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undergone gastroscopy because LyGa shows no gastric symptoms. Therefore, although these concomitant occurrences appear coincidental, further studies are required for a better understanding of LyGa and its relationship with adenocarcinoma.

Whether LyGa is monoclonal proliferation or not remains a matter of debate. Unlike B or T cells, NK cells do not undergo any specific gene rearrangement, rendering it difficult to determine whether the proliferation of EBV-free NK cells is monoclonal or not. Vega et al[23] indicated that the NK-cell proliferation in their study appeared polyclonal because of the heterogeneous expression of the immunoglobulin-like receptors CD158a, CD158b, and CD158e; nevertheless, they could not exclude the possibility of a low-grade neoplasm. Siu et al[26] reported that the p73 gene was methylated in 94% of the NK-cell malignancies and that other methylated genes included *hMLH1* (63%), *p16* (63%), *p15* (48%), and *RAR* β (47%). We analyzed the methylation status of several genes, including *p16*, *p73*, *DAPK*, *MGMT*, *CDH1*, and *hMLH1*, in 2 heterochronically biopsied specimens from case 3 to obtain evidence of monoclonality. No aberrant methylation, however, was found in the examined genes (data not shown). These results reconfirmed that LyGa is different from extranodal NK/T-cell lymphoma, but the results did not serve as evidence for the monoclonality of LyGa. Further investigation with a larger sample size is required to clarify this distinction. Cytogenetic analyses and studies involving the identification of genetic loss/gain (eg, studies involving single nucleotide polymorphism microarray analysis) or point mutations (eg, studies involving next-generation genome sequencing) may be helpful to clarify the biologic natures of LyGa, especially whether LyGa is monoclonal proliferation or not. Procurement of fresh materials for these studies is impeded by spontaneous regression of lesions after the index biopsy; the biopsy specimen is usually fixed in formalin and embedded in paraffin for routine pathologic diagnosis.

LyGa should be regarded as a distinctive clinicopathologic entity and be observed without treatment. However, if not well recognized, LyGa is probably to be histopathologically misdiagnosed as lymphoma. For example, Kikuchi-Fujimoto disease, a self-limiting disorder of unknown cause, is still often mistakenly diagnosed as lymphoma,[4] although > 30 years have passed since it was first described in 1972. If LyGa is misdiagnosed as NK/T-cell lymphoma, it might be treated with radical therapeutic procedures, including chemotherapy, radiotherapy, gastrectomy, and stem cell transplantation. In fact, 2 patients of the present series underwent gastrectomy. The remaining 8 patients did not receive any treatment because the staging procedures followed by the initial diagnosis showed that the lesions regressed spontaneously. For 1 patient, however, the first biopsy specimen diagnosed as lymphoma was suspected to have been mistakenly identified to the patient. Fortunately, LyGa shows highly conserved and characteristic features in terms of clinical presentation, morphology, and immunophenotype (immunohistochemistry for CD3, CD5, CD7, CD56, and cytotoxic molecule(s) and EBER in situ hybridization are required to diagnose LyGa). Therefore, as long as LyGa is recognized as a distinct disease concept, there is no scope of misdiagnosis as malignancy.

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Authorship

Contribution: K.T. and K.O. conceived the study, collected and analyzed the data, and drafted the paper; M.Y., Y.T., K. Marutsuka, M.N., N.F., T.Y., H.N., F.A., K. Hoshi, K. Matsue, and K. Hatake contributed patient materials and analyzed the data; and S.I. and K.N. performed special studies and analyzed the data.

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Sorafenib-induced erythema multiforme for metastatic renal cell carcinoma

Sorafenib is an active agent for cytokine-refractory renal cell carcinoma (RCC) patients [1]. Skin toxicity such as hand–foot syndrome (HFS) is one of the frequent adverse events of sorafenib. In the phase II study conducted in Japan, grade 3 skin toxicity occurred in 13.7% of 131 patients with RCC, but all of those skin toxic effects were HFS or rash/desquamation [2]. We here report three cases of erythema multiforme (EM) associated with sorafenib therapy. EM, Stevens–Johnson syndrome, and toxic epidermal necrolysis are mucocutaneous diseases associated with significant morbidity and mortality. The term 'Stevens–Johnson syndrome' has been widely accepted as a synonym for EM major [3].

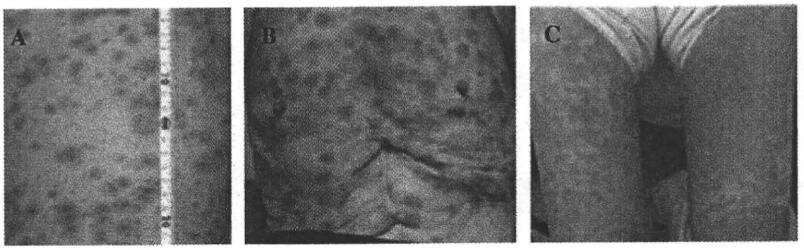

Figure 1. Erhythema multiforme of three cases. Target-like erythematous skin rash of left femoral lesion of case 1 (A), case 2 (B), and case 3 (C).

case 1

A 25-year-old female with pulmonary metastases of papillary RCC received sorafenib 800 mg/day. At day 8, erythema appeared on lower legs and spread over 50% of body surface area in 2 days (Figure 1A) with HFS. She also suffered intermittent fever up to 39.5°C from day 8. Serum examination excluded the viral infection, including measles, herpes simplex, or herpes zoster. Skin biopsy of femoral region at day 12 revealed superficial and perivascular lymphocyte infiltration and necrotic keratinocytes, compatible with EM. Skin rash disappeared within days after discontinuation of sorafenib without steroid treatment or antimicrobial treatment.

case 2

An 80-year-old man with multiple pulmonary metastases of papillary RCC was treated with sorafenib 800 mg/day. From the 8th to 12th day of sorafenib, erythema spread over his whole body (Figure 1B) with grade 2 HFS, mild stomatitis, and grade 3 fatigue. EM and HFS disappeared within 2 weeks after discontinuation of sorafenib and oral predonisolone 10 mg/day. When sorafenib (400 mg/day) was rechallenged, EM with high fever reappeared within 24 h and the sorafenib was discontinued at once.

case 3

A 70-year-old female with metastatic clear cell RCC developed erythema spread to whole body 15 days after starting sorafenib 800 mg/day (Figure 1C). Eruption disappeared within 2 weeks after discontinuation of sorafenib and topical treatment without steroid. She was treated with sunitinib 50 mg/day and EM has not appeared.

Rash, desquamation, and HFS are most frequent skin symptoms with sorafenib. They are dose dependent and disappear with discontinuation of sorafenib. In most cases, restart with the same dose is possible and the symptoms may resolve without dose modification. MacGregor et al. [4] reported sorafenib-induced EM in malignant melanoma patient. They carried out skin biopsy and showed the same findings with that of our case. In that report, the patient was rechallenged with the reduced dose of sorafenib (100 mg) but developed the same eruption within 24 h, and they discontinued sorafenib. There have been only two other reports about sorafenib-induced EM [5, 6], but we experienced three EM patients, one of which was confirmed by biopsy, of 16 RCC patients we have treated with sorafenib in a year.

Furthermore, postmarketing surveillance of sorafenib-treated patients in Japan reported that 108 cases of so-called EM occurred in 2889 cases from February 2008 to October 2009, so occurrence rate of EM might be different between Japanese and Caucasians. A recent study suggests that polymorphisms in specific genes encoding for metabolizing enzymes, efflux transporters, and drug targets are associated with toxic effects of sunitinib, another angiogenesis inhibitor [7]. In addition, the population-related pharmacogenomics might contribute to differences in adverse events and responses of antitumor agents between patients in Japan and those in the United States [8]. Further study is necessary to elucidate this discrepancy of EM occurrence rate between Japanese and Caucasians.

In clinical practice, it is very difficult to diagnose whether the drug-induced skin rash is caused by allergic or toxic mechanisms. To use sorafenib safely, restarting of sorafenib needs careful monitoring because the possibility of an allergic mechanism cannot be ruled out. Patients with skin lesions due to allergic mechanisms may not have benefits from sorafenib treatment because of early treatment failure. At present, we cannot recommend the rechallenge of sorafenifb for these patients as two of three patients including our case recurred EM.

Sorafenib is now one of few standard agents for metastatic RCC. Molecular mechanism of this type of toxicity remains unknown. Further investigation is necessary to disclose the mechanism and establish the effective therapy for sorafenib-induced EM, which might not be a rare adverse event in Japanese patients.

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disclosure

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CORRESPONDENCE

Is Statin Use Really Associated With Efficacy of Rituximab?

TO THE EDITOR: We would like to raise several issues regarding the recent article in *Journal of Clinical Oncology* by Nowakowski et al,[1] "Statin Use and Prognosis in Patients With Diffuse Large B-Cell Lymphoma and Follicular Lymphoma in the Rituximab Era."

This report focused on the clinical impact of statin use on outcomes of patients with diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL) receiving rituximab treatment based on the experimental results that statins impair the efficacy of rituximab. However, we do not think it can be concluded with certainty that statin use was associated with the efficacy of rituximab.

First, the authors have mentioned the limitation of medication compliance, the timing of the opening for statin use, and the type of statin, which was collected retrospectively using medical records; however, they do not provide any information about the serum cholesterol level. In a laboratory study, statins were found to significantly decrease rituximab-mediated complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity against B-cell lymphoma cells.[2] This study suggested that statins, through the depletion of serum cholesterol, induce conformational changes in CD20 molecules that result in impaired binding of rituximab.[2] Thus, we think the difference in serum cholesterol levels in the statin group versus no statin group should be compared. If the statin use group had an advantage for higher total cholesterol level despite statin use, it would be difficult to know whether statin use with lowered cholesterol levels may have a different impact. Our recent analysis showed that statin use was not correlated with the prognosis of patients with DLBCL receiving rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone therapy by adjusting serum cholesterol level.[3]

Second, although the authors have analyzed event-free survival in all patients with FL as a whole group, regardless of whether or not they received rituximab, there are still heterogeneous groups of patients as a result of the disparate approaches to the initial care. Because rituximab significantly improved the outcomes of patients with FL,[4-8] we think the authors should analyze outcomes separately according to statin use, with or without the addition of rituximab.

Third, previous studies have shown that statin use reduces the risk of cardiovascular disease[9] and cerebral vascular attack, and the influence of death from these diseases should be ruled out to evaluate the correlation between statin use and prognosis of lymphoma. Detailed information about death or the evaluation of progression-free survival is encouraged.

Fourth, in patients treated on phase III adjuvant colon clinical trials, disease-free survival (DFS) and overall survival are highly correlated, both within patients and across trials. Although the correlation between DFS and overall survival in patients with FL treated with rituximab-containing chemotherapy has still not been proven, these results suggest that DFS after 3 years of median follow-up is an appropriate end point for adjuvant colon cancer clinical trials of fluorouracil-based regimens.[10]

The influence of statin use on rituximab efficacy is a significant clinical problem. Further studies and follow-up are warranted to confirm the prognostic significance of statins for patients with DLBCL and FL receiving rituximab-containing chemotherapy.

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Implications for Differential Diagnosis of Lung Cancer–Associated Lymphadenopathy in Lymphoepithelioid Cell Lymphoma (Lennert's Lymphoma) Arising Simultaneously with Lung Cancer

A Case Report

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Background

Lymphoepithelioid cell lymphoma (LCL) is a rare morphologic variant of peripheral T-cell lymphoma, and its cytologic features have not been well characterized. We describe details from fine needle aspiration cytology (FNAC) of LCL in a patient simultaneously suffering from lung cancer, in whom extensive lymph node metastasis was suspected clinically.

Case

A 54-year-old man had a lung nodule diagnosed as an adenocarcinoma by biopsy. 18F-fluoro-deoxyglucose positron emission tomography showed high uptake in the lung nodule as well as interlobar, supraclavicular and axillary lymph nodes. FNAC from interlobar and supraclavicular lymph nodes revealed abundant lymphoid cells intermingled with epithelioid cell clusters. Most lymphoid cells were small, with teardrop-shaped nuclei. Occasionally, large lymphoid cells with hyperconvoluted nuclei and prominent nucleoli were observed. An extensive sarcoid reaction was suspected on cytology, and lobectomy was performed. LCL with lung adenocarcinoma was diagnosed on the immunohistochemical findings.

Conclusion

Detailed observation of lymphoid cells with FNAC is important even in patients with lung cancer and massive regional lymphadenopathy. Presence of a teardrop nuclear shape and nuclear irregularities of lymphoid cells provides important information for cytologic diagnosis of LCL when epithelioid cell clusters are evident. (Acta Cytol 2010;54:197–201)

It is important to carefully examine the morphology of lymphoid cells on FNAC even if metastasis from a malignant tumor such as lung cancer is highly suspected.

Keywords: aspiration cytology, fine-needle; lung cancer; lymph node metastasis; lymphoepithelioid cell lymphoma; sarcoid reaction.

Lymphoepithelioid cell lymphoma (LCL) considered to be a rare variant of peripheral T-cell lymphoma, unspecified, in the World Health Organization (WHO) classification.[1,2] Its major cytologic feature is the presence of epithelioid cell clusters intermingled with atypical lymphoid cells.[3-17] Although several case reports of LCL have appeared in the literature, detailed cytologic features have yet to be established.[11-17]

The frequency of synchronous nodal lymphomas and lung cancer is in the range of 0.13% to ~0.43% of all lung cancers in Japan.[18] To our knowledge, however, simultaneous LCL with lung cancer has never been reported. Many nodal lesions seen in patients with lung cancer are sarcoid reactions and nodal reactive changes, including granulomatous lymphadenitis,[13,19] and need to be distin-

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guished from LCL cytologically.[3-17]

We describe a rare case of simultaneous LCL with lung cancer. Extensive lymph node metastasis was suspected on imaging, but a sarcoid reaction was indicated by fine needle aspiration cytology (FNAC). We discuss cytologic pitfalls and detailed characteristics of LCL.

Case Report

A 54-year-old Japanese man was referred to our hospital for further examination of a right lung nodule found on chest radiography at a regular health check-up. Chest computed tomography (CT) showed a 24-mm nodule with spiculation and pleural indentation in the right upper lung field and lymphadenopathy involving the mediastinal, subcarinal, interlobar and left axillary lymph nodes. Transbronchial FNA (TBAC) and biopsy from the nodule revealed a primary lung adenocarcinoma. FNAC from interlobar and subcarinal lymph nodes was then performed for diagnosis of the apparent metastasis. Cytology for interlobar lymph nodes revealed a few atypical cells, but no unequivocal malignancy. Whole-body positron emission tomography revealed increased 18F-fluoro-deoxyglucose uptake in the right lung nodule and interlobar, right supraclavicular and left axillary lymph nodes; FNAC and biopsy of a supraclavicular lymph node were performed. This revealed atypical lymphoid cells, again with uncertainty as to whether they were malignant or reactive in nature. Examination of biopsy material from interlobar, subcarinal and supraclavicular lymph nodes resulted in a diagnosis of sarcoid reactions in all cases.

The presence of teardrop-shaped nuclei and nuclear irregularity of small lymphoid cells admixed with epithelioid cell clusters could be helpful for cytologic diagnosis of LCL.

With the clinical diagnosis of stage I disease, the patient underwent right upper lobectomy with mediastinal lymph node dissection. The interlobar lymph node was diagnosed as demonstrating granulomatous lymphadenitis from examination of intraoperative frozen sections. Finally, the pulmonary nodule was diagnosed as a stage I poorly differentiated adenocarcinoma. Based on further immunohistochemical studies, the interlobar lymph node was diagnosed as LCL. Thus we reviewed the biopsy material obtained from a supraclavicular lymph node and added immunohistochemical investigation. The final diagnosis was LCL. As a result of 6 cycles of chemotherapy with Adriamycin, cyclophosphamide, vincristine and prednisone, he is now in complete remission after 10 months of follow-up. Unfortunately, he had lung cancer recurrence locoregionally 2 years after the surgery, but is now alive after 3 years of follow-up.

Right Upper Lobe Lung Cancer

Cytologic Findings. The smears of TBAC were moderately cellular, with cohesive clusters of large tumor cells on a necrotic background (Figure 1). The tumor cells were polygonal to cuboidal, with abundant homogenously staining cytoplasm, hyperconvoluted nuclei with a finely reticular chromatin pattern, thickened nuclear membranes and a single to several and prominent nucleoli. There was no evidence of glandular structures.

Histopathologic Findings. Grossly, the resected pulmonary tumor was a solid, gray-white, firm nodule, 21 mm in diameter, located in the peripheral portion of the right upper lobe. Microscopically, the tumor consisted of sheets of large polygonal cells with occasional mucin vacuoles (Figure 2). The tumor cells had hyperconvoluted nuclei, and markedly cellular pleomorphism was evident. Papillary structures were observed within the lesion focally.

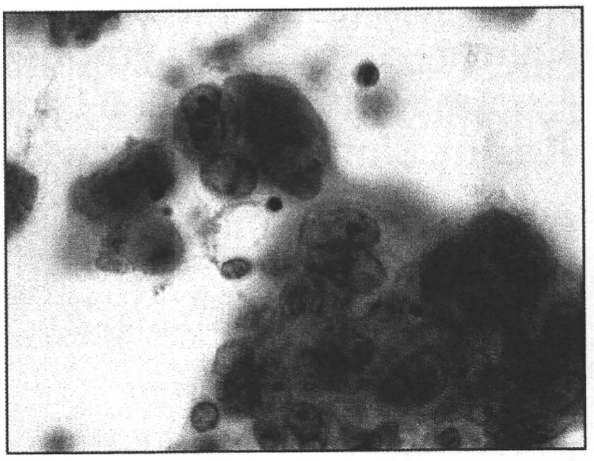

Figure 1 TBAC smear from the lung cancer. The cohesive clusters of polygonal to cuboidal tumor cells (Papanicolaou stain, × 1,000).

Lymphoepithelioid Cell Lymphoma

Cytologic Findings. The smears of TBAC from an interlobar lymph node and FNAC from a supraclavicular lymph node were highly cellular, with numerous lymphoid cells and occasional dispersed epithelioid cell clusters (Figure 3). Most of the lymphoid cells were small, with frequent teardrop-shaped nuclei (Figure 4). Occasionally, isolated and large lymphoid cells were evident (Figure 5) with a round shape and pale or clear cytoplasm. These cells had hyperconvoluted nuclei with a finely granular chromatin pattern and thin nuclear membranes. Single to several large, round to irregularly shaped nucleoli were seen.

The epithelioid cell clusters consisted of small, monolayered and loose aggregates of 10–20 spindle cells with abundant, pale-staining

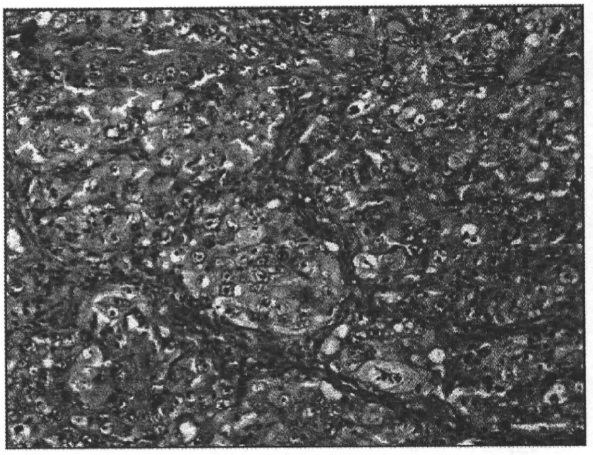

Figure 2 Histologic findings of the resected lung cancer. Sheets of large and polygonal cells with marked cellular pleomorphism (hematoxylin-eosin, × 200).

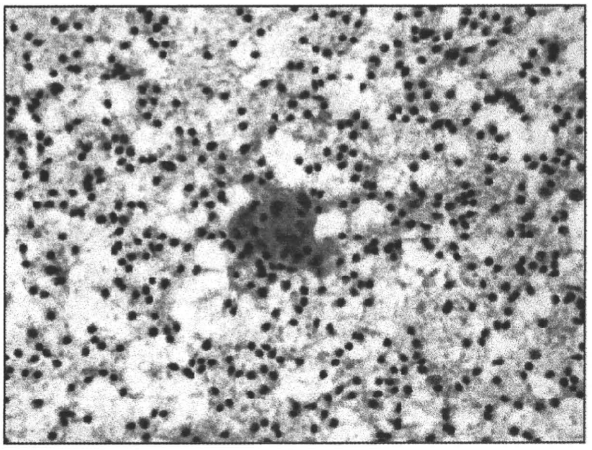

Figure 3 TBAC smear of LCL. The cohesive clusters of epithelioid cells with many atypical lymphoid cells (Papanicolaou stain, ×400).

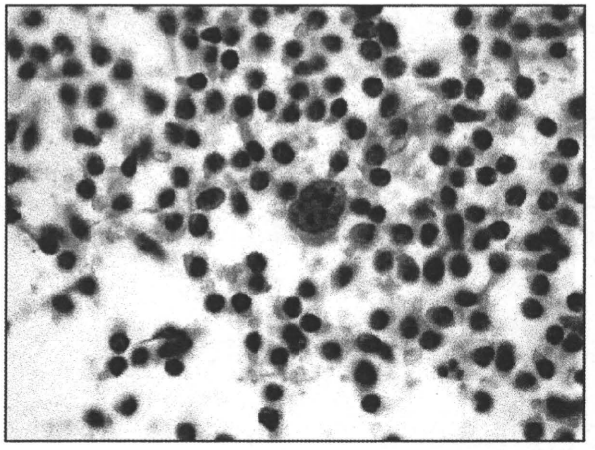

Figure 5 TBAC smear of LCL. Occasional atypical large lymphoid cells with hyperconvoluted nuclei and prominent nucleoli (Papanicolaou stain, ×1,000).

and homogeneous cytoplasm. Oval to elongated nuclei with single small nucleoli were evident.

Histopathologic Findings. Dissected interlobar, hilar and mediastinal lymph nodes were all enlarged. Microscopically, nodal structures in the obtained materials were precluded by the presence of epithelioid histiocytes usually grouped in small clusters (Figure 6A). The lymphoid component was heterogeneous in population, including small and medium-sized to large cells, eosinophils and plasma cells. Most of the lymphoid cells had irregularly shaped nuclei, granular chromatin and prominent nucleoli. Occasionally, large cells resembling Reed-Sternberg cells were observed. Immunohistochemical studies of the lymph nodes were performed with a labeled streptavidin-biotin staining kit (Dako, Carpinteria, California, U.S.A.) according to the manufacturer's instructions. The results were classified as follows: negative—the absence of positive-stained tumor cells; weakly positive—the presence of tumor cells expressing the antigen more weakly than normal T-cells of positive controls; and positive—the presence of tumor cells expressing the antigen as strongly as normal T-cells of positive controls. Immunohistochemically, the lymphoid cells were positive for CD8 (Nichirei, Tokyo, Japan) (Figure 6B) and weakly positive for CD3 (Dako, Glostrup, Denmark), CD7 (Novocastra, Newcastle upon Tyne, U.K.) and CD30 (Dako). Immunostaining for CD4 (Nichirei), CD5 (Novocastra), CD15 (Becton-Dickinson, Mountain View, California, U.S.A.), CD20 (Dako), perforin (Novocastra) and Granzyme B (Dako) was negative (Table I). These findings indicate a T-cell lineage. Because large cells resembling Reed-Sternberg cells showed positivity for CD8, as did small and medium-sized lymphoid cells, Hodgkin's disease was excluded. In situ hybridization using a fluorescein-conjugated oligonucleotide probe for Epstein-Barr virus–encoded RNA was performed with paraffin-embedded specimens.[20,21] As a result, the Epstein-Barr virus–encoded RNA probe detected no evident Epstein-Barr virus infection. The histologic features of biopsy material obtained from a supraclavicular lymph node were similar to those described earlier. Finally, these lymph nodes were diagnosed as LCL by the WHO classification.[1]

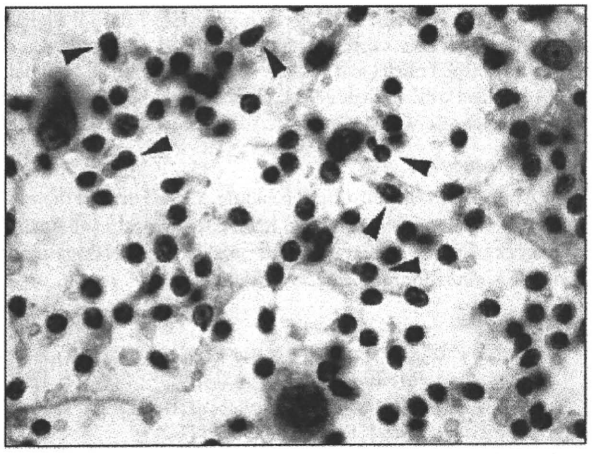

Figure 4 TBAC smear of LCL. Atypical small lymphoid cells with teardrop-shaped nuclei (arrowheads) (Papanicolaou stain, ×1,000).

Discussion

Complete and accurate mediastinal staging of patients with lung cancer is essential for determining prognosis and guiding optimal treatment strategies. Therefore, for patients with lung cancer accompanied by lymphadenopathy, it is important to indicate whether the cause of the lymphadenopathy is metastasis. For differential diagnosis of lymphadenopathy in patients with lung cancer, several reports have indicated that sarcoid reactions require particular attention in this regard.[22,23] For staging lung cancer, FNAC has demonstrated a sensitivity of 76.0–95.7%, a specificity of 100% and a diagnostic accuracy of 98.0%, with fewer false positive results than with CT and PET.[24,25] In our case, because extensive lymphadenopathy was apparent, FNAC was performed and a sarcoid reaction was diagnosed cytologically. On pathologic examination and immunohistochemical analysis of resected materials, however, lymph nodes were diagnosed as LCL.

LCL is now included in the category of peripheral T-cell lymphomas, unspecified, by the WHO classification.[1] Atypical lymphoid cells express variable T-lineage markers, such as CD2, CD3, CD4, CD5, CD7, CD8, CD43 and CD45RO, and lack expression of B-lineage markers.[9-17,26,27] Previously the immunocytochemical characteristics of LCL cells were thought to be CD4-positive and CD8-negative. In some cases of LCL, however, the neoplastic cells were CD8-positive and CD4-negative, a well-known variant of LCL.[28] Moreover, Epstein-Barr virus infection is occasionally

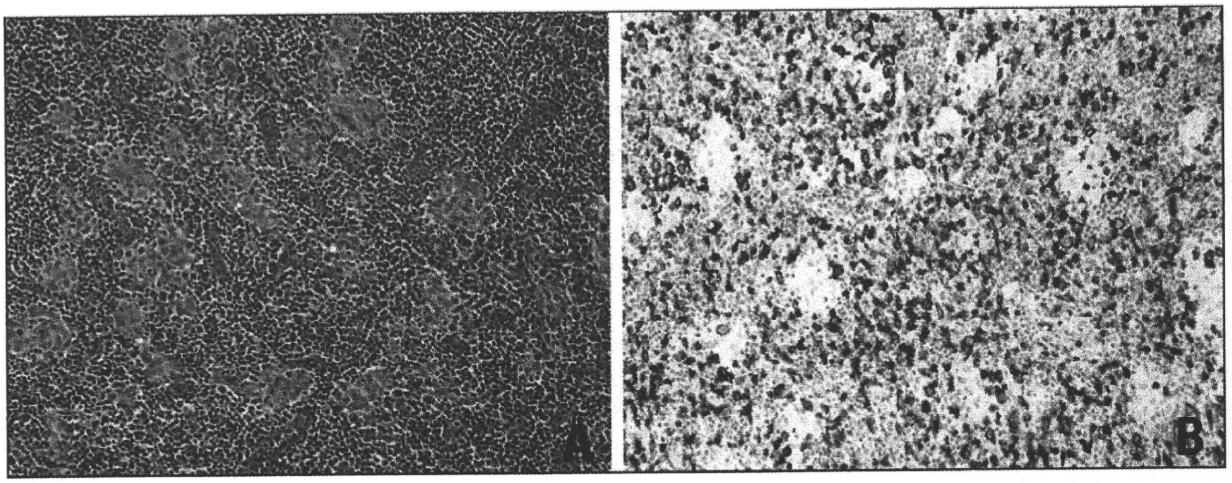

Figure 6 Histologic findings of LCL. (A) Aggregates of epithelioid cells interspersed between atypical lymphoid cells. (B) Immunoreactivity of lymphoid cells for CD8 (A, hematoxylin-eosin, ×200; B, immunostain, ×200).

demonstrated in reactive bystander B cells in LCL and is thought to be associated with a poor prognosis.[28] LCL patients usually present with generalized disease, often involving systemic lymph nodes.[29] In spite of morphologic features mimicking granulomatous disease, LCL is considered to show aggressive behavior clinicopathologically.[26,29] Therefore it is important to alert the clinician to the possibility of this disease.

Although several cytologic reports of LCL have appeared in the literature, simultaneous LCL with lung cancer has not described. Cytologic materials obtained from lymph nodes of our case included a lymphoid population consisting of a few large cells intermingled with numerous small cells. Moreover, these large lymphoid cells had hyperconvoluted nuclei and prominent nucleoli, raising the possibility of metastasis from co-present lung cancer. Cytologic discrimination of the large lymphoid cells was facilitated by the following characteristics: absence of adhesive clusters, round shape, scant and clear cytoplasm with distinct cell borders, round nuclei with thin nuclear membranes and finely granular and homogeneous nuclear chromatin. In spite of these distinctive features, however, we could not diagnose initially LCL in this case because of concern regarding the possibility of metastasis.

Review of the literature[11-17] shows the presence of atypical lymphoid cells with irregular nuclei to be a cytologic feature of non-Hodgkin's lymphoma (NHL), including LCL, on FNAC. As confirmed by the present case, it is important for cytologic diagnosis to focus on nuclear irregularity. In addition, the nuclei of small lymphoid cells frequently showed a teardrop shape in the smears of our case. To our knowledge, teardrop-shaped nuclei are rarely seen in smears obtained from normal lymph nodes or benign lesions such as granulomatous lymphadenitis. Therefore this feature may be of assistance for cytologic diagnosis of NHL, including LCL. Because many cases of NHL present as homogeneous populations of medium to large lymphocytes,[11-16,27] the finding of heterogeneity may point to an LCL.

In earlier cytology reports, the presence of epithelioid cell clusters showing small, monolayered and loose aggregates of spindle cells was a major feature on FNAC of LCL.[11-17] They were also present in our case. However, the existence of epithelioid cell clusters may in fact be a pitfall for cytologic diagnosis, because such clusters are also featured in sarcoid reactions.[13,30,31]

Several reports have indicated that Hodgkin's disease needs to be differentiated from LCL.[13,14,17] In the present case, the finding that atypical large lymphoid cells were smaller than Reed-Sternberg cells, together with the teardrop-shaped nuclei and binuclei, made for a clear diagnosis.

In conclusion, it is important to carefully examine the morphology of lymphoid cells on FNAC even if metastasis from a malignant tumor such as lung cancer is highly suspected. The presence of teardrop-shaped nuclei and nuclear irregularity of small lymphoid cells admixed with epithelioid cell clusters could be helpful for cytologic diagnosis of LCL.

Table I *Immunohistochemical Findings*

Primary antibodies	Source	Results
CD3	Dako	Weakly +
CD4	Nichirei	–
CD5	Novocastra	–
CD7	Novocastra	Weakly +
CD8	Nichirei	+
CD15	Becton-Dickinson	–
CD20	Dako	–
CD30	Dako	Weakly +
Perforin	Novocastra	–
Granzyme B	Dako	–

+ = Tumor cells expressed the antigen as strongly as normal T-cells of positive controls, weakly + = the tumor cells expressed the antigen more weakly than normal T-cells of positive controls.

Acknowledgments

We thank Mr. Masafumi Tsuzuku and Dr. Yasuo Hirai for their advice with cytology; Mss. Tomoko Kakita, Reimi Asaka and Kazuko Yokokawa for their technical expertise; and Drs. Ken Nakagawa and Sakae Okumura for their clinical assistance.

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Sensitive immunohistochemical detection of WT1 protein in tumors with anti-WT1 antibody against WT1 235 peptide

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The Wilms' tumor 1 (WT1) gene is overexpressed in leukemia and various types of solid tumor, such as lung and colorectal cancer, and plays an oncogenic role in their tumorigenesis. Recent studies have demonstrated the potential of WT1-targeting cancer immunotherapy in clinical settings. As expression of WT1 protein in tumor cells is a prerequisite for WT1-targeting immunotherapy, immunohistochemical methods to detect WT1 protein with high sensitivity and specificity are required. In the present study, we developed a rabbit polyclonal antibody (WT1-R) against the 9-mer WT1 235 peptide, which is used for vaccination. The specificity of WT1-R was confirmed by immunoprecipitation, western blotting analysis, and competitive enzyme-linked immunosorbent assay. Immunocytochemistry showed the same reactivity against five cell lines (K562, Daudi, HT-180, SW480, and PC-14), whereas levels of WT1 mRNA expression determined by real-time qPCR (RT-PCR) analysis were not equivalent. Next, we examined the reactivity of WT1-R in tissue samples compared with a previously developed anti-WT1 antibody, 6F-H2. WT1-R showed greater sensitivity for detecting WT1 protein expression in samples from four different breast cancer patients than 6F-H2 antibody. The discrepancy in WT1 expression between these methods suggested that immunohistochemical detection of WT1 peptide may be advantageous for predicting the efficacy of WT1 vaccine compared to RT-PCR, and the highly sensitive WT1 antibody, WT1-R, may be useful to detect WT1 protein in tumors. (*Cancer Sci* 2010; 101: 1089–1092)

Wilms' tumor 1 (WT1) mRNA is expressed at high levels in hematological malignancies and various cancers, while normal tissue shows only low levels of its expression.[1–6] The specific overexpression of WT1 in malignant cells makes it an attractive potential target for immunotherapy, including WT1-targeting vaccine therapy.[7–11] A peptide has already been developed as a vaccine and its clinical efficacy has been evaluated.[12,13]

Precise determination of WT1 protein expression in tumors would be useful to predict the efficacy of WT1 vaccine. Although the real-time quantitative PCR (RT-PCR) method is commonly used to measure WT1 expression, it is not a direct method to evaluate expression of the target of WT1 vaccine, which is a small part of the WT1 protein. In this regard, immunohistochemical analysis using antibodies that recognize peptide sequences in WT1 protein corresponding to the peptide target of WT1 vaccine may be better correlated with efficacy of the vaccine compared to the RT-PCR method. In addition, immunohistochemical analysis is sometimes preferred to RT-PCR as most solid tumors are diagnosed by histopathological analysis of paraffin-embedded tissues. Immunohistochemical analysis has a number of benefits for estimating the efficacy of WT1 vaccine, but no antibodies for this purpose are commercially available.[14–19] Here, we developed an antibody that is specific for a peptide corresponding to the region recognized by the WT1 vaccine and examined its specificity and reactivity.

Materials and Methods

Primary antibodies. A peptide corresponding to the wild-type human WT1 (WT1 235 peptide, CMTWNQMNL) was synthesized[11] and coupled to bovine thymoglobulin as a carrier protein. This complex of peptide and protein was used as an immunogen and used to immunize three rabbits four times every 2 weeks. The sera from all rabbits showed a high anti-WT1 peptide antibody titer 1 week after the last immunization (Immuno-Biological Laboratories, Gunma, Japan). A sufficient amount of blood volume was collected from the rabbits 9 weeks after the last immunization, and the serum samples were purified using Thiol Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ, USA) coupled with antigen peptide. This purified solution was used as anti-WT1 antibody (WT1-R). In addition, mouse monoclonal antibodies to WT1 (clone 6F-H2; Dako Cytomation, Carpinteria, CA, USA) and to glutathione *S*-transferase (GST; clone 40B3; BioPortfolio, Dorset, UK) were also used for comparison.

Purification of recombinant WT1. GST-WT1 and GST-delWT1 were produced according to the methods described previously.[20] Briefly, full-length WT1 and part of the *WT1* gene corresponding to amino acids 180–324 were ligated into the pGEX-5X-3 vector (GE Healthcare, Buckinghamshire, UK) and transfected into *E. coli*. After collection of bacterial cells and extraction of the protein, GST-WT and GST-delWT1 protein were purified and used for western blotting analysis.

Immunoprecipitation. Aliquots of 25 μg of GST-WT1 or GST-delWT1 and GST protein were incubated with 3 μg of purified anti-WT1 polyclonal antibody at 4°C overnight, and the immune complexes were collected by incubation with protein G-Sepharose beads at 4°C for 1 h. The beads were washed with TNE buffer (10 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 1 mM EDTA), and the proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer.

Western blotting analysis. Denatured proteins were fractionated by SDS–polyacrylamide gel electrophoresis and subsequently blotted onto polyvinylidene difluoride membranes. The filters were blocked with PBS containing 3.3% nonfat milk, 1% bovine serum albumin, and 0.05% NaN_3 at 37°C for 2 h, and

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then incubated overnight with the primary antibody. After binding of relevant peroxidase-conjugated secondary antibodies, the filters were developed with ECL (GE Healthcare).

Competitive enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with GST-WT1 protein (50 ng/well) at 4°C overnight, and then blocked with 0.1% casein in PBS at 4°C overnight. Serial dilutions of WT1 peptide were incubated with anti-WT1 antibody (WT1-R) at a concentration of 3 μg/mL at 4°C overnight. The mixtures were added to GST-WT1-coated plates and incubated at 37°C for 30 min. After incubation with peroxidase-conjugated antirabbit IgG, absorbance was determined at 492 nm with an ELISA reader.

Real-time quantitative-PCR (RT-PCR). For RT-PCR, total RNA was extracted from each cell line and reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Tokyo, Japan). Quantitative PCR was performed using a LightCycler (Roche Diagnostics) with LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. The primers used for PCR were as follows. Human GAPDH forward: 5′-TGAACGGGAAGCTCACTGG-3′, reverse: 5′-TCCACCACCCTGTTGCTGTA-3′; Human WT-1 forward: 5′-CCAGGCTGCAATAAGAGATA-3′, reverse: 5′-TCTTTTGAGCTGGTCTGAA-3′. PCR for human GAPDH was performed for 40 cycles consisting of 95°C for 10 s, 60°C for 10s, and 72°C for 12 s. Human WT-1 was amplified by 40 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 5 s.

Immunohistochemistry. For immunohistochemical staining, cells were fixed in PBS with 10% formaldehyde and then these fixed cells were embedded in agar blocks and sections were then cut using a microtome for immunostaining. Breast cancer and normal gastric mucosa specimens were obtained with informed consent (Pathology Institute Corporation, Toyama, Japan), and antigen retrieval was performed after deparaffinization of the slides by heating the sections in Tris-buffered saline, pH 9.0, in a water bath at 95°C for 40 min. Sections were allowed to cool to room temperature and endogenous peroxidase activity was blocked with 3% H_2O_2. The sections were then incubated with WT1-R or 6F-H2 as the primary antibody at a dilution of 1:500 (WT1-R) or 1:50 (6F-H2) for 30 min, followed by detection using the Dako EnVision/Polymer System (Dako, Ely, Cambridgeshire, UK). Sections were lightly counterstained with hematoxylin.

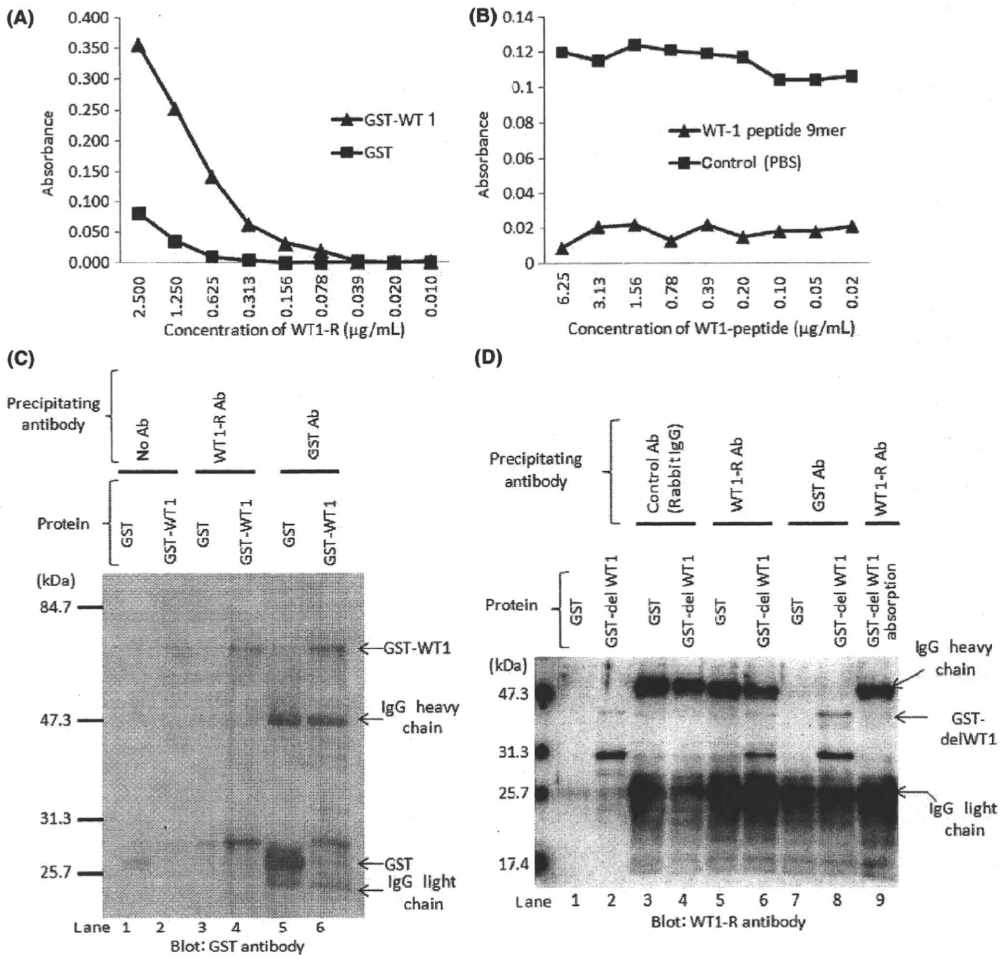

Fig. 1. Reactivity of WT1-R antibody against 9-mer peptide region of Wilms' tumor 1 (WT1) protein targeted by WT1 vaccine. (A) Dilution curves of WT1-R. Plates coated with WT1-GST or GST protein were incubated with serial dilutions of WT1-R antibody and the binding of WT1-R antibody to plates was determined. (B) Results of competitive ELISA. Serially diluted WT1 peptide was incubated with or without 3 mg/mL of WT1-R antibody, and this mixture was then added to GST-WT1-coated plates. (C,D) Western blotting analysis with GST or WT1-R antibody. Proteins were immunoprecipitated with the antibodies indicated above before electrophoresis.

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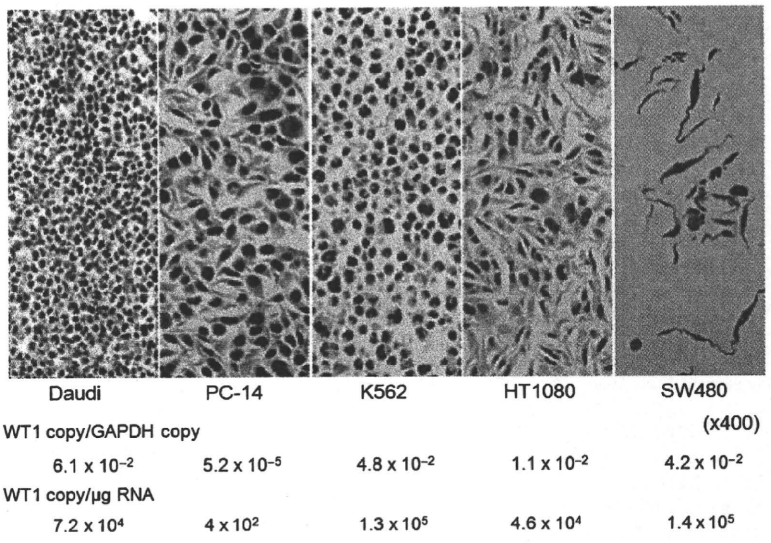

Fig. 2. Comparison between immunohistochemical staining with WT1-R and real-time quantitative PCR (RT-PCR) to detect Wilms' tumor 1 (WT1) expression in five cell lines (Daudi, PC-14, K562, HT1080, and SW480). RT-PCR results are shown beneath the photographs. Magnification, ×400.

Results

Anti-WT1 polyclonal antibody WT1-R binds specifically to GST-WT1 protein and 9-mer peptide corresponding to WT1 vaccine antigen. To assess antibody reactivity, WT1-R was serially diluted and its binding to plates coated with GST-WT1 protein and GST protein was examined. WT1-R bound strongly to GST-WT1-coated plates, whereas no binding was observed on GST-coated plates (Fig. 1A). To evaluate the specificity of WT1-R antibody, we performed competitive ELISA with WT1 peptide. As shown in Figure 1(B), antibody binding to GST-WT1 was markedly decreased by preincubation with WT1 peptide compared to control (PBS). Next, we examined antibody binding by immunoprecipitation and Western blotting analysis. As shown in Figure 1(C), GST-WT1 protein was immunoprecipitated by WT1-R and anti-GST antibodies and detected by anti-GST antibody (lanes 4 and 6). Figure 1(D) shows the results of western blotting analysis using WT1-R as the primary antibody. In this experiment, we used GST-delWT1 protein, which included the amino acid sequence targeted by WT1-R. Purified GST-delWT1 protein (lane 2) was detected by WT1-R, whereas WT1-R did not react with GST protein (lane 1). GST and GST-delWT1 protein were immunoprecipitated with control (rabbit IgG), anti-GST, or anti-GST antibody before western blotting, and WT1-R reacted only with GST-delWT1 immunoprecipitated by WT1-R (lane 6) and anti-GST antibody (lane 8). These bands disappeared when the antibodies were preincubated with 9-mer WT1-peptide before immunoprecipitation (lane 9). These results indicated that WT1-R antibody could detect WT1 protein and the binding was specific for part of WT1 protein corresponding to the 9-mer peptide used for WT1 vaccine.

Immunohistochemical analysis of WT1-R antibody. Immunohistochemical analysis of five cell lines (K562, Daudi, HT-1080, SW480, and PC-14) was performed and the results were

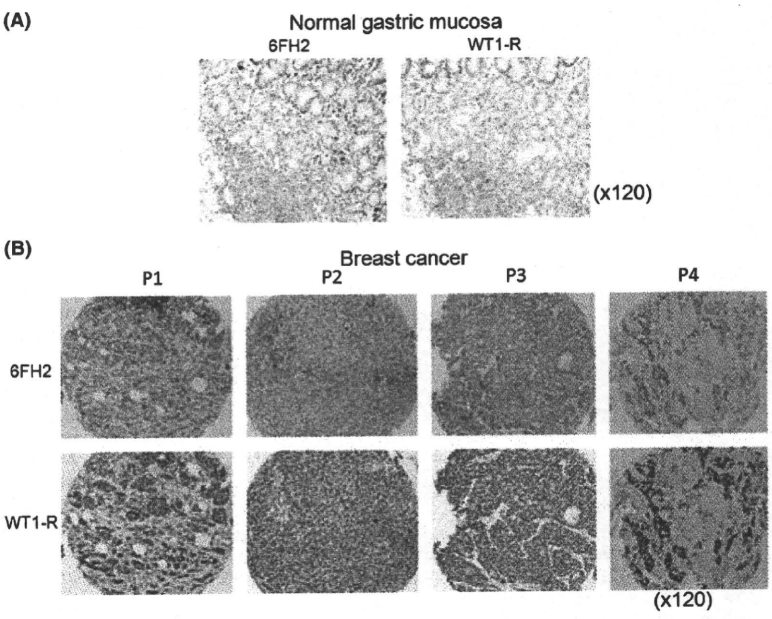

Fig. 3. Immunohistochemical analysis of normal gastric mucosa (A) and breast cancer tissue samples from four different patients (B). In (B), the upper and lower photographs show the results of immunohistochemical staining with 6F-H2 and with WT1-R, respectively. Magnification, ×120.

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compared with those of RT-PCR. As shown in Figure 2, K562 (WT1 copy number/GAPDH copy number: 0.048), Daudi (0.061), and SW480 (0.042) showed about 5–6-fold higher levels of expression than HT-1080 (0.01), and the expression level in PC-14 was almost 1000-fold lower (0.000052) than in the other lines. These results indicated that the expression was not equivalent in these cell lines although they expressed WT1. On the other hand, in contrast to the difference in WT1 mRNA expression between cell lines, immunohistochemical analysis with WT1-R showed almost the same binding intensity in these cell lines (Fig. 2).

Next, we compared the reactivity of WT1-R with that of 6F-H2 antibody in normal gastric mucosa tissue and breast cancer tissue samples obtained from four different patients (Fig. 3). In normal gastric mucosa, plasma cells showed nonspecific staining with 6F-H2 antibody, but were completely negative for staining by WT1-R with no nonspecific binding (Fig. 3A). In breast cancer tissues, immunostaining with 6F-H2 showed weak positive reactivity in breast cancer samples, while staining with WT1-R was clearly positive in both the nucleus and cytoplasm of breast cancer cells and the staining intensity was significantly higher with WT1-R than in 6F-H2 (Fig. 3B). These observations indicated that WT1-R antibody is more sensitive for detection of WT1 protein in breast cancer than 6F-H2 antibody.

Discussion

RT-PCR is widely employed for determination of WT1 expression.[11,21] However, the results of RT-PCR are not always correlated with protein expression[22] and this method is not suitable for tumors that consist of cells of various types, including malignant and non-malignant cells, because the results are dependent on the proportion of malignant to normal cells. Therefore, immunohistochemical analysis of WT1 expression in solid tumors may be a better option than RT-PCR. In the present study, the results of immunohistochemical analysis of WT1 expression were not correlated with those of RT-PCR. The level of WT1 mRNA transcript expression in PC-14 was very weak, but protein expression level was almost the same as in the other cell lines. This observation suggested that WT1 mRNA expression is not equivalent to its protein expression. A discrepancy between WT1 mRNA and protein expression was reported previously in childhood leukemia.[22] These results suggested that immunohistochemical analysis is important to predict the efficacy of WT1 vaccines. The WT1-R antibody developed in the present study shows sensitivity for detection of WT1 protein and may be useful for immunohistochemical analysis.

Currently, there is no standard method for immunohistochemical analysis of WT1 because of a lack of appropriate antibodies. It has already been reported that staining results with 6F-H2 and another antibody against WT1 showed marked differences in some types of tumor.[21] The WT1-R antibody developed in the present study showed high sensitivity for detection of WT1 protein in breast cancer samples compared with 6F-H2 antibody, and may be appropriate for immunohistochemical analysis of WT1. Further studies of the sensitivity and specificity of WT1-R antibody in various types of cancer are required.

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Disclosure Statement

The authors have no conflict of interest.

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Ocular Involvement by Epstein-Barr Virus–Positive Diffuse Large B-Cell Lymphoma of the Elderly: A New Disease Entity in the World Health Organization Classification

The new World Health Organization classification of lymphoma places great emphasis on the definition of real biological disease entities in the category of diffuse large B-cell lymphoma (DLBCL). Epstein-Barr virus (EBV)–positive DLBCL of the elderly is a new subtype of DLBCL according to the 2008 World Health Organization classification. This is an extremely rare tumor, and no case of ocular EBV-positive DLBCL of the elderly has been reported to our knowledge. Here we describe the first case of EBV-positive DLBCL of the elderly with involvement of the eyelid and orbit.

Report of a Case. An 83-year-old woman was referred with an eyelid tumor of her left eye. Serologically, human immunodeficiency virus antigen and anti–human T-cell lymphoma virus 1 antigen were both negative. The left upper and lower eyelids were affected by a hyperemic tumor with a rough surface and a small scab, which caused ectropion (**Figure 1**A and B). Physical examination revealed that her left submandibular, parotid, and cervical lymph nodes were enlarged. Staging examination showed that she had stage III B-cell malignant lymphoma according to the Ann Arbor classification. Orbital magnetic resonance imaging revealed a cystic mass in the left periorbital region, invading the orbit and the anterior ethmoid sinus (Figure 1F and G). Incisional biopsy of the left eyelid lesion was performed, revealing a tumor composed of pleomorphic large cells and marked by the presence of mononuclear Hodgkin-like cells and multinucleated Reed-Sternberg–like cells (**Figure 2**A and B). Immunohistochemistry revealed that the tumor cells expressed CD20 (Figure 2C), Pax-5 (Figure 2D), CD30 (Figure 2E), and multiple myeloma oncogene 1 protein (not shown). The tumor cells were also positive for latent membrane protein 1 (Figure 2F) but negative for EBV nuclear antigen 2 (not shown), indicating that the EBV infection pattern in this case was type 2 latency. Staining results for CD5, CD10, and the follicular B-cell lymphoma marker bcl-6 were negative (not shown). Based on these findings, a diagnosis of EBV-positive DLBCL of the elderly was made according to the new World Health Organization lymphoma classification. Cervical lymph node involvement was also confirmed by biopsy. Immediately after the biopsy, the patient received standard chemotherapy with rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, and prednisone, followed by rituximab and

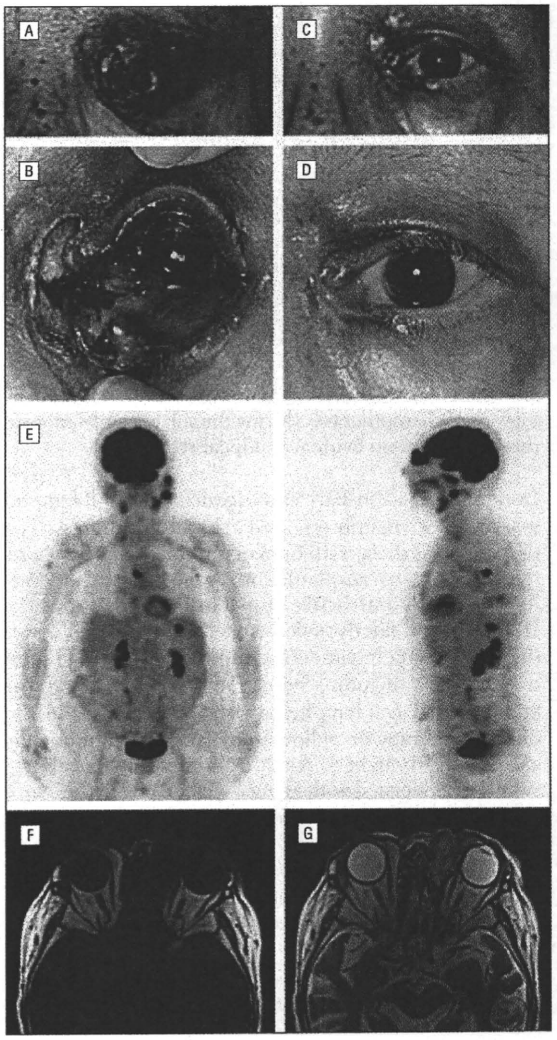

Figure 1. Images from a patient with Epstein-Barr virus–positive diffuse large B-cell lymphoma of the elderly. A and B, Before starting chemotherapy. C and D, After completion of chemotherapy. E, Positron emission tomography confirms marked left orbital uptake. There is also uptake in the spleen, mediastinum, and involved lymph nodes. F and G, Orbital magnetic resonance imaging shows a cystic mass in the left periorbital region that invades the orbit and anterior ethmoid sinus and is accompanied by eyelid ulceration. The mass shows heterogeneous low signal intensity on a T1-weighted axial image (F) and high signal intensity on a T2-weighted axial image (G).

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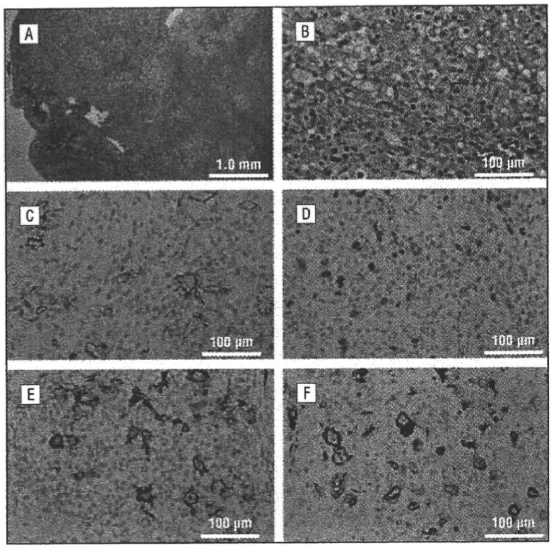

Figure 2. Histological examination findings from the eyelid of a patient with Epstein-Barr virus–positive diffuse large B-cell lymphoma of the elderly. A, A diffuse lymphoid infiltrate with necrosis can be seen at low power (hematoxylin-eosin). B, At higher power, the infiltrate comprises large and pleomorphic tumor cells, including Hodgkinlike cells and Reed-Sternberg–like giant cells, with a background of smaller reactive inflammatory cells (hematoxylin-eosin). The tumor cells express B-cell markers CD20 (C) and Pax-5 (D), while CD30 (E) and latent membrane protein 1 (F) are also positive.

etoposide. She achieved near complete remission after 4 months of chemotherapy. During the subsequent 4 months, there has been no evidence of local recurrence.

Comment. Epstein-Barr virus–associated B-cell lymphomas have been mainly reported in immunosuppressed patients such as those with human immunodeficiency virus infection, organ transplantation, or methotrexate therapy for rheumatoid arthritis.[1] Epstein-Barr virus–positive DLBCL of the elderly is defined as a lymphoproliferative disorder arising in patients without predisposing immunodeficiency, including human immunodeficiency virus and human T-cell lymphoma virus 1 infection, a history of chemotherapy or radiotherapy, and autoimmune disease, and is thought to result from immunological deterioration associated with aging.[2,3] According to the World Health Organization classification, EBV-positive DLBCL of the elderly is a rare DLBCL that accounts for 8% to 10% of DLBCL among patients without predisposing immunodeficiency in Asian countries.[4] Interestingly, EBV-positive DLBCL of the elderly frequently involves extranodal sites.[3,5] Most patients with extranodal disease also have nodal disease. In fact, 70% of patients have extranodal disease affecting sites such as the skin, lung, tonsils, or stomach, while 30% of patients have lymph node involvement alone.[4] However, no case of ocular involvement, which is an extranodal site, has been reported. To our knowledge, our case is the first report of EBV-positive DLBCL of the elderly involving the eyelid and orbit.

Epstein-Barr virus–positive DLBCL of the elderly with ocular involvement is totally distinct, both clinically and pathologically, from extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, an indolent tumor that is the most common form of lymphoma in the orbital region. Although relatively uncommon, EBV-positive DLBCL of the elderly should be considered in the differential diagnosis of ocular lymphoma since it needs to be treated appropriately as a highly aggressive lymphoma.

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Severe Retinal Vascular Infarction After Photodynamic Therapy With Verteporfin Using the Standard Protocol

Photodynamic therapy (PDT) with verteporfin has been widely used for the treatment of choroidal neovascularization associated with age-related macular degeneration, with both the efficacy and safety of PDT considered to be at tolerable levels. Herein, we describe a patient who experienced severe retinal vascular infarction exactly corresponding to the irradiated spot following PDT using the standard protocol.

Report of a Case. A 60-year-old man was referred because of decreased vision in the right eye. He had a history of diabetes mellitus, systemic hypertension, arteriosclerosis obliterans, and renal failure requiring dialysis. The right

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Activation status of receptor tyrosine kinase downstream pathways in primary lung adenocarcinoma with reference of *KRAS* and *EGFR* mutations

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ABSTRACT

The activation status of signal transduction pathways involving receptor tyrosine kinases and its association with *EGFR* or *KRAS* mutations have been widely studied using cancer cell lines, although it is still uncertain in primary tumors.

To study the activation status of main components of growth factor-induced pathways, phosphorylated Akt (pAkt), extracellular signal-regulated kinases 1 and 2 (pERK) and other downstream proteins were immunohistochemically examined using surgical samples of 193 primary lung adenocarcinomas. Also, thyroid transcription factor-1 (TTF-1) expression and mutation status of *EGFR* and *KRAS* were examined.

Advanced tumor stages ($p < 0.001$), negative TTF-1 expression ($p < 0.001$) and Akt activation ($p = 0.015$) were independent and significant poor prognostic markers. Akt activation related to advanced stage ($p = 0.021$), invasiveness ($p = 0.004$), and not to mutations. TTF-1 expression associated with never-smoker ($p = 0.013$), pre- or minimally invasiveness ($p < 0.001$) and *EGFR* mutations ($p = 0.017$) as well as with pERK ($p = 0.039$) expression. *EGFR* mutations did not correlated with pAkt and pERK expression, which was different from the results based on cultured cells, while *KRAS* mutations were solely and significantly linked to ERK activation ($p = 0.009$).

In lung adenocarcinoma, tumors with TTF-1 expression have distinct characteristics regarding mutations, signal protein activation and clinical issues. Moreover, this property was revealed to be important in outcome estimation at any tumor stage, whereas Akt activation is abnormally affected according to the tumor stage regardless of their cell origin. The signal proteins were differently related to mutation status from cultured cells.

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1. Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide [1] and adenocarcinoma is recently becoming a frequent histologic type among non-small-cell lung cancers (NSCLCs) in many countries. Gefitinib, an inhibitor of epidermal growth factor receptor (*EGFR*) tyrosine kinase, has shown remarkable efficacy for control of a subset of lung adenocarcinomas, reflecting improved understanding of the underlying biology [2]. Especially the detection of somatic mutations in *EGFR* shed light on the mechanisms of acquisition of tumor growth advantage, featuring dysregulated signal transduction in tumor cells [3,4].

Akt, a serine/threonine kinase, and extracellular signal-regulated kinases 1 and 2 (ERK) are major target proteins, downstream of EGFR and various other oncoproteins such as Ras and Raf. They are known to be activated in a wide spectrum of human cancer together with various downstream substrates such as glycogen synthase kinase 3-beta (GSK3B), mammalian target of Rapamycin (mTOR), p70 ribosomal protein S6 kinase (S6K) and forkhead proteins FKHR/FKHRL1 (FKHR) [5–8] and to play central roles in tumorigenesis or cell proliferation. The present study was performed to elucidate, by immunohistochemistry (IHC), whether there might be selective activation of downstream pathways of receptor tyrosine kinases (RTKs) in lung adenocarcinomas, depending on tumor-cell lineage or with/without *EGFR* and *KRAS* mutations. We further evaluated the clinicopathological and prognostic significance of such activation in various adenocarcinoma subtypes. Since we earlier revealed by expression profiling that

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adenocarcinoma cell lines might have different characteristics of gene expression from clinical adenocarcinomas [9], we here used tissue materials of surgically resected adenocarcinomas rather than cell lines.

2. Materials and methods

2.1. Patients and pathological review

A series of 193 Japanese cases with primary lung adenocarcinoma surgically resected between 1998 and 2001 at the Department of Thoracic Surgical Oncology, The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, were selected for the present study. Informed consent was obtained from all the subjects at the time of surgery. This study was approved by the Institutional Review Board of the JFCR. All patients were staged pathologically according to the 5th edition of the UICC-TNM staging system [10]. For accuracy of survival analysis, only death of lung cancer was counted as cause-specific death. Smoking history was ascertained with all patients in detail.

Histological diagnosis was made according to the WHO classification [11], using sections through the largest cut surface of each tumor stained by hematoxylin–eosin and alcian-blue methods and PAS reaction. However, with its subdivision of lung adenocarcinomas, more than 80% tumors fell into the mixed subtype category. We therefore additionally used an noninvasive/invasive dichotomy as well as a predominance classification for invasive carcinomas, which is mostly based on the WHO classification except for the mixed subtype, such as bronchioloalveolar carcinoma (BAC) predominant, papillary predominant, acinar predominant, etc. The noninvasive carcinoma includes BAC. In the predominance classification of invasive carcinomas, we diagnosed by a component that makes up the predominant portion in the largest cut surface, or the cut surface containing a solid part shown by CT scans, of a tumor. Also, we employed a concept of "minimally invasive adenocarcinomas", which were defined to be lesions where an invasive area of less than 5 mm in diameter or less.

2.2. Tissue microarrays

Tumor tissues were fixed in 15% neutral formalin and embedded in paraffin. Three histologically representative sites were selected per tumor, considering the well-known heterogeneity of lung adenocarcinomas (including the peripheral boundary and the central part of each tumor) and tissue microarrays were constructed as follows. Selected points of the donor paraffin blocks of the largest cut surface were punched with a 2-mm-diameter coring needle, and transferred to the array in the recipient block using a manual tissue arrayer (KIN-1, Azumaya, Inc, Japan). 48 human tissue rods (16 tumors) were embedded in one tissue array block. Based on our preliminary examinations using whole sections of tumor for several cases, we adopted 2 mm needles and three points to take tissues, rather than smaller needles and only one or two points. As controls, 3 mouse xenografts were selected from the panel of 39 cell lines (termed JFCR 39) [12] and embedded together with clinical samples in each array block as detailed below.

2.3. Protein expression analysis

Phosphorylated protein levels of Akt, ERK, GSK3B, mTOR, S6K and FKHR were immunohistochemically examined using antibodies for phosphorylated proteins designated by pAkt, etc. Also, thyroid transcription factor-1 (TTF-1) was examined for cell lineage analysis. The primary antibodies and citrate buffer used in this study are listed in Suppl. Table 1 and details of our immunohistochemical technique are also available on this. Antigen–antibody complexes were detected by labeling with the Envision+/HRP system (DAKO, Carpinteria, CA, USA), using 3,3′-diaminobenzidine tetrachloride as the chromogen and hematoxylin as counterstain. As well as using some normal cells as internal controls, mouse xenografts of the three cancer cell lines (PC-3, a prostate cancer line, showing high pAkt and low pERK expression, U251, a brain tumor line, showing moderate pAkt and low pERK expression and HTB26, a breast cancer line, showing low pAkt and high pERK expression) were included as external controls (Fig. 1), because we sought better quantification of immunoreactivity of each antibody by using well-known cell lines with well-documented reactivity. Immunoreactivity of each case was evaluated for all the tumor cells (or other cells of interest) appearing in all the three portions, applying the staining results for these xenografts. Essentially, for pAkt and pFKHR cytoplasmic staining (Fig. 1 and Suppl. Fig. 1), and for pS6K and pGSK3B whole cell staining were evaluated respectively, referring to the U251 levels. For pmTOR staining, comparison was with HTB26. All these were recorded as dichotomous parameters categorized as "negative" (weaker than or equal to xenograft staining) and "positive" (stronger than xenograft staining). For pERK immunoreactivity, the percentages of cells with positive staining were recorded and a score of 10% or less was categorized as "negative" and a score of more than 10% as "positive".

2.4. Mutation analysis of EGFR and KRAS

The mutation status of four exons of the *EGFR* gene and three codons of the *KRAS* gene was evaluated in the subset ($n=93$) of the 193 cases. The primer sequences for exons 18 and 21 of *EGFR* were as follows (forward and reverse, respectively), exon 18 (5′-TCCAAATGAGCTGGCAAGTG-3′ and 5′-TCCCAAATACTCAGTGAACAAA-3′), exon 21 (5′-GATGCAGAGCTTCTTCCCAT-3′ and 5′-ATACAGCTAGTGGGAAG GCA-3′). For *KRAS*, codon 12 and codon 13 (5′-CCTTATGTGTGACATGTTCT-3′ and 5′-CTATTGTTGGATCATATTCG-3′), codon 61 (5′-TTCCTACAGGAAGCAAGTA-3′ and 5′-GGCAAATACACAAAGAAAGC C-3′). All PCR assays were carried out in a 20 μL volume that contained 0.2 μL of Taq DNA polymerase (NEB Phusion TM High Fidelity DNA polymerase sets, Finnzymes Oy, Finland). DNA was amplified for 35 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a 7 min extension at 72 °C. All PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (USB corporation, Exo SAP-IT, OH, USA) according to the manufacturer's instructions and then sequenced directly by a cycle sequencing method (Beckman Coulter Inc, DTLS-Quick Start Kit, CA, USA). All sequence variants were confirmed by sequencing the products of independent PCR amplifications in both directions. To detect deletion in exon 19 and insertion in exon 20 of *EGFR*, common fragment analysis was used. Sample DNA was amplified with a Cy5-labeled primer set as follows: exon 19 (5′ Cy5-GTCTTCCTTCTCTCTCTGTCAT-3′ and 5′-TGTGGAGA GTGAGCAGGGTCT-3′), exon 20 (5′ Cy5-A CCATGCGAAGCCACACTGA-3′ and 5′-TCCTTATCTCCCCTCCCCGTAT-3′) and any deletion or insertion mutation was detected as a new peak of amplified products in the electrophoregram.

2.5. Statistical analysis

Statistical analyses were accomplished with STATA software, version 9 (Stata Corp. LP, College Station, TX, USA) and statistical programming language of R [13]. We studied the relationships between the survival and other clinicopathological factors and phospho-protein expression by univariate analyses of log-rank test. Then multivariate analyses using Cox regression model together with an AIC (Akaike Information Criteria) stepwise selection were applied to those factors to evaluate their relative risks. Then, correlation coefficients between each clinicopathological or