

Immunocytochemical staining for stratifin and OCIAD2 in bronchial washing specimens increases sensitivity for diagnosis of lung cancer

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Objective: Brushing or washing cytology taken at bronchoscopy is a standard diagnostic procedure for lung cancer. The present study evaluated the sensitivity of immunocytochemical diagnosis of lung cancer using bronchial washing materials.

Methods: We collected bronchial washing samples taken at bronchoscopy between July 2012 and July 2013 at Tsukuba University Hospital and studied 106 cases that were finally diagnosed as lung cancer. We collected exfoliated cells using a thin-layer advanced cytology assay system (TACAS™) and performed cytological diagnosis using Papanicolaou staining. As controls, we randomly selected 30 tumour-negative cases from among samples collected during the same period. Using these materials, we also examined the expression of stratifin (14-3-3 sigma) ($n = 92$) and OCIAD2 ovarian immunoreactive antigen domain 2) ($n = 106$) by immunocytochemistry, as these are considered to be broad spectrum immune markers for lung adenocarcinoma including early invasive lung adenocarcinoma.

Results: Using Papanicolaou staining, 52 out of 106 lung cancers (49.1%) were diagnosed as positive. However, positivity was increased to 63.0% by immunocytochemistry using anti-stratifin or anti-OCIAD2 antibodies. Biopsies were taken in 103/106 cases and cancer was diagnosed in 60/103, (58.3%). The sensitivity of stratifin or OCIAD2 was significantly higher than that of Papanicolaou staining ($P = 0.027$), but immunocytochemistry detected false-positive cells in 3/30 cases (10%) for stratifin and 2/30 cases (7%) for OCIAD2.

Conclusion: Immunocytochemical staining for stratifin and OCIAD2 improved diagnostic sensitivity for lung cancers but diagnostic specificity was lower than that for cytology alone. The immunostains carried up to a 10% risk of a false-positive result and therefore positive staining must be confirmed by morphological evidence of malignancy.

Keywords: stratifin, OCIAD2, bronchial washing cytology, liquid-based cytology, lung cancer

Introduction

Lung cancer is the most common cancer in terms of both incidence and mortality, accounting for more than 1.4 million deaths annually worldwide.¹ The

prognosis of lung cancer is poor, as most patients are diagnosed at an advanced stage. Early detection of lung cancer is therefore important, as it increases the likelihood of curative treatment.

Brushing or washing cytology taken at bronchoscopy is one of the standard diagnostic procedures for lung cancer, but the diagnostic rate based on cytology (43–61%) is lower than that based on histology (57–74%).² Also, the high frequency of suspicious cases is problematic. In contrast, tissue sampling is not always possible and sometimes cytological diag-

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nosis may be the only option. Therefore, cytology plays an important role in lung cancer diagnosis, and new innovative diagnostic methodologies are required in order to increase the sensitivity of cytological diagnosis.

Many tumour markers such as p53, carcinoembryonic antigen (CEA), cytokeratin 19 fragments (CYFRA 21-1) and squamous cell carcinoma (SCC) antigen have been examined as immunomarkers for lung cancer, but none of them have shown adequate specificity and sensitivity. In contrast, we have recently reported two new biomarkers of early lung adenocarcinoma: stratifin (14-3-3 sigma) and OCIAD2 (ovarian carcinoma immunoreactive antigen domain 2).^{3,4} Stratifin, one of the 14-3-3 family proteins, was originally identified as a p53-inducible gene responsive to DNA-damaging agents.⁵ Stratifin changes the structure of binding protein through bond dissociation, controls a wide range of physiological processes, and has been implicated in variety of diseases, including cancer and neurological disorders.⁶ In the lung, the normal epithelium does not express stratifin, but aberrant stratifin overexpression is detectable in early invasive adenocarcinoma.³ Its expression is regulated by tumour-associated CpG demethylation in lung adenocarcinoma, independently of p53 alteration.⁷ The other biomarker, OCIAD2, was identified by Strausberg *et al.*⁸ in 2002 on the basis of its sequential similarity to OCIAD1, the latter having been originally immuno-screened from ascites of a patient with ovarian cancer, and found to be a tumour-specific protein.⁹ The expression of OCIAD2 increases during the course of malignant progression of ovarian mucinous tumours.¹⁰ In the lung, OCIAD2 shows higher expression in early invasive adenocarcinoma than in *in situ* adenocarcinoma.⁴ Thus, both stratifin and OCIAD2 are tumour-specific genes expressed by early invasive lung adenocarcinoma. Interestingly, on the basis of immunohistochemical staining, stratifin and OCIAD2 are expressed in most invasive lung adenocarcinomas including early invasive ones, but are nearly always negative in normal lung tissue.³ These findings suggest that stratifin and OCIAD2 could be strong and specific biomarkers for both cytological and histological diagnosis of lung adenocarcinoma.

Liquid-based cytology (LBC) is a thin-layer or monolayer slide preparation technique that has been introduced as a potential solution for improving the sensitivity and specificity of cytological assessment.^{11,12} LBC has gained favour over conventional

smear and cytospin techniques in recent years, and has been shown to yield equivalent or better diagnostic accuracy, particularly in specimens containing abundant mucus and/or blood.¹³ LBC is also more suitable for immunocytochemistry and for further investigations such as DNA analysis.

In the present study, we examined the diagnostic applicability of stratifin and OCIAD2 immunocytochemistry in lung carcinoma using bronchial washing cytology specimens prepared using the LBC method.

Methods

Samples and patient characteristics

A total of 219 bronchial washing samples were collected between July 2012 and July 2013 at Tsukuba University Hospital (Ibaraki, Japan). Among them, 106 samples obtained by bronchoscopy were finally diagnosed as lung cancer histologically or cytologically. These included 57 cases of adenocarcinoma, 23 cases of squamous cell carcinoma, six cases of small cell carcinoma and 20 cases of other cancer types. The patients ranged in age from 37 to 89 years with an average age of 69 years. Sixty-five patients were male and 41 were female. As negative controls, we also randomly selected and reviewed 30 tumour-negative samples during the same period. All patients gave informed consent before collection of the samples.

Liquid-based cytology preparation and immunocytochemistry

We received the bronchial washing samples in ethanol-based fixative solution (TACAS™ Ruby), and prepared the LBC specimens using the thin-layer advanced cytology assay system (TACAS™; Medical and Biological Laboratories Co. Ltd, Nagoya, Japan). Initially, we prepared a single LBC slide for Papanicolaou staining and then, using residual bronchial washing specimens, prepared other slides for immunocytochemistry, soaking the slides in 95% ethyl alcohol for at least 10 minutes and then autoclaving them in 10 mM citrate buffer (pH 6.0) at 121 °C for 10 minutes for antigen retrieval. After cooling, these slides were incubated with a monoclonal antibody against stratifin ($n = 92$) diluted 1 : 40 (Immunobiological Laboratories Co., Ltd., Gunma, Japan) or a polyclonal antibody against OCIAD2 ($n = 106$) diluted 1 : 120 (Sigma-Aldrich, Saint Louis, MO, USA) for 30 minutes at room temperature.

Subsequently, the slides were incubated with the secondary antibody (EnVision™ +DualLink; Dako, Tokyo, Japan) for 30 minutes at room temperature. Immunoreactivity was detected using 3,3'-diaminobenzidine (DAB) and the slides were counterstained with haematoxylin. This immunocytochemistry method, including antibody dilution, was similar to that used for histology, except for the DAB incubation time. The concentrations of the first antibodies for stratifin and OCIAD2 were based on the manufacturers' recommendations.

Assessment and statistical analysis

All samples were screened by two certified cytoscreeners (T.N. and Y.M.) and confirmed by one certified pathologist (N.I.). We scored stratifin and OCIAD2 immunoreactivity according to the intensity of cytoplasmic staining. The staining was assessed as positive when more than one cell was stained more strongly than bronchial epithelial cells, regardless of the presence or absence of recognizable carcinoma cells, as bronchial epithelial cells are slightly immunoreactive for both stratifin and OCIAD2. We defined positive cases as those showing immunocytochemical staining for at least one antigen (stratifin or OCIAD2).

The relationship between cytological diagnoses and cytoplasmic staining for stratifin and OCIAD2 was examined using Fisher's exact test for 2 × 2 contingency tables. The level of significance was set at $P < 0.05$.

Results

Cytological diagnosis based on Papanicolaou staining was performed routinely, and the results are shown in Table 1. The specimens were classified as 'positive', 'suspicious' or 'negative'. We classified a specimen as 'suspicious' when several atypical cells requiring further evaluation were observed according to the former study.¹⁴ The proportions of cases thus classified were 49.1% (52/106), 12.3% (13/106) and 38.7% (41/106), respectively. In addition, biopsies were obtained in most cases (103/106 cases), and malignancy was diagnosed in 60/103 cases (58.3%).

Table 2 shows the numbers of cases that were positive for stratifin, OCIAD2 and the combination of the two markers (stratifin or OCIAD2). Among 44 cytologically positive cases, cells positive for stratifin or OCIAD2 were detected in 35. In contrast, among 48 cytologically suspicious or negative cases, immu-

Table 1. Cytological classification

	Number (%)
Negative	41 (38.7)
Suspicious	13 (12.3)
Positive	52 (49.1)
Total	106

nopositive cells were found in 14. Among the histological subtypes, cells positive for stratifin or OCIAD2 were detected in 28 of 50 cases of adenocarcinoma, 10 of 20 cases of squamous cell carcinoma, four of five cases of small cell carcinoma and seven of 17 cases of other cancer types.

Finally 67 tumours were surgically resected and we performed immunohistochemistry for 62 of them. Most of the tumours were positive for stratifin (61/62) and OCIAD2 (61/62), but focal (<66% of tumour cells) or weak expression was observed in 10 cases for stratifin and in 29 cases for OCIAD2. The cases showing focal or weak expression tended to be non-adenocarcinomas. Among adenocarcinomas, 44 tumours were surgically resected after cytological or histological diagnosis, and of these 40 were diagnosed as invasive adenocarcinoma and four as minimally invasive adenocarcinoma as reported in a former study.¹⁵ In the four minimally

Table 2. Numbers of immunocytochemically positive cases (a) stratifin ($n = 92$), (b) OCIAD2 ($n = 106$) and (c) Combination (stratifin or OCIAD2) ($n = 92$)

	Histological type of carcinoma				Total
	Adeno	Squamous	Small cell	Others	
(a)					
Negative	1/19	1/7	0/1	1/8	3/35
Suspicious	2/10	0/2	NA	0/1	2/13
Positive	14/21	7/11	3/4	4/8	28/44
Total	17/50	8/20	3/5	5/17	33/92
(b)					
Negative	4/22	1/10	0/1	1/8	6/41
Suspicious	6/10	½	NA	1/1	8/13
Positive	19/25	6/11	5/5	7/11	37/52
Total	29/57	8/23	5/6	9/20	51/106
(c)					
Negative	4/19	1/7	0/1	1/8	6/35
Suspicious	6/10	1/2	NA	1/1	8/13
Positive	18/21	8/11	4/4	5/8	35/44
Total	28/50	10/20	4/5	7/17	49/92

NA, not available.

invasive adenocarcinomas, we were unable to find any malignant cells or cells positive for stratifin or

OCIAD2 in slides subjected to Papanicolaou or immunocytochemical staining.

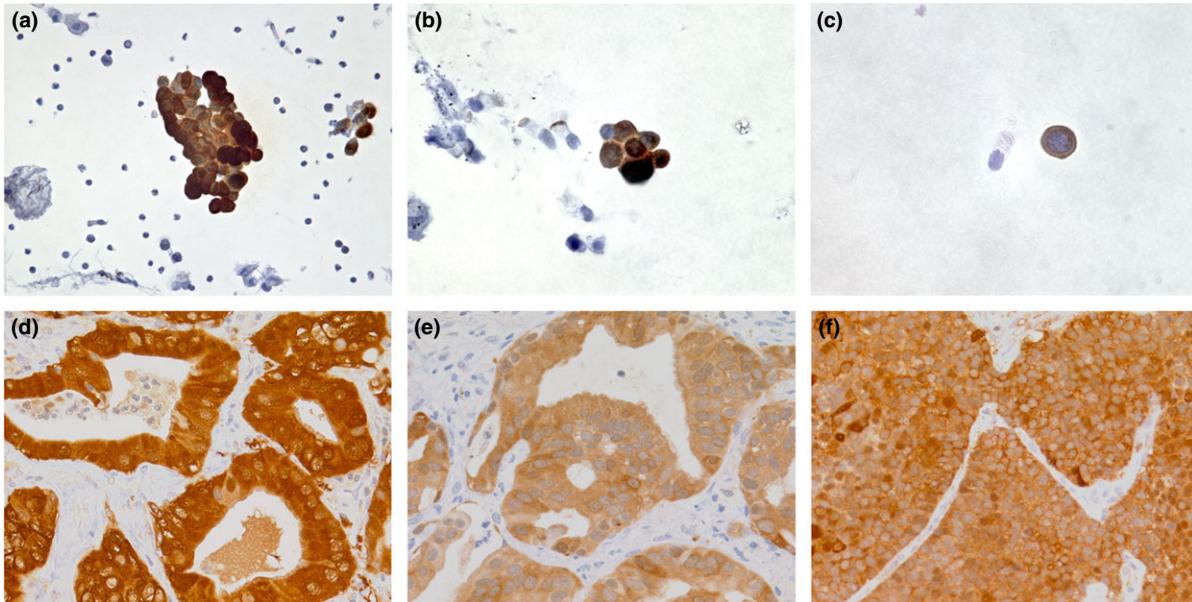


Figure 1. Cytological and histological findings for anti-stratifin (14-3-3 sigma). (a–c) Cytological findings in bronchial washing specimens prepared using a liquid-based cytology (LBC) method: immunocytochemistry $\times 400$ (a); $\times 1000$ (b, c). (d–f) Histological findings on surgical resections from the same patients: immunohistochemistry $\times 400$ (d, e, f).

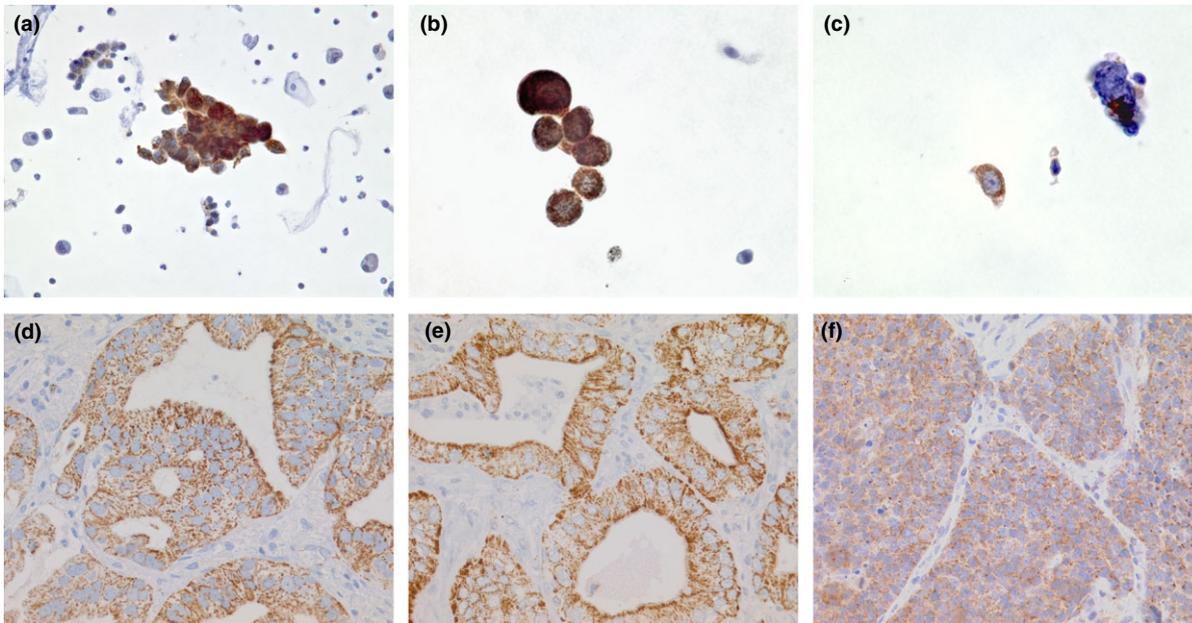


Figure 2. Cytological and histological findings for Anti-OCIAD2 ovarian immunoreactive antigen domain 2). (a–c) Cytological findings in bronchial washing specimens prepared by a liquid-based cytology (LBC) method: immunocytochemistry $\times 400$ (a); $\times 1000$ (b, c). (d–f) Histological findings on surgical resections from the same patients: immunohistochemistry $\times 400$ (d, e, f).

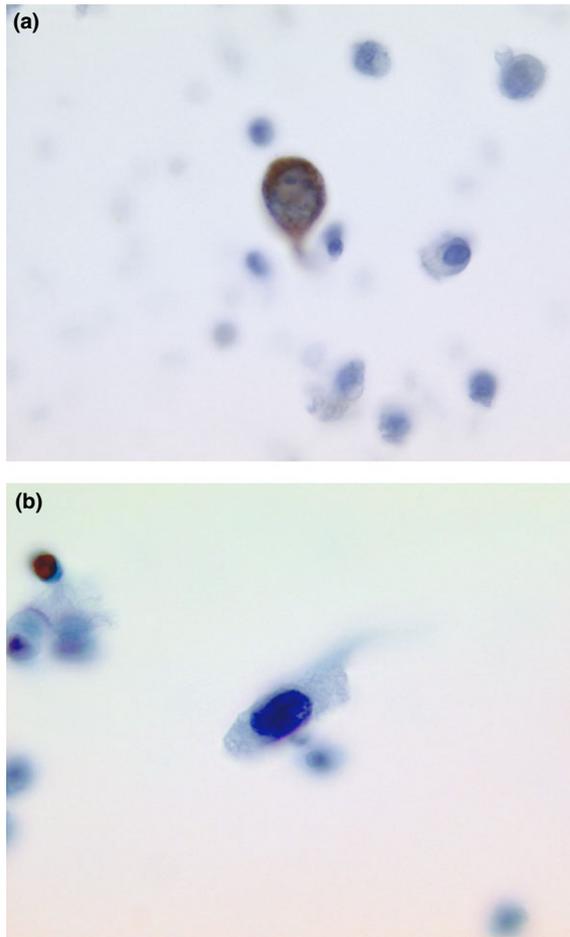


Figure 3. OCIAD2-positive/cytologically negative case finally diagnosed as squamous cell carcinoma. (a) A few OCIAD2-positive tumour cells were present (immunocytochemistry $\times 1000$); (b) very few but certainly atypical cells were detected upon re-examination of the cytology (Papanicolaou $\times 1000$).

Figures 1 and 2 show examples of immunocytochemical staining in representative cases. Stratifin and OCIAD2 were mainly positive in the cytoplasm of the tumour cells, similar to the cancer cells in the resected specimens. Stratifin was stained diffusely, whereas OCIAD2 showed a granular staining pattern. Tumour cells in the LBC specimens showed a significantly stronger immunoreaction for stratifin and OCIAD2 than bronchial epithelial cells (Figures 1a and 2a). It was noteworthy that a few immunocytochemically positive cells were detectable even in cytologically suspicious or negative cases (Figures 1b,c and 2b,c). After immunostaining, we re-examined the cytologically negative cases that included cells positive for stratifin or OCIAD2, and found a very small number of cells that certainly appeared atypical in Papanicolaou slides in some cases (3/6). One of the representative cases those were cytologically negative is shown in Figure 3. We also reviewed all of the cytologically suspicious cases that were positive for stratifin or OCIAD2, but none of them had any obviously positive tumour cells. Figure 4 shows the proportions and actual numbers of cases positive for stratifin, OCIAD2 and the combination of the two markers (stratifin or OCIAD2). OCIAD2-positive cases outnumbered stratifin-positive cases, but some cases were immunoreactive only for stratifin.

In contrast, cells showing false-positivity for stratifin and OCIAD2 were found in three (10%) and two (7%) of 30 cases, respectively. However, these cells showed only very mild atypia, and were morphologically distinguishable from cancer cells.

We then compared the differences in sensitivity and specificity for diagnosis of lung carcinoma between Papanicolaou staining and Papanicolaou

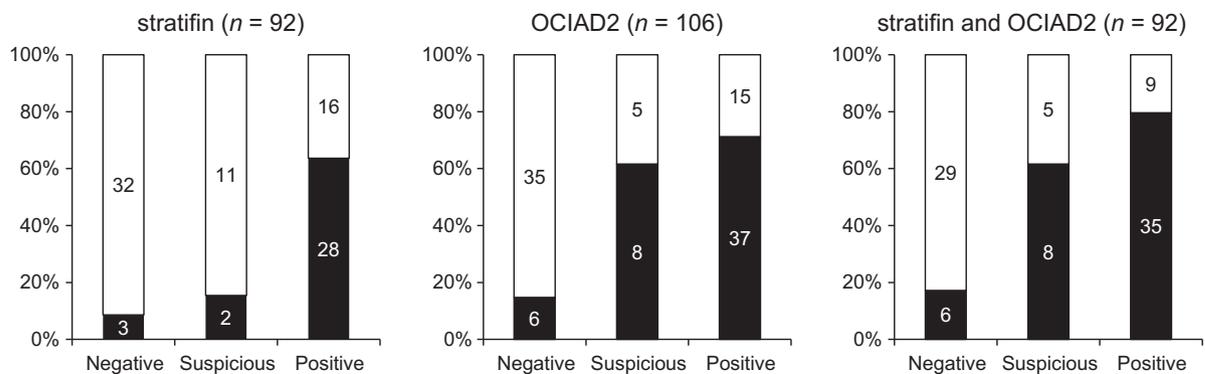


Figure 4. Numbers of immunocytochemically positive cases related to cytology result (white, negative; black, positive).

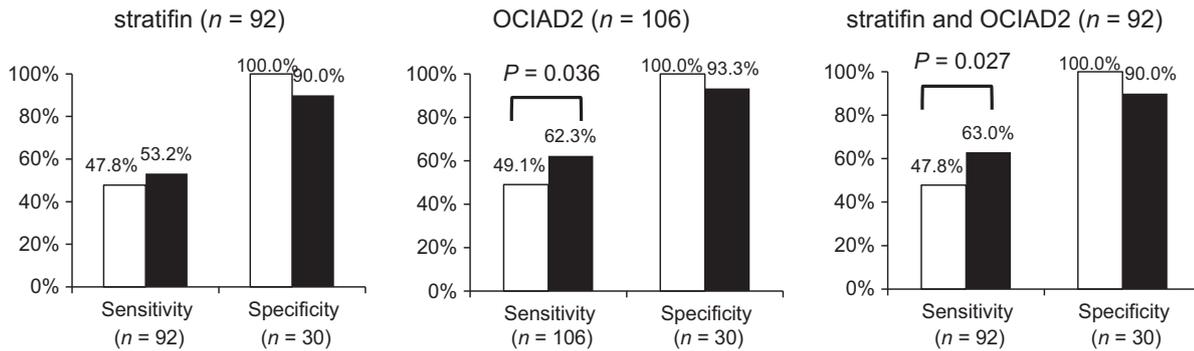


Figure 5. Immunocytochemical sensitivity and specificity in comparison with Papanicolaou staining only (white, Papanicolaou staining only; black, Papanicolaou staining plus immunocytochemistry).

staining plus immunocytochemistry for stratifin, OCIAD2 and the combination of the two markers (stratifin or OCIAD2). As shown in Figure 5, sensitivity in terms of stratifin or OCIAD2 immunocytochemistry was significantly higher than that of Papanicolaou staining alone ($P = 0.027$). The use of stratifin or OCIAD2 immunocytochemistry increased the diagnostic yield of washing cytology from 47.8% to 63.0%, and was thus slightly better than that for biopsy (58.3%; 60/103).

Discussion

In the present study, we demonstrated that bronchial washing cytology specimens prepared using the LBC method were successfully immunostained for stratifin and OCIAD2, which are reported to be proteins expressed particularly in early invasive lung adenocarcinoma.^{3,4}

Although the rates of immunohistochemical positivity for stratifin/OCIAD2 in surgical specimens of invasive adenocarcinoma were more than 95%, immunocytological sensitivity for adenocarcinoma was 66.7% (14/21) for stratifin and 76.0% (19/25) for OCIAD2 in this series.

For a number of reasons, there are some discrepancies between immunohistochemistry and immunocytochemistry for diagnosis of not only adenocarcinoma but also non-adenocarcinoma. One of the most likely is immune-heterogeneity in histological specimens. Cytological specimens from cases showing heterogeneous expression of stratifin and OCIAD2 on histology might not contain representative positive tumour cells. As an alternative in the present series, we prepared slides for immunocytochemistry using residual bronchial washing

specimens after preparing a sufficient number of Papanicolaou-stained slides. We expected that in some instances no immunoreactive cancer cells might remain on the slide for immunocytochemistry, although Papanicolaou-stained slides contained tumour cells for cytological diagnosis.

Unexpectedly, we found that immunocytochemical sensitivity was highest for small cell carcinoma, and was relatively high for squamous cell carcinoma, even although we had initially identified stratifin and OCIAD2 as tumour-specific genes that were expressed specifically in both definitely invasive adenocarcinoma and also early-stage adenocarcinoma. These findings suggested that stratifin and OCIAD2 might be tumour-specific genes expressed in a wide range of cancers. In fact, it has been reported that stratifin shows high expression in squamous cell carcinoma of the lung,¹⁶ head and neck cancer,^{17,18} gastrointestinal cancer,¹⁹ pancreatic cancer,^{20,21} cervical squamous cell carcinoma, and urinary squamous cell carcinoma.²² In contrast, OCIAD2 is expressed in ovarian mucinous tumours¹⁰ and small-cell lung carcinoma.²³ Therefore, the present approach may be useful for screening of cytological materials such as pancreatic juice, urine, pleural effusion or ascites. Future studies will need to focus on tumour-specific expression of stratifin and OCIAD2 in various types of carcinoma.

Interestingly, the present series included 14 cases that were immunocytochemically positive for stratifin or OCIAD2 among cytologically negative or suspicious cases. Six cases were negative and eight were suspicious. These cases were finally confirmed to be lung cancer on the basis of histology or additional cytological examination, thus indicating that cytological negativity or suspicious diagnoses may be

attributable to carcinoma cells showing only slight atypia, or being present in only small numbers in bronchial washing fluid. However, immunocytochemical positivity cannot be regarded as diagnostically definitive, and additional sampling (by needle aspiration or biopsy) should be performed, because false-positive cells were also detected in up to 10% of our control cases.

LBC provides a cleaner background, a smaller area for screening and well-preserved distinctly stereoscopic original cellular structures in comparison with conventional cytology. Furthermore, LBC specimens can be used for additional evaluations such as immunocytochemistry for stratifin and/or OCIAD2. Such additional examinations might increase the sensitivity of cytological screening. For example, immunocytochemistry for stratifin and/or OCIAD2 in addition to Papanicolaou staining might reduce the work burden on screeners, and thus prevent oversights, as well as being very useful for lung cancer diagnosis, especially in suspicious cases. Immunocytochemistry has been widely employed for routine cytological screening, and is easily applicable at most pathology laboratories. In spite of the presence of immunocytological false positivity, the sensitivity of cytological screening could be increased significantly.

Biologically, overexpression of stratifin is strictly regulated by tumour-associated CpG demethylation in lung adenocarcinoma.⁷ Stratifin demethylation is a very specific feature of invasive adenocarcinoma. Therefore, analysis of DNA methylation in bronchial washing specimens may represent a new approach for the detection of cancer cells in such specimens. Although the function of OCIAD2 is still unclear, it is located mainly in mitochondria and mitochondria-associated ER membranes (MAM).²⁴ At the same time, OCIAD2 has high homology with OCIAD1, which was originally immunoscreened from ascites of a patient with ovarian cancer. Therefore, serological testing of bronchial washing specimens may represent a new approach for the diagnosis of lung cancer. In this study, we showed that stratifin and OCIAD2 were applicable as tumour markers for cytological diagnosis of lung carcinoma. Of course, immunostaining for stratifin alone or OCIAD2 alone is helpful for cytological diagnosis, but positivity for both markers would be more diagnostically reliable because the biological significance of each marker differs.

In conclusion, LBC is a useful diagnostic tool for application of immunocytochemical staining. Stratifin

and OCIAD2 are useful immunomarkers and suitable targets for cytological screening of lung cancer. Their immunocytochemical staining shows adequate specificity, as long as positive staining is confirmed morphologically, and its high sensitivity increases the likelihood of lung cancer cell identification.

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