid carcinoma of the bronchus as well as high-grade mucoepidermoid carcinoma. All five patients in our series underwent lobectomy plus lymph node dissection or sampling, and all are currently alive without evidence of disease at an average of 50.4 months after surgery (range, 15–82 months; median, 57 months).

5. Conclusions

We reviewed the HRCT and pathologic findings in five cases of mucoepidermoid carcinoma of the lung. Mucoepidermoid carcinoma is often visualized as marked heterogeneous contrast enhancement on HRCT images. The presence of abundant microvessels, detected immunohistochemically by microscopic examination, may affect the enhancement pattern on HRCT. However, examinations of HRCT images of mucoepidermoid carcinoma of the lung are insufficient because of the rarity of the tumour. The HRCT characteristics of the tumour must therefore be evaluated in more cases.

Conflict of interest

None declared.

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Association of KRAS polymorphisms with risk for lung adenocarcinoma accompanied by atypical adenomatous hyperplasias

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The *pulmonary adenoma susceptibility 1* (*Pas1*) gene affects susceptibility to the development of lung adenomas in mice with a subset of the adenomas progressing to adenocarcinoma (ADC). In this study, genotype distributions for 10 polymorphisms in the human counterparts for three mouse candidate *Pas1* genes, *KRAS*, *CASC1/LAS1* and *LRMP*, were examined in a hospital-based case–control study consisting of 364 lung ADC cases and 253 controls. All the ADC cases were subjected to lobectomy and subsequent pathological investigation of atypical adenomatous hyperplasia (AAH), a putative precursor for peripheral lung ADC, including bronchioloalveolar carcinoma, in the resected lobes. Eighty-one (22%) of the ADC cases carried at least one AAH lesion in addition to the primary ADC and 34 (9%) of them carried multiple AAH lesions. None of the 10 polymorphisms examined showed significant associations with overall lung ADC risk ($P > 0.05$). However, minor allele carriers for two polymorphisms in the *KRAS* gene, KRAS-1 and -6, showed significantly increased odds ratios (ORs) for ADC accompanied by multiple AAHs [OR = 3.0; 95% confidence interval (CI) = 1.4–6.2, $P = 0.004$ and OR = 2.4; 95% CI = 1.1–4.7, $P = 0.02$, respectively]. Minor haplotypes including the minor allele for the KRAS-6 polymorphism showed increased ORs for ADC accompanied by multiple AAHs, and *KRAS* transcripts from the minor allele for this polymorphism were more abundantly detected in lung tissues than those from the major allele. Thus, *KRAS* polymorphisms were indicated to be involved in risk for the development of AAHs that progress to ADC by causing differential *KRAS* oncogene expression in the lungs.

Introduction

Adenocarcinoma (ADC) is now the most common type of non-small cell lung carcinoma, followed by squamous cell carcinoma and small cell carcinoma (1,2). Development of lung ADC is less associated with smoking compared with squamous cell carcinoma and small cell carcinoma. Thus, effective ways of preventing ADC are being searched for (1,2). Identification of genes responsible for susceptibilit to lung ADC is considered to be indispensable to establish novel an efficient ways of preventing the disease. However, only a few meta bolic and DNA repair genes, such as *CYP1A1* and *OGG1*, have bee shown to be associated with risk for lung ADC (1,3–6), and therefor genes responsible for lung ADC susceptibility are largely unknown

Inbred strains of mice exhibit a difference in their susceptibilitie to both spontaneous and carcinogen-induced lung adenoma devel opment (7). To date, dozens of genetic loci have been shown to b linked to mouse lung adenoma susceptibility (*Las*) through linkag analyses, including pulmonary adenoma susceptibility (*Pas*), pulmo nary adenoma resistance and susceptibility to lung cancer (8–12 Recently, we reported that a non-synonymous (associated wit amino acid change) single-nucleotide polymorphism (SNP) in *PG L1*, the human counterpart for a candidate pulmonary adenoma re sistance 2 gene, was associated with lung ADC risk (13). Thus, i was suggested that human counterparts for mouse *Las* genes als play a role in human lung ADC risk. *Pas1* is a major locus account ing for ~50% of variances in *Las* in mice, and three genes of Kirste rat sarcoma oncogene (*Kras*) 2, cancer susceptibility candidat (*Casc*) 1/*Las1* and lymphoid-restricted membrane protein (*Lrmp* have been identified from this locus as strong candidates determin ing the susceptibility (11,14–18). A case–control study of lung AD in an Italian population showed that a SNP in the *KRAS* gen (KRAS-1 in Table I) was associated with lung ADC risk (19); how ever, the association was not reproduced in the following studie (20,21). SNPs in the *LRMP* and *CASC1* genes did not show associ ations with lung ADC risk in a recent study (22). Instead, the abov SNP in *KRAS* and a SNP in *LRMP* (LRMP-6 in Table I) showe associations with prognosis of lung ADC patients (19,20,22). Thu it is still controversial how the human counterparts for the mous *Pas1* genes are involved in the development and progression of lun ADC in humans.

The mouse *Pas1* gene affects susceptibility to lung adenoma de velopments in mice, and a subset of the adenomas progress to AD that is analogous to bronchioloalveolar carcinoma (BAC) in human (7). Thus, it is possible that variations in human counterparts fo *Pas1* are also involved in susceptibility to the development of lun adenoma and/or BAC (18). Atypical adenomatous hyperplasi (AAH) is a lesion with a monoclonal nature (23) and has bee considered as a precursor for peripheral lung ADC, includin BAC, the adenoma in an adenoma–carcinoma sequence in the pe ripheral lung (24). AAH is a frequent incidental histologic finding i lungs bearing primary lung ADC (24,25). Such an accompanimer of AAH was detected in 16–35% of ADC cases. AAH was als detected in the lungs of autopsy cases without cancer by histologic examination. In two studies, AAH was examined in hospital autops cases and was detected in 2.0 and 3.4% of the cases without cance respectively (26,27). In another study, administration autopsy case were examined to estimate the prevalence of AAH in the gener population, and AAH was detected in 2.8% of the cases (28). There fore, AAH is indicated to be present in the lungs of individual without cancer; however, the frequency of having AAH in thos individuals is considerably lower than that in ADC patients (24 The results indicate that the susceptibility to the development c AAH is also associated with that of lung ADC in humans, an therefore, the lungs of humans with primary ADCs accompanie by AAH, particularly those accompanied by multiple AAHs, ar analogous to those of mice with the susceptible *Pas1* allele. Thu in the present study, polymorphisms of the *KRAS*, *CASC1* and *LRM* genes were examined for association with risk for the developmer of lung ADC after subclassification of the subjects according t AAH accompaniment and BAC component involvement.

Abbreviations: AAH, atypical adenomatous hyperplasia; ADC, adenocarcinoma; BAC, bronchioloalveolar carcinoma; Casc, cancer susceptibility candidate; cDNA, complementary DNA; CI, confidence interval; *Kras*, Kirsten rat sarcoma oncogene; *Las*, lung adenoma susceptibility; LD, linkage disequilibrium; LRMP, lymphoid-restricted membrane protein; OR, odds ratio; *Pas1*, pulmonary adenoma susceptibility 1; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

Table I. Ten polymorphisms in the KRAS, CASC1 and LRMP genes, and primers and conditions for pyrosequencing

Polymorphism	Accession no. in dbSNP	Nucleotide substitution[a]	Location	Amino acid change	PCR			Sequencing
					Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Annealing temperature (°C)	Primer
KRAS								
12	rs2955407	A/G	Gene upstream		AACATGGGGAATTTGGCTTT	TTCACAGGCACTTCATTCTCA	63	TTGGACAGGCATTTG
1[b]	rs11836509	A/C	Intron 3		GAGTCTTTGCTAATGCCATGC	CACTGCTCTAATCCCCCAAG	61	TGCCATGCATATAATATTT
6	-/T		Exon 4b (3′-UTR)		GGCCATACTTCAGGAACTGC	TTTGTTAAACCATTCAAAGTTCACA	63	GGGCATTTTTTTAAGGTAG
CASC1								
1[b]	rs10842501	A/G	Intron 1		AACTTTTAGGGTCCCAAAGC	CTGTTCATACACTGTAAGTG	55	GTTCATACACTGTAAGTGAT
4[b]	rs10842496	C/A	Exon 3	Arg33Ser	AAATTATTTTGGTTCTTTAGAGGAAG	GCTTCAAGTCGATGCCATTT	55	GGTTCTTTAGAGGAAGCC
5	rs2352782	T/C	Intron 8		AAGAAGACCTTTCGTACTTACCAG	TGGGGTATAACCCCTTGTCA	61	TCGTACTTACCAGTGCAT
LRMP								
7[b]	rs1908946	C/G	Exon 13	Ser197Cys	AGAGAGGTCCCGTGACTTGG	CCCAAATGTTCTGATTCTCACC	61	ATTTGAAGAAAGAAATCACT
6[b]	rs7969931	G/C	Exon 11	Val141Leu	GTGGTTTCCCCTCTTCCTGT	CATGCTACATGAAGAAGCACTTA	61	TTACTTCTCACTTTGTCTAA
4	rs2291801	C/T	Intron 5		AGTTGGTGCGTGCTGGTTAT	TCCACATTTGGGCTCTTTTC	61	TGCTGGTTTAAATAGAGG
1	rs1497259	G/A	Intron 1		CTTCACGGCCCAATAGATTC	TTTGGGCAGCAAAAGATACA	61	GGCAGCAAAAGATACAA

[a]Shown in the direction of genes.
[b]Association with lung ADC risk was examined in previous studies.

Materials and methods

Case and control subjects

All cases and controls were Japanese. The cases consisted of 364 ADC patients treated at the National Cancer Center Hospital, Tokyo. All ADC cases, who received lobectomies from 1999 to 2004 and from whom informed consents as well as blood samples were obtained, were consecutively included in this study without any particular exclusion criteria. All the cases were diagnosed as ADC by histological examinations according to the World Health Organization classification (24,29). Information on the AAH in the ADC patients was obtained by routine surgical pathology examination as follows. Resected lungs were inflated with 10% formalin through bronchial cut ends and after fixation for a few days were serially sliced at intervals of 5 mm, and each cut surface was macroscopically examined. Sliced lungs containing a lesions suspected for AAH were further examined microscopically. Even in cases without macroscopic lesions, at least one tissue block was prepared from all sliced lungs and subjected to microscopic examination. The criteria for AAH were as follows as described previously (27,28): (i) a localized lesion with well-defined boundaries; (ii) an alveolar wall slightly thickened with mild infiltration of inflammatory cells but without scar formation; (iii) proliferating atypical epithelial cells abutting each other but not as compact as in ADC; (iv) atypical epithelial cells that were cuboidal to low columnar or peg shaped in appearance, resembling either type II pneumocytes or non-ciliated bronchiolar epithelial cells (Clara cells) and (v) the presence of some atypical cells with two or more nuclei, most of which had relatively smaller and smoother contours than those of ADC. These criteria are compatible with those described in the reference of World Health Organization classification of lung tumors as a proposal (24,30). The controls consisted of cancer-free patients of National Cancer Center Hospital. All the control subjects were selected with a criterion of no history of cancer. Smoking history of cases and controls was obtained via interview using a questionnaire. Smoking habit was expressed by pack-years, which was defined as the number of cigarette packs smoked daily multiplied by years of smoking, both in current smokers and former smokers. Smokers were defined as those who had smoked regularly for 12 months or longer at any time in their life, whereas non-smokers were defined as those who had not. From each individual, a 20 ml whole-blood sample was obtained. The study was approved by the Institutional Review Boards of the National Cancer Center.

DNA extraction, polymorphism search and genotyping

Genomic DNAs were isolated from whole-blood samples using a QIAamp DNA Blood Maxi kit (Qiagen, Tokyo, Japan). DNAs from 24 lung ADC cases and 24 controls, respectively, were subjected to a search for polymorphisms in exons of the *KRAS*, *CASC1* and *LRMP* genes by resequencing according to the procedure described previously (13). SNPs in introns of these three genes with minor allele frequencies >0.1 in the Japanese population were selected from SNPs deposited in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Genotyping was performed with 10 ng of genomic DNA by the pyrosequencing method according to the procedure described previously (13).

Statistical analysis

Hardy–Weinberg equilibrium tests were performed using the SNPAlyze version 3 software (DYNACOM Co., Ltd, Chiba, Japna). SNPs with a P value for deviation >0.01 were considered to be in Hardy–Weinberg equilibrium. Calculation of the D' values and haplotype estimation were undertaken using the expectation-maximization (EM) algorithm using the same software. The strength of association of genotypes with ADC risks was measured as crude odds ratios (ORs) and ORs adjusted for gender, age (<49, 50–59, 60–69 and >70) and smoking dosage (pack-years: 0, 1–49 and >50) with 95% confidence intervals (CIs) by unconditional logistic regression analysis (31). Statistical analyses were performed using the JMP version 6.0 software (SAS Institute, Cary, NC). ORs for carrying a copy of a haplotype were also calculated by the bootstrap method with 5000 resampling. The statistical analyses were performed using the SAS version 9 software (SAS Institute). Statistically, a level of $P < 0.05$ for an OR was considered significant, whereas a level of $0.05 \leq P < 0.10$ for an OR was considered marginally significant.

Analysis of KRAS transcripts

Genomic DNA and total RNA were extracted from eight non-cancerous lung tissues of eight lung ADC patients heterozygous for an insertion–deletion polymorphism, KRAS-6 (see Table I). Complementary DNA (cDNA) was synthesized by reverse transcription of 1 mg of total RNA using the Superscript First-Strand Synthesis System. Ten nanograms of genomic DNA and cDNA corresponding to 50 ng of total RNA were subjected to polymerase chain reaction (PCR) in duplicate. The PCR was performed using a set of primers, 5′-CAGGAACTGCAGTGCTTATG-3′ and 5′-TTAAGGCTGTAATAATTAGGTAAC-3′ (fluorescein isothiocyanate labeled), for 30 cycles consisting of

denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR products were electrophoresed using an ABI PRISM 3700 Genetic Analyzer and analyzed by Gene Scan software (Applied Biosystems, Foster City, CA). Ratios of the minor allele (i.e. T allele) to the major allele (i.e. – allele) products in each sample were calculated from the height of peaks corresponding to the minor and major alleles, respectively. The ratio for each sample was expressed by the mean ratios of the two independent PCRs. The difference in the mean ratios between genomic DNA and cDNA in eight cases was tested by the paired *t*-test.

Results

We searched for polymorphisms located in exons of the *KRAS*, *CASC1* and *LRMP* genes by the resequencing of their exons in 48 Japanese individuals, and identified a 1 bp insertion–deletion polymorphism in the *KRAS* gene and three non-synonymous SNPs in the *CASC1* and *LRMP* genes (Table I). The insertion–deletion polymorphism was novel, while the three SNPs had been deposited in the dbSNP database. Six other SNPs in introns of these three genes whose minor allele frequencies were >0.1 in the Japanese population were selected from SNPs deposited in the dbSNP database (Table I). In total, 10 polymorphisms dispersed in the *KRAS*, *CASC1* and *LRMP* genes were selected for the present study (Figure 1).

We prepared 364 ADC cases and 253 hospital-based controls (Table II). The ADC cases received lobectomies at National Cancer Center Hospital and were subjected to the pathological search for AAH in the resected lobes. In the lobes, one or more (i.e. multiple) AAH lesions were detected in 81 cases (22%), while no AAH lesion was detected in the remaining 283 cases (78%). The frequency of AAH accompaniment in these ADC cases was consistent with those in previous studies (24). Representative microphotographs of multiple AAH lesions detected in an ADC patient are shown in supplementary Figure 1 (available at *Carcinogenesis* Online). In 34 of the 81 cases (9%), 2 or more (i.e. multiple) AAH lesions were detected. The 364 ADC cases included 173 cases of small-sized ADC (i.e. <2 cm in maximum diameter), and the information on the presence of BAC components in the tumor was available (Table II). One hundred and fifty-two cases (88%) contained BAC components in the tumor, whereas the remaining 21 cases (12%) did not. It was difficult to histologically define AAHs in primary ADC lesions, even in ADCs with BAC components. Thus, it was not possible to pick up cases of ADCs in AAH lesions, so-called 'carcinoma in adenoma'. Accordingly, cases with AAHs were defined as having AAHs that existed independently from primary ADCs in the present study.

All the cases and controls were subjected to genotyping for the 10 polymorphisms, and all the polymorphisms were in Hardy–Weinberg equilibrium both in the cases and controls. Genotypic differentiation for the 10 polymorphisms was examined between cases and controls. The differentiation was examined for all ADC cases as a whole and cases categorized by AAH accompaniment. The differentiation wa also examined for small-sized ADC cases with BAC components. Th number of small-sized ADC cases without BAC components wa small; therefore, they were excluded from the analysis. The differen tiation was also examined after dividing ADC subjects into smoker and non-smokers. To increase statistical power, genotypic differenti ation was examined by assessing OR of minor allele carriers agains homozygotes for the major allele (i.e. non-carriers).

Minor allele carriers for a SNP, KRAS-12, showed a marginall significant increase in the OR for the risk for overall ADC (P = 0.06 while none of the other nine polymorphisms showed significant in creases or decreases in the OR (Table III). When the ADC cases wer categorized by AAH accompaniment, ORs of minor allele carriers fc six polymorphisms, KRAS-1, -6, CASC-1, -4, -5 and LRMP-7 against non-carriers were higher for ADC with AAH than for tha without. The ORs were even higher for ADC with ≥2 AAHs. In creases in adjusted ORs for two of the six polymorphisms, KRAS- and -6, were statistically significant (OR = 3.0; 95% CI = 1.4–6. and 2.4; 95% CI = 1.1–5.1, respectively), and those for three othe polymorphisms, CASC-1, -4 and LRMP-7, were marginal (Table III

We next calculated ORs for the risk for small-sized ADC with BA components. Minor allele carriers for the KRAS-1 SNP, whic showed the most significantly increased OR of 1.3 for ADC wit ≥2 AAHs, showed a slightly increased OR against non-carriers fc the risk for ADC with BAC components, but the increase was nc statistically significant (P = 0.3) (Table III). ORs for the other nin polymorphisms were not significant, either. When the ADC case were divided into smokers and non-smokers, ORs were not apparentl different between them. Increases or decreases in ORs were not sig nificant, either.

Linkage disequilibrium (LD) among the 10 polymorphisms wa then estimated. Five polymorphisms, KRAS-1, -6, CASC1-1, -4 an -5, were in LD with one another ($D' > 0.9$) (Figure 1), and the size c the region with LD was 94 kb. Thus, the distribution of haplotype consisting of these five polymorphisms was evaluated in cases an controls (Table IV). Three haplotypes were deduced to compris ~97% of chromosomes among controls and cases. The major haplc type consisting of major alleles for all the five polymorphisms (i.e haplotype 1 in Table IV) was less prevalent in ADC cases with ≥ AAHs than in controls, whereas a minor haplotype consisting c minor alleles for the five polymorphisms (i.e. haplotype 2) was mor prevalent. Haplotype 3 was almost evenly distributed in cases an controls. Adjusted ORs for the risk for ADC accompanied by ≥ AAHs by carrying one copy of haplotypes 2 and 3 was calculate based on the estimated copy number of haplotypes for each subject b the bootstrap method. The ORs for haplotypes 2 and 3 were 2.6 (95% CI = 1.1–5.9, P = 0.02) and 1.8 (95% CI = 0.6–5.6, P = 0.3), re spectively. Therefore, both haplotypes showed increased ORs, whil only increase in the OR for haplotype 2 was statistically significant

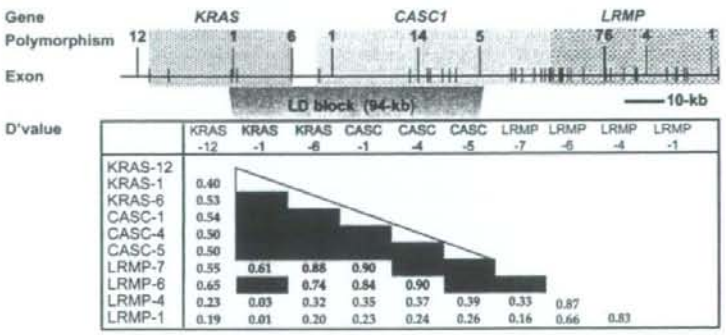

Fig. 1. Polymorphisms in the human *Pas1* locus and their LD. Ten polymorphisms are shown on top, and D' values between the SNPs are shown below. D' values >0.9 are marked in red.

Table II. Lung ADC cases and controls used in this study

Subject and category	All	Sex (%)		Age	Smoking habit (%)[a]	
		Male	Female	(Mean ± SD)	Smoker	Non-smoker
Case	364 (100)	196 (54)	168 (54)	61 ± 10	174 (48)	189 (52)
AAH accompaniment						
Absent	283 (77)	157 (55)	126 (45)	62 ± 11	135 (48)	147 (52)
Present	81 (23)	39 (48)	42 (52)	61 ± 8	36 (44)	45 (56)
1 AAH	47 (14)	20 (43)	27 (57)	61 ± 8	22 (47)	25 (53)
≥2 AAHs	34 (9)	19 (56)	15 (44)	62 ± 7	14 (41)	20 (59)
Tumor size						
≤2 cm	173 (47)	94 (54)	79 (46)	61 ± 10	93 (54)	79 (45)
BAC component (+)	152 (41)	83 (55)	69 (45)	61 ± 10	85 (56)	66 (43)
BAC component (−)	21 (6)	11 (52)	10 (48)	61 ± 11	8 (38)	13 (62)
>2 cm	191 (53)	114 (60)	77 (40)	62 ± 10	110 (58)	81 (42)
Control	253 (100)	144 (57)	109 (43)	64 ± 11	134 (53)	117 (46)

[a]Data were not available for one case and two control subjects.

In mice, it was shown that *KRAS* transcripts from the susceptible *Pas1* allele in lung tissue are more abundant than those from the resistant allele, and such a difference was proposed to underlie the difference in the susceptibility to adenoma development (18). As described above, hapolytypes 2 and 3, which showed significantly and insignificantly increased ORs for ADC accompanied by multiple AAHs, respectively, contained a minor allele for KRAS-6, a 1 bp insertion–deletion polymorphism in the 3′-untranslated region (3′-UTR) of the *KRAS* gene. This result prompted us to compare the amounts of *KRAS* transcripts between susceptible and resistant alleles by using the KRAS-6 polymorphism. Genomic DNA and cDNA prepared from non-cancerous lung tissues of eight heterozygotes for this polymorphism were subjected to PCR using a set of primers that amplifies the same DNA/cDNA fragments encompassing the KRAS-6 site. Relative amounts of the susceptible (i.e. T) allele products to the resistant (i.e. −) allele products were greater in cDNA than in genomic DNA in all the eight cases (Figure 2). The mean for the ratio of the minor allele products to the major allele products in genomic DNA and cDNA was 1.02 and 1.13, respectively, and the difference was statistically significant ($P = 0.005$ by the paired *t*-test). Thus, it was indicated that transcripts from the KRAS-6 minor allele were more abundant than those from the major allele in lung tissues.

Discussion

In the present study, we examined the association of polymorphisms in the human counterparts for three mouse candidate *Pas1* genes with risk for lung ADC. None of the 10 polymorphisms examined showed significant associations with risk for lung ADC as a whole. This result was consistent with recent case–control studies (20,22). However, when ADC subjects were categorized according to AAH accompaniment, two polymorphisms, KRAS-1 and -6, showed associations with risk for ADC accompanied by multiple AAHs. Haplotypes containing the minor allele for the KRAS-6 polymorphism also showed increased ORs for ADC accompanied by multiple AAHs. The results strongly suggested that *KRAS* is a determinant for susceptibility to ADC accompanied by multiple AAHs in humans. Namely, individuals with minor alleles for these two *KRAS* polymorphisms could have a higher risk than those without for the development of lung tumors analogous to the tumors in mice with a susceptible *Pas1* allele; i.e. multiple adenomatous lesions (multiple AAHs) with a subset of lesions that have progressed to primary ADC. *KRAS* transcripts from the risk haplotypes were more abundant than those from the resistant haplotype. This result was also analogous to the status of the mouse *Pas1* locus (18). *KRAS/Kras* is an oncogene that is activated by somatic mutations in lung ADC both of humans and mice (32–35). Thus, an abundant *KRAS/Kras* expression due to polymorphisms might make the lung epithelial cells more susceptible to the development of adenomas, a subset of which progress to ADC, both in humans and mice.

KRAS minor allele carriers showed only a slightly increased and insignificant OR for the risk for development of lung ADC as a whole and even of lung ADC with BAC components, which is considered to develop from AAH. This result is in contrast to the finding in mice that *Pas1* has a major role in predisposition to not only lung adenoma but also lung ADC (36). The result may imply that *KRAS* polymorphisms are responsible for the development of AAH; however, their effects on the development of lung ADC, including BAC, are limited. In this context, however, we should consider the difference in the process of ADC development between mice and humans. In the studies of experimental mouse models, lung ADCs in mice could be exclusively developed through adenomas; therefore, *Pas1* might have been judged as having a role in the predisposition to not only lung adenoma but also lung ADC. In humans, in contrast, a subset of ADC, including BAC, may not be developed through AAH. This could be a reason why the *KRAS* minor allele has not been defined as a risk allele for lung ADC development in several studies, including this one. In fact, fractions of minor allele carriers for KRAS-1 and -6 polymorphisms in ADC cases with multiple AAHs were significantly higher than those in ADC cases without AAH (OR = 3.0; 95% CI = 1.4–6.2, $P = 0.004$ and OR = 2.2; 95% CI = 1.1-4.7, $P = 0.03$, respectively). This result further supports that *KRAS* polymorphisms are responsible for the development of AAH but not of lung ADC. In addition, progression of AAH to ADC in mice can be influenced by several other genetic factors, as indicated by the pulmonary adenoma progression loci, which determine the susceptibility to the progression of adenoma to ADC in the lungs (21,36). Thus, association of polymorphisms in the *KRAS* gene with risk for lung AAH and ADC should be further examined in a larger number of samples in conjunction with polymorphisms of human counterparts for such modifier loci for *Pas1*.

Two SNPs in the *KRAS* gene were in LD with three other SNPs in a nearby gene, *CASC1*. A haplotype consisting of minor alleles of these five polymorphisms were significantly associated with risk for ADC with multiple AAHs. Genotypes with minor alleles for polymorphisms of the *CASC1* gene also showed increased ORs for ADC accompanied by multiple AAHs, although they were not statistically significant. Thus, it was also suggested that *CASC1* polymorphisms could also be involved in the susceptibility to ADC with multiple AAHs. Similar results were also shown in mice; *Casc1* polymorphisms are in LD with *KRAS* polymorphisms, and *Casc1* polymorphisms also showed associations with lung adenoma risk (14,15,37). The *Casc1* gene has a polymorphism associated with amino acid substitution. Casc1 protein has a growth-suppressive activity on lung cancer cells, and the activity was indicated to be different between the polymorphic proteins (15). However, differential expression of *Casc1* was not observed between polymorphic alleles (18). Thus, it is

Table III. KRAS, CASC1 and LRMP genotypes and risks for ADC

SNP	Genotype	Control	Case												
			All			AAH accompaniment									
						Absent			Present						
									All			1 AAH			≥2 AAHs		
		No. (%)	No. (%)	OR[a]	*P*	No. (%)	OR[a]	*P*	No. (%)	OR[a]	*P*	No. (%)	OR[a]	*P*	No. (%)	OR[a]	*P*
KRAS-12	A/A	85 (34)	99 (27)	Ref.		78 (28)	Ref.		21 (26)	Ref.		12 (26)	Ref.		9 (26)	Ref.	
	A/G + G/G	168 (66)	265 (7)	1.4	0.06	205 (72)	1.4	0.09	60 (74)	1.4	0.3	35 (74)	1.3	0.4	25 (74)	1.4	0.4
KRAS-1	A/A	203 (80)	279 (7)	Ref.		224 (79)	Ref.		55 (68)	Ref.		36 (77)	Ref.		19 (56)	Ref.	
	A/C + C/C	50 (20)	85 (23)	1.2	0.4	59 (21)	1.1	0.8	26 (32)	1.8	0.04	11 (23)	1.2	0.7	15 (44)	3.0	0.004
KRAS-6	-/-	163 (64)	217 (60)	Ref.		171 (61)	Ref.		43 (53)	Ref.		29 (62)	Ref.		14 (41)	Ref.	
	-/T + T/T	90 (36)	147 (40)	1.2	0.4	109 (39)	1.1	0.7	38 (47)	1.5	0.1	18 (38)	1.0	0.9	20 (59)	2.4	0.02
CASC1-1	A/A	161 (64)	211 (58)	Ref.		169 (60)	Ref.		42 (52)	Ref.		27 (59)	Ref.		15 (44)	Ref.	
	A/G + G/G	92 (36)	153 (42)	1.2	0.3	114 (40)	1.1	0.6	39 (48)	1.5	0.1	19 (41)	1.2	0.6	19 (56)	2.0	0.06
CASC1-4	Arg/Arg	157 (62)	210 (58)	Ref.		168 (59)	Ref.		42 (52)	Ref.		27 (57)	Ref.		15 (44)	Ref.	
	Arg/Ser + Ser/Ser	96 (38)	154 (42)	1.1	0.4	115 (41)	1.1	0.7	39 (48)	1.4	0.2	20 (43)	1.1	0.7	19 (56)	2.0	0.08
CASC1-5	T/T	157 (62)	207 (57)	Ref.		165 (58)	Ref.		42 (82)	Ref.		26 (55)	Ref.		16 (47)	Ref.	
	T/C + C/C	96 (38)	157 (43)	1.2	0.3	118 (42)	1.1	0.5	9 (18)	1.4	0.2	21 (45)	1.3	0.5	18 (53)	1.7	0.1
LRMP-7	Ser/Ser	172 (68)	231 (63)	Ref.		183 (65)	Ref.		48 (59)	Ref.		31 (66)	Ref.		17 (50)	Ref.	
	Ser/Cys + Cys/Cys	81 (32)	133 (37)	1.2	0.4	100 (35)	1.1	0.6	33 (41)	1.3	0.3	16 (34)	1.0	1.0	17 (50)	1.9	0.09
LRMP-6	Val/Val	201 (79)	288 (79)	Ref.		223 (79)	Ref.		65 (80)	Ref.		37 (79)	Ref.		28 (82)	Ref.	
	Val/Leu + Leu/Leu	52 (21)	76 (21)	1.0	0.9	60 (21)	1.0	1.0	16 (20)	1.0	0.9	10 (21)	1.1	0.8	6 (18)	0.8	0.7
LRMP-4	C/C	91 (36)	139 (38)	Ref.		107 (38)	Ref.		32 (40)	Ref.		18 (38)	Ref.		14 (41)	Ref.	
	C/T + T/T	162 (64)	225 (62)	0.9	0.6	176 (62)	0.9	0.6	49 (60)	0.9	0.7	29 (62)	0.9	0.8	20 (59)	0.8	0.6
LRMP-1	G/G	81 (32)	133 (37)	Ref.		105 (37)	Ref.		28 (35)	Ref.		17 (36)	Ref.		11 (32)	Ref.	
	G/A + A/A	172 (68)	231 (63)	0.8	0.2	178 (63)	0.8	0.2	53 (65)	1.0	0.9	30 (64)	0.9	0.8	23 (68)	1.1	0.9

SNP	Genotype	Case							
		Small-sized ADC with BAC components			Smoking habit				
					Non-smoker			Smoker		
		No. (%)	OR[a]	*P*	No. (%)	OR[b]	*P*	No. (%)	OR[b]	*P*
KRAS-12		49 (32)	Ref.		44 (25)	Ref.		54 (29)	Ref.	
		103 (68)	1.1	0.8	130 (75)	1.4	0.1	135 (71)	1.3	0.2
KRAS-1		115 (76)	Ref.		135 (77)	Ref.		146 (77)	Ref.	
		37 (24)	1.3	0.3	41 (23)	1.2	0.5	44 (23)	1.2	0.5
KRAS-6		94 (62)	Ref.		106 (61)	Ref.		110 (58)	Ref.	
		58 (38)	1.1	0.6	68 (39)	1.1	0.8	79 (42)	1.3	0.2
CASC1-1		93 (61)	Ref.		104 (60)	Ref.		106 (56)	Ref.	
		59 (39)	1.1	0.7	70 (40)	1.1	0.6	83 (44)	1.3	0.2
CASC1-4		93 (61)	Ref.		102 (59)	Ref.		107 (57)	Ref.	
		59 (39)	1.1	0.8	72 (41)	1.1	0.7	82 (43)	1.2	0.3
CASC1-5		92 (61)	Ref.		100 (57)	Ref.		106 (56)	Ref.	
		60 (39)	1.1	0.7	74 (43)	1.2	0.5	83 (44)	1.2	0.3
LRMP-7		105 (69)	Ref.		109 (63)	Ref.		121 (64)	Ref.	
		47 (31)	1.0	0.8	65 (37)	1.2	0.4	68 (36)	1.2	0.5
LRMP-6		127 (84)	Ref.		138 (79)	Ref.		149 (79)	Ref.	
		25 (16)	0.8	0.4	36 (21)	1.0	0.9	40 (21)	1.0	0.9
LRMP-4		67 (44)	Ref.		75 (43)	Ref.		65 (34)	Ref.	
		85 (56)	0.7	0.2	99 (57)	0.8	0.3	125 (66)	1.1	0.6
LRMP-1		61 (40)	Ref.		70 (40)	Ref.		63 (33)	Ref.	
		91 (60)	0.7	0.2	104 (60)	0.8	0.3	126 (67)	0.9	0.8

[a]Adjusted for gender, age and smoking dosage.
[b]Adjusted for gender and age.