transcription signaling pathways: altogether, these promote cell survival [4,6].

EGFR-TKIs are competitive inhibitors of the adenosine triphosphate-binding clefts within the tyrosine kinase domain of *EGFR* [7]; they effectively inhibit the critical antiapoptotic signals transduced by the mutant receptors [6]. The clinicopathologic parameters of female gender, East Asian ethnicity, adenocarcinoma histology, and nonsmoking status are strong predictors of the response to EGFR-TKIs [4,5,8,9]. Moreover, the DEL and L858R mutations were also revealed to be strong predictors [10–14]. Therefore, the detection of such mutations provides both patients and physicians with important information regarding the optimal choice for therapy.

Direct sequencing is the gold standard method to detect *EGFR* mutations. However, to obtain precise data, high-quality DNA extracted from an adequate amount of pure tumor cells is required, and this is expensive and time-consuming. Recently, other indirect methods were developed to detect *EGFR* mutations, including Scorpion ARMS, the peptide nucleic acid-locked nucleic acid PCR clamp, mutant-enriched PCR, the smart amplification process, and high-resolution melting analysis (HRMA) [15,16]. These methods have high sensitivities, and can be applied to specimens in which cancer cell content is low. However, they invariably require technical labor and sophisticated instruments, and are therefore, not applied in most pathology laboratories.

Compared to molecular techniques, immunohistochemistry (IHC) is a fast and cost-effective method that can be performed in most pathology laboratories on not only fresh, but also archival, formalin-fixed tissue samples. Recently, some authors revealed the correlation between EGFR mutations and EGFR phosphorylation detected by IHC [17,18]. Additionally, EGFR phosphorylation antibodies exhibited a correlation with response to EGFR-TKIs [18]. However, these antibodies recognize EGFR phosphorylation regardless of mutational status. More recently, highly sensitive and specific rabbit monoclonal antibodies against the 2 most common mutations were developed for detecting *EGFR* mutations [19–24].

The main purpose of the present study was to explore the use of the 2 mutation-specific antibodies for DEL and L858R for detecting *EGFR* mutations. Additionally, we compared the molecular-based and the IHC-based *EGFR* mutational status to the response to EGFR-TKI.

## 2. Materials and methods

### 2.1. Case selection

After obtaining institutional review board approval, the specimens used in the present study were obtained from 577 Japanese patients who underwent a surgical resection for primary lung adenocarcinoma at the National Cancer Center Hospital, Tokyo, Japan, between 1993 and 2009. Histological diagnosis was based on the latest World Health Organization classification of lung tumors [25].

### 2.2. Analysis of EGFR mutational status by molecular technique

The materials analyzed for the molecular-based mutational status were as follows: fresh frozen (in liquid nitrogen), surgically resected tissue specimens from 505 patients (88%); methanol-fixed, paraffin-embedded, surgically resected tissue specimens from 36 patients (6%); and ethanol-fixed, imprint cytologic smears obtained from the fresh-cut surface of resected tumor specimens from 36 patients (6%). We used HRMA for detecting the DEL and L858R mutations, routinely performed at our institution. HRMA is well validated, and has been previously shown to accurately reflect *EGFR* mutational status [15].

### 2.3. Tissue microarray construction

The representative tumor regions to be sampled for the tissue microarray (TMA), were carefully selected and marked on a hematoxylin-eosin-stained slide. The TMAs were assembled using a manual tissue-arraying instrument (Azumaya, Tokyo, Japan). Considering tumor heterogeneity, 2 replicate 2-mm cores were routinely sampled from different regions of each tumor.

### 2.4. Immunohistochemistry

For the immunohistochemical staining, the 4-μm-thick TMA sections were deparaffinized. A heat-induced epitope retrieval with Target Retrieval Solution (Dako, Carpinteria, CA, USA) was performed. The primary antibody used were a rabbit monoclonal antibody against human *EGFR* with the DEL (E746-A750del) mutation (1:100, clone 6B6, Cell Signaling Technology, Danvers, MA, USA) and a rabbit monoclonal antibody against human *EGFR* with the L858R mutation (1:200, clone 43B2, Cell Signaling Technology). The antibodies were diluted in SignalStain (Cell Signaling Technology), and slides were incubated with each primary antibody for 1 h, at room temperature. The immunoreactions were detected using the EnVision Plus system (Dako) and 3,3′-diaminobenzidine, followed by counterstaining with hematoxylin. We used positive and negative controls for the IHC that previously confirmed the mutational status by using molecular analyses.

### 2.5. Immunohistochemical scoring system for mutation-specific antibodies

The immunoreactivity for each mutation-specific antibody was evaluated by using light microscopy at magnifications of 4 and 10× with objective lenses. Immunoreactivity was classified on the basis of cytoplasmic intensity. The following scoring system was used: negative intensity, 0 (defined as no immunoreactivity with any intensity); weak intensity, 1 (defined as the immunoreactivity only observed in 10× objective lenses); moderate intensity, 2 (defined as the immunoreactivity easily detected in 4× objective lenses, but less intense than the positive control); and strong intensity, 3 (defined as immunoreactivity equal to or stronger than the positive control; Fig. 1A and B). We also evaluated the extent of each intensity as a percentage (0–100%). Next, an expression score was obtained by multiplying the intensity by the percentage values (range, 0–300) for each core. Finally, the staining scores obtained in 2 cores were averaged, and the result was used as the representative score for each case. In the case of loss of tumor cells in 1 of the 2 cores during IHC, the staining score for the other core was used. We set the threshold at a staining score of 10; therefore, a staining score <10 was categorized as negative and a score ≥10 was categorized as positive. Additionally, we set another threshold for positive cases, defined as >50% of immunopositive tumor cells with any intensity.

### 2.6. Evaluation of the response to EGFR-TKI

Of the 577 patients, 116 received systemic therapy with EGFR-TKI gefitinib (250 mg daily) after tumor relapse. The therapeutic effect of gefitinib was complete response (CR) in 3, partial response (PR) in 61, stable disease (SD) in 13, and progressive disease (PD) in 37. Two patients were not evaluable for the clinical response due to the withdrawal of gefitinib caused by drug-induced liver dysfunction. The clinical response to gefitinib was determined using standard bidimensional measurements [26]. Responders were defined as patients with CR or PR, and non-responders were defined as patients with SD or PD.

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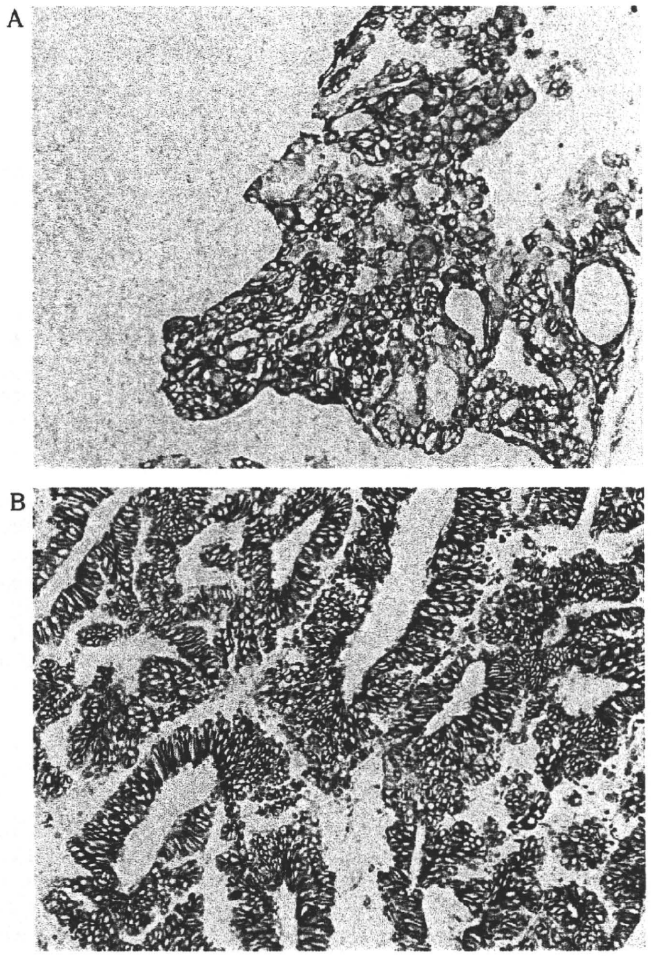


**Fig. 1.** A representative immunohistochemistry staining of intensity 3 for the DEL-specific antibody (1A, top) and the L858R-specific antibody (1B, bottom). The case 1A/1B harbored the molecular based DEL/L858R status.

### 2.7. Statistical analyses

Statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Chi-square tests for categorical variables were used and $p < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Clinicopathologic parameters

There were 319 males and 258 females with median age at surgery being 60 years (range, 30–82). A total of 343 patients had never/light smoking status with Brinkman index of <400, and 234 patients had smoking status with Brinkman index of ≥400. The pathological tumor stage (p-stage) was I in 331, II in 74, III in 164, and IV in 8 cases.

### 3.2. Molecular-based EGFR mutational status

After analyzing the *EGFR* mutational status by HRMA, DEL (m-DEL) was detected in 135 cases (23%), and L858R (m-L858R) was detected in 172 cases (30%). The remaining 270 cases (47%) were regarded as wild-type (m-WT), because neither the DEL nor the L858R mutation was detected.

**Table 1A**
Usefulness of DEL-specific antibody in detecting *EGFR* mutation of DEL under the threshold for the mutation-positive defined as staining score ≥10 and >50% of immunopositive tumor cells.

| IHC-based *EGFR* mutation of DEL | Molecular-based *EGFR* mutation of DEL | |
|---|---|---|
| Staining score ≥10 | (+) | (−) |
| (+) | 57 | 2 |
| (−) | 78 | 440 |
| Sensitivity = 42.2%; specificity = 99.5% | | |
| >50% of immunopositive tumor cells | | |
| | (+) | (−) |
| (+) | 28 | 0 |
| (−) | 107 | 442 |
| Sensitivity = 20.7%; specificity = 100.0% | | |

EGFR, epidermal growth factor receptor; DEL, deletions in exon 19; IHC, immunohistochemistry.

### 3.3. IHC-based EGFR mutational status

Although the tumor tissues of 52 of the 2308 cores (2.3%) were lost during the IHC procedure, at least 1 of the 2 cores contained tumor tissue in all cases. A positive immunoreactivity for the DEL-specific antibody was observed in 59 cases (10%). A positive immunoreactivity for the L858R-specific antibody was observed in 139 cases (24%). The remaining 379 cases were regarded as negative because neither the DEL- nor the L858R-specific antibody was positive. The immunohistochemical expression using DEL- and L858R-specific antibodies was mutually exclusive.

### 3.4. Correlation between the molecular-based and the IHC-based EGFR mutational status

We compared the molecular-based and IHC-based mutational status using molecular-based mutational status as the gold standard. The 59 cases that were positive for the DEL-specific antibody consisted of 57 cases with m-DEL, and 2 cases with m-WT. The sensitivity and specificity for the DEL-specific antibody was 42.2% and 99.5%, respectively (Table 1A). The 139 cases that were positive for the L858R-specific antibody consisted of 130 cases with m-L858R, and 9 cases with m-WT. The sensitivity and specificity for the L858R-specific antibody was 75.6% and 97.8%, respectively (Table 1B). Combining the results using these 2 antibodies, the overall sensitivity and specificity were 60.9% and 98.7%, respectively.

**Table 1B**
Usefulness of L858R-specific antibody in detecting *EGFR* mutation of L858R under the threshold for the mutation-positive defined as staining score ≥10 and >50% of immunopositive tumor cells.

| IHC-based *EGFR* mutation of L858R | Molecular-based *EGFR* mutation of L858R | |
|---|---|---|
| Staining score ≥10 | (+) | (−) |
| (+) | 130 | 9 |
| (−) | 42 | 396 |
| Sensitivity = 75.6%; specificity = 97.8% | | |
| >50% of immunopositive tumor cells | | |
| | (+) | (−) |
| (+) | 83 | 5 |
| (−) | 89 | 400 |
| Sensitivity = 48.3%; specificity = 98.8% | | |

EGFR, epidermal growth factor receptor; L858R, L858R mutation in exon 21; IHC, immunohistochemistry.

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**Table 2**
Correlation between the clinicopathologic parameters of 577 patients and the response to EGFR-TKI.

| | Responder (CR + PR, $n = 64$) | Non-responder (SD + PD, $n = 50$) | $p$-Value |
|---|---|---|---|
| Age | | | |
| ≥65 | 20 | 18 | 0.690 |
| <65 | 44 | 32 | |
| Gender | | | |
| Male | 35 | 27 | 1.000 |
| Female | 29 | 23 | |
| Smoking status | | | |
| Brinkman index <400 | 45 | 33 | 0.687 |
| Brinkman index ≥400 | 19 | 17 | |
| p-Stage | | | |
| IA–IIB | 26 | 19 | 0.848 |
| IIIA–IV | 38 | 31 | |

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

### 3.5. Correlation between the molecular-based and IHC-based EGFR mutational status under another threshold

Positive immunoreactive cases for the DEL- or the L858R-specific antibody exhibited lower sensitivities and higher specificities when the threshold for the mutation-positive cases was restricted to >50% of the immunopositive tumor cells with any intensity. The incidence of positive immunoreactive cases for the DEL-specific antibody decreased from 59 to 28 cases—all of which were m-DEL (sensitivity, 20.7%; specificity, 100.0%; Table 1A). The incidence of positive immunoreactive cases for the L858R-specific antibody decreased from 139 to 88 cases, with 83 m-L858R cases and 5 m-WT cases (sensitivity, 48.3%; specificity, 98.8%; Table 1B).

### 3.6. Comparison of the molecular-based and IHC-based EGFR mutational status and the response to EGFR-TKI

A total of 114 patients were evaluable for the clinical response to EGFR-TKI. They consisted of 38, 39, and 37 patients with tumors harboring m-DEL, m-L858R, and m-WT, respectively; therefore, 68% of patients harbored the molecular-based *EGFR* mutations, and the remaining 32% harbored wild-type *EGFR*. The correlation between the conventional clinicopathologic parameters and the response to EGFR-TKI is shown in Table 2. In the present study, none of these parameters were significantly correlated with the response to EGFR-TKI.

Among the 77 patients harboring the molecular-based *EGFR* mutations, 59 (77%) were responders. In contrast, among the 37 patients without molecular-based *EGFR* mutations, only 5 (14%) were responders. Among the 55 patients with the IHC-based *EGFR* mutations, 40 (73%) were responders. In contrast, among the 59 cases without IHC-based *EGFR* mutations, 24 (41%) were responders (Table 3). Both the molecular- and IHC-based mutational statuses were significantly correlated with the response to EGFR-TKI ($p < 0.001$ and $p = 0.001$, respectively). We analyzed another threshold of the mutation-specific antibodies, defined as mutation-positive in >50% of the immunopositive tumor cells with any intensity. However, this threshold resulted in a slightly weaker correlation between the IHC-based mutational status and the response to EGFR-TKI ($p = 0.012$, Table 3).

### 3.7. Multivariate analysis of the response to EGFR-TKI

A multivariate analysis of the response to EGFR-TKI with 2 variables (molecular-based mutational status and IHC-based mutational status), which showed a significant correlation by univariate analysis, was performed; only the molecular-based mutational status was significantly correlated with the response to EGFR-TKI ($p < 0.001$). The IHC-based mutational status ($p = 0.211$) was not significantly correlated (Table 4).

**Table 3**
Comparison of the molecular-based and IHC-based *EGFR* mutational status and the response to EGFR-TKI.

| | Responder (CR + PR, $n = 64$) | Non-responder (SD + PD, $n = 50$) | $p$-Value |
|---|---|---|---|
| Molecular-based *EGFR* mutation | | | |
| (+) | 59 | 18 | <0.001 |
| (−) | 5 | 32 | |
| IHC-based *EGFR* mutation | | | |
| Staining score ≥10 | 40 | 15 | 0.001 |
| Staining score <10 | 24 | 35 | |
| Immunopositive tumor cells >50% | 24 | 8 | 0.012 |
| Immunopositive tumor cells ≤50% | 40 | 42 | |

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; IHC, immunohistochemistry.

## 4. Discussion

In the present study, we investigated the clinical usefulness of IHC using 2 rabbit monoclonal antibodies against specific mutant *EGFRs* in lung adenocarcinomas. We found that the IHC-based *EGFR* mutational status detected by these antibodies was significantly correlated with the molecular-based *EGFR* mutational status. Furthermore, the IHC-based mutational status showed a significant correlation with the clinical response of tumors in conjunction with EGFR-TKI therapy.

The overall specificity of the 2 mutation-specific antibodies was 99%, and this specificity was consistent with that reported previously [19–24]. There were 11 cases in which the results of IHC examination were positive and those of molecular testing were negative. These false-positive cases might harbor other types of mutations that induce conformational changes in the *EGFR* protein, similar to DEL and L858R [24]. Since none of these 11 patients received EGFR-TKI therapy, the clinical significance of these mutations was not analyzed in the present study.

Despite the significant correlation between clinical response and immunoreactivity, the overall sensitivity of the 2 mutation-specific antibodies was 61%. This sensitivity was the lowest compared to values previously reported by others, which ranged from 78% to 92% [19,21–23]. One possible reason for the lower sensitivity in the present study was the methodological difference in the analysis of the molecular-based *EGFR* mutational status. HRMA, which was used for the molecular *EGFR* mutation analysis in the present study, was more sensitive than direct sequencing. HRMA has been shown to be a highly sensitive method for detecting DEL and L858R in prospective studies, and the detection sensitivity of this assay was reported to be 0.1–10%[15,27,28]. Conversely, the direct sequencing used in previous reports [19,21–23] required the presence of at least 20–25% *EGFR*-mutant cells to detect the DEL and L858R mutations. In the other 2 reports that validated the mutation-specific antibodies by correlating them with the *EGFR* mutational status by using highly sensitive molecular assays (mass spectrometry-based DNA analysis, cycleave PCR, and frag-

**Table 4**
Multivariate analysis of the response to EGFR-TKI.

| | Odds ratio | 95% CI | $p$-Value |
|---|---|---|---|
| Molecular based mutation | 40.533 | 8.691–189.035 | <0.001 |
| IHC based mutation | 0.421 | 0.109–1.632 | 0.211 |

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CI, confidence interval; IHC, immunohistochemistry.

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ment analysis), the reported sensitivities of IHC-based mutations were lower and partially similar to ours. Brevet et al. have reported that the sensitivity of the DEL-specific antibody was 67%, and that of the L858R-specific antibody was 76%, with the threshold for positive cases defined as moderate staining [20]; Kitamura et al. have reported that the overall sensitivity of these 2 mutation-specific antibodies was 47%, with almost the same threshold for positive cases as our staining score of 10 [24]. Although highly sensitive methods sometimes elicit false positive results, we showed that the response rate to EGFR-TKI in patients with lung tumors harboring HRMA-detected *EGFR* mutations was 77%, and this was consistent with 2 previous reports (82% and 83%) [12,29]. Therefore, HRMA was not likely to have overestimated the *EGFR* mutations.

Most of the extracted DNA in the present study was isolated from fresh frozen tissues or ethanol-fixed imprint cytologic smears, whereas in other reports concerning mutation-specific antibodies, DNA extracted from formalin-fixed, paraffin-embedded tissues was used for molecular *EGFR* mutation analysis [19–22]. Formalin-fixed tissues exhibit non-reproducible sequence alterations more frequently than DNA isolated from frozen tissues. This is because formalin can cross-link cytosine nucleotides on either strand [30]. However, ethanol causes very little chemical change, and therefore preserves nucleic acids better than formalin [30]. Taken together, these data suggest that using a highly sensitive molecular assay and high-quality DNA can reduce false-negative cases. Therefore, the sensitivity of the 2 novel mutation-specific antibodies used in the present study, was decreased.

Another possibility was that the immunopositive tumor cells for the mutation-specific antibodies were not diffusely distributed. When the threshold for mutation-positive was set as >50% of immunopositive tumor cells, the positive cases for the DEL- and L858R-specific antibodies decreased from 59 to 28 cases (47%), and from 139 to 88 cases (63%), respectively. From these decreased rates, the immunopositive tumor cells for DEL were distributed more sparsely and/or focally than those for L858R. These findings, detected by IHC analysis, suggested the presence of heterogeneity in the *EGFR*-mutant cells. Other molecular methods for detecting *EGFR* mutations also revealed the heterogeneous distribution of *EGFR* mutant cells [31–33].

In the present study, the predictors of the EGFR-TKI response were molecular-based (HRMA) *EGFR* mutations ($p < 0.001$), and IHC-based *EGFR* mutations ($p = 0.001$). Two novel mutation-specific antibodies served as the predictors of EGFR-TKI response in the univariate analysis. However, the multivariate analysis revealed that only molecular-based *EGFR* mutations were significantly correlated with the response to EGFR-TKI. Among 6 previous reports on mutation-specific antibodies, 3 analyzed the correlation of IHC-based *EGFR* mutational status with the response to EGFR-TKI, and a significant correlation was found in 2 of these studies [21,24]. The sensitivity and specificity of IHC-based *EGFR* mutations to the EGFR-TKI response calculated in this study were 63% and 70%, respectively. In 2 previous reports, IHC-based *EGFR* mutations showed a sensitivity ranging from 59% to 89% and a specificity ranging from 73% to 96% to the EGFR-TKI response. The last report showed an insignificant correlation between these parameters [22]. The role of IHC in predicting response to EGFR-TKI remains controversial [34]. It is necessary to prospectively study a larger number of cases to determine the usefulness of IHC for the response to EGFR-TKI.

The amount of immunopositive tumor cells did not affect the EGFR-TKI response in the present study. The threshold for mutation-positive, defined as >50% of immunopositive tumor cells, was less significantly correlated with the clinical response to EGFR-TKI than when using judgments by the expression score of 10 ($p = 0.012$). Further discussion regarding whether the percentage of immunopositive tumor cells is correlated with the response to EGFR-TKI, is necessary. The present results showed, for the first time, that the presence of diffusely immunopositive cells does not necessarily predict a response to EGFR-TKI therapy. Therefore, in clinical practice, a threshold for mutation-positive of expression score of 10 should be adopted.

Although the mutation-specific antibodies are not superior to the highly sensitive molecular techniques in detecting *EGFR* mutations, they have some potential advantages. Their excellent specificities [19–24] will serve as the first screening for *EGFR* mutational status, including the human epidermal growth factor 2 status for breast carcinoma [35,36]. In clinical settings, the first screening of IHC enables the omission of molecular *EGFR* mutational analysis in IHC-positive cases. IHC saves time, is cost-effective, and can be performed in most pathology laboratories. Another advantage of IHC over molecular techniques is that it can distinguish between tumor morphology and mutation-bearing cells by light microscopy.

In summary, the mutation-specific antibodies exhibited extremely high specificities, but did not show high sensitivities compared to the highly sensitive molecular method. In clinical practice, IHC using these 2 antibodies is a cost-effective and simple method for detecting *EGFR* mutations in most pathology laboratories, and can quickly evaluate patients for EGFR-TKI therapy.

## Conflict of interest statement

All authors have no financial or personal relationship with other people or organization that could inappropriately influence our work.

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Original Article

# Common Arm Comparative Outcomes Analysis of Phase 3 Trials of Cisplatin + Irinotecan Versus Cisplatin + Etoposide in Extensive Stage Small Cell Lung Cancer

## Final Patient-Level Results From Japan Clinical Oncology Group 9511 and Southwest Oncology Group 0124

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**BACKGROUND:** Southwest Oncology Group 0124 was a large North American phase 3 trial that failed to confirm a survival benefit for cisplatin/irinotecan over cisplatin/etoposide in patients with extensive stage small cell lung cancer (SCLC). These results were contrary to Japan Clinical Oncology Group 9511, a phase 3 trial exclusively in Japanese patients. Because 0124 and 9511 used identical treatment regimens and similar eligibility criteria, patient-level data were pooled from both trials, and a common arm analysis was performed to explore potential reasons for the divergent results. **METHODS:** Patients with documented extensive stage SCLC and adequate end-organ function were randomized to intravenously receive either cisplatin 60 mg/m$^2$ Day 1 + irinotecan 60 mg/m$^2$ Days 1, 8, and 15 every 4 weeks or cisplatin 80 mg/m$^2$ Day 1 + etoposide 100 mg/m$^2$ Days 1-3 every 3 weeks. Demographic and outcome data were compared among 805 patients enrolled in 9511 and 0124 receiving identical treatment using a logistic model adjusted for age, sex, and performance status (PS). **RESULTS:** Of 671 patients in 0124, 651 eligible patients were included, as were all 154 patients from 9511. Significant differences in sex and PS distribution as well as toxicity were seen between trials. There were also significant differences in response rates (87% vs 60%, $P<.001$) and median overall survival (12.8 vs 9.8 months, $P<.001$) when the cisplatin/irinotecan arms from both trials were compared. **CONCLUSIONS:** Significant differences in patient demographics, toxicity, and efficacy were identified in the 9511 and 0124 populations. These results, relevant in the current era of clinical trials globalization, warrant: 1) consideration of differential patient characteristics and outcomes among populations receiving identical therapy; 2) utilization of the common arm model in prospective trials; and 3) inclusion of pharmacogenomic correlates in cancer trials where ethnic/racial differences in drug disposition are expected. ***Cancer* 2010;116:5710–5.** © *2010 American Cancer Society.*

**KEYWORDS:** small cell lung cancer, chemotherapy, extensive stage, cisplatin, irinotecan.

**Lung** cancer represents the most common cause of malignant disease globally. Almost 1.4 million new cases of lung cancer are diagnosed annually worldwide, with nearly 1.2 million deaths.[1] Small cell lung cancer (SCLC) is a unique subtype of lung cancer that accounts for approximately 15% of all new cases.[2] Unfortunately, most SCLC patients die from the disease, due commonly to systemic metastasis (defined as "extensive stage").[3,4] Over the past 20 years, standard therapy for most patients with extensive stage SCLC has been either carboplatin or cisplatin in combination with etoposide.[5]

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This paradigm was challenged in 2002, when the results of the Japanese phase 3 study Japan Clinical Oncology Group 9511, comparing etoposide/cisplatin with cisplatin/irinotecan in 174 patients, demonstrated that tumor response, progression-free survival (PFS), and overall survival (OS) rates were significantly higher in the cisplatin/irinotecan group.[6] It must be noted that 9511 was stopped early at interim analysis by its data safety monitoring board when prospectively prespecified efficacy parameters were met.

Subsequently, the Southwest Oncology Group conducted a large phase 3 trial (0124) involving 671 patients that used virtually the same eligibility criteria and treatment regimens as the Japanese trial to confirm the results of 9511 in North American patients.[7] As reported previously, 0124 found no statistical differences in tumor response, PFS, and OS between the 2 arms, contrary to the results of 9511.

To explore potential reasons for the divergent results of these identically designed phase 3 trials, a pooled comparative outcomes analysis inclusive of patient-level data from both trials was conducted.

## MATERIALS AND METHODS

Patients in both trials had cytologically or histologically confirmed SCLC and clinical evidence of extensive stage disease (defined by distant metastasis, contralateral hilar-node metastasis, or malignant pleural effusion). Eligibility criteria for both trials were similar and have been previously reported. Patients were randomly assigned to receive either etoposide/cisplatin or cisplatin/irinotecan. The cisplatin/irinotecan regimen consisted of 4 cycles of 60 mg of irinotecan per square meter of body surface area on Days 1, 8, and 15 and 60 mg of cisplatin per square meter on Day 1. Cycle length for this arm was 4 weeks. The etoposide/cisplatin regimen consisted of 4 cycles of 100 mg of etoposide per square meter on Days 1, 2, and 3 and 80 mg of cisplatin per square meter on Day 1. Cycle length for this arm was 3 weeks.

All patients underwent evaluations every cycle that included an assessment of symptoms, a physical examination, a complete blood count, and blood chemistry studies. Tumor response was assessed after every 2 cycles. In the 0124 trial, tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors, whereas in the 9511 trial, the World Health Organization criteria were used.[8] OS was calculated as the time between trial registration and death or date of last contact. PFS was calculated as the time between trial registration and death or progression, with censoring if alive without progression at last contact.

### *Study Design and Statistical Analysis*

The primary objective of both studies was to compare the survival in patients with extensive SCLC treated with etoposide/cisplatin (standard arm) with that in comparable patients treated with the cisplatin/irinotecan (experimental) on an intent-to-treat basis. As 0124 and 9511 protocols used identical treatment regimens and similar eligibility criteria, patient-level data from both trials were pooled to explore potential reasons for the divergent results. Final results of both trials have previously been reported. Of 671 patients in 0124, 651 were eligible and included in this analysis, as were all 154 patients from 9511. Patient demographics, toxicity, and outcomes were compared among 805 patients receiving identical treatment using a common arm analysis. OS and PFS as compared between the Japan and US trials for both treatment arms in the combined sample were analyzed using Cox proportional hazards regression, adjusted for age, sex, and performance status. A logistic model adjusted for age, sex, and performance status was used to compare response to treatment between the 2 trials for the 2 treatment arms. The existence of possible interactions between trial (Japan Clinical Oncology Group vs Southwest Oncology Group) and treatment arm was evaluated for all endpoints, using data pooled over both arms. Significance was set at $P<.05$.

## RESULTS

### *Patient Demographics*

Median age in 9511 and 0124 was 61 and 62 years, respectively. There were proportionally more men in 9511 (86%, n = 132) compared with 0124 (57%, n = 370). There were more patients with Zubrod performance status 0 in 0124 (211, 32%) compared with 9511 (19, 12%). Demographics are summarized in Table 1.

### *Toxicity*

Common arm comparisons of select attributable hematologic toxicities are summarized in Table 2. Regardless of treatment arm, patients in 9511 experienced significantly more hematologic toxicity consisting of neutropenia, leucopenia, and anemia than 0124. Other than a difference in infection rates in the cisplatin/etoposide arm, no

**Table 1.** Patient Demographics

| Characteristic | JCOG-9511 | | | SWOG-0124 | | |
|---|---|---|---|---|---|---|
| | Cisplatin + Etoposide | Cisplatin + Irinotecan | Total | Cisplatin + Etoposide | Cisplatin + Irinotecan | Total |
| **Age, y** | | | | | | |
| Median | 63 | 63 | 63 | 63 | 62 | 63 |
| Minimum | 41 | 30 | 30 | 35 | 22 | 22 |
| Maximum | 70 | 70 | 70 | 86 | 86 | 86 |
| Male sex | 90% | 82% | 86% | 56% | 58% | 57% |
| **PS** | | | | | | |
| 0 | 12% | 13% | 12% | 33% | 32% | 32% |
| 1 | 75% | 79% | 77% | 66% | 67% | 66% |
| 2 | 13% | 8% | 10% | 0% | 0% | 0% |

JCOG-9511 indicates Japan Clinical Oncology Group 9511 trial; SWOG-0124, Southwest Oncology Group 0124 trial; PS, performance status.
Comparisons of the JCOG and SWOG populations with respect to differences in sex and PS were significant by chi-square test ($P < .0001$ for both comparisons).

**Table 2.** Common Arm Comparative Toxicity Analysis

| ≥Grade 3 Toxicity | Cisplatin + Etoposide | | | Cisplatin + Irinotecan | | |
|---|---|---|---|---|---|---|
| | JCOG-9511 | SWOG-0124 | *P* | JCOG-9511 | SWOG-0124 | *P* |
| Infection | 3 (4%) | 52 (16%) | .01 | 4 (5%) | 36 (11%) | .23 |
| Neutropenia | 71 (92%) | 220 (68%) | <.001 | 49 (65%) | 107 (34%) | <.001 |
| Leukopenia | 41 (53%) | 109 (34%) | .006 | 20 (27%) | 57 (18%) | .04 |
| Anemia | 25 (32%) | 39 (12%) | <.001 | 21 (28%) | 18 (6%) | <.001 |

JCOG-9511 indicates Japan Clinical Oncology Group 9511 trial; SWOG-0124, Southwest Oncology Group 0124 trial.

differences in nonhematologic toxicities between the 2 trials were seen.

### *Treatment Delivery and Dose Intensity*

In the original 9511 and 0124 papers, there were no significant differences reported between the 2 arms in terms of treatment delivery. A preliminary common arm comparison of treatment delivery and dose intensity (DI) was performed in the current analysis. These results are summarized in Table 3. There were no clear differences in the proportion of patients completing all 4 cycles of therapy. However, a higher proportion of patients completed all 4 cycles of etoposide/cisplatin in 9511 versus 0124 (38% vs 29%). A more modest difference was seen in the cisplatin/irinotecan arm (29% vs 23%). When comparing the published DI data (9511 vs 0124), there was a numerical difference in the proportion of irinotecan (80.4% vs 66%) and cisplatin (95.3% vs 78%) DI.

### *Efficacy*

Common arm comparisons of efficacy endpoints including response rate, PFS, OS are summarized in Table 4 and Figure 1. Ten patients (2 from Japan Clinical Oncology Group and 8 from Southwest Oncology Group) were excluded from the analysis of treatment response because they did not receive treatment. Significant differences in response rates were seen in the common arm comparisons when evaluated in multivariate logistic regression models, which enabled adjustment for age, sex, and performance status. Specifically, for the etoposide/cisplatin arm, response rates were 68% in 9511 and 57% in 0124 ($P = .02$). For the cisplatin/irinotecan arm, response rates were 87% for the 9511 and 60% in 0124 ($P<.001$). In an expanded logistic regression model that pooled the data for both treatment arms, there was a significant arm by trial interaction, indicating that the difference in response between the Japanese and US patients is significantly greater in the cisplatin/irinotecan arm patients. ($P$ value for interaction = .03)

There were no differences in PFS and OS for the etoposide/cisplatin arm across trials. However, significant differences were seen for OS for the cisplatin/irinotecan arm. Specifically, median OS was 12.8 months for 9511 and 9.9 months for 0124 ($P<.001$, adjusted for age, sex, and performance status via Cox proportional hazards regression). Similarly, 1-year survival rates were 58% and 41%, respectively. The 1-month numerical difference in PFS in the cisplatin/irinotecan arm was not statistically significant. Kaplan-Meier survival curves of OS common arm comparisons in the cisplatin/irinotecan arm are shown in Figure 1. In a multivariate proportional hazards regression model including trial (Japan vs United States) treatment arm, age, sex, and performance status, the interaction between trial and treatment arm is significant, confirming that the survival difference by site (Japan vs United States) depends on treatment arm ($P$ value for interaction term = .01). A performance status of 0 (vs 1 or 2) was also independently prognostic for increased survival in multivariate modeling ($P<.001$). Age and sex were not.

**Table 3.** Common Arm Analysis of Treatment Delivery and Dose Intensity

| Treatment Arm | P + E | P + I |
|---|---|---|
| **Completed all 4 cycles** | | |
| JCOG-9511 | 55/77 (71.4%) | 53/77 (68.8%) |
| SWOG-0124 | 218/327 (66.6%) | 213/324 (65.8%) |
| **Completed 4 cycles without dose modification** | | |
| JCOG-9511 | 29/77 (38%) | 22/77 (29%) |
| SWOG-0124 | 94/327 (29%) | 76/324 (23%) |
| **Reported average dose intensity[a]** | | |
| JCOG-9511 | E: 83.9%; P: 84.6% | I: 80.4%; P: 95.3% |
| SWOG-0124 | E: 78%; P: 81% | I: 66%; P: 78% |

P indicates cisplatin; E, etoposide; I, irinotecan; JCOG-9511, Japan Clinical Oncology Group 9511 trial; SWOG-0124, Southwest Oncology Group 0124 trial.

[a] Percentage of total planned dose.

## DISCUSSION

This common arm comparison of 9511 and 0124 using pooled patient-level data provides unique insights into potential reasons for the divergent results of these trials. In addition, this analysis highlights the issue of whether in the current era of clinical trials globalization, the results of randomized oncology studies conducted outside the United States are directly translatable to North American populations.[9] These issues obviously have regulatory implications.

This analysis is unparalleled because 0124 was designed a priori as a confirmatory trial for 9511, albeit accruing from a different ethnic patient pool. The design of the 0124 protocol was modeled directly on 9511, including similar eligibility criteria and identical treatment dose schedules. The observed differences in demographics, toxicity, and efficacy outcomes between these trials can be attributed to many factors, some of which were previously discussed in the 0124 paper. With the pooled multivariate analysis, we were able to investigate (and rule out) the possibility that the different outcomes between trials in the cisplatin/irinotecan arms were attributable to clear differences in patient populations with respect to sex and performance status. Our analysis of both survival and response showed that although performance status was prognostic for survival, the differences between trials in the cisplatin/irinotecan arm persisted even after adjusting for this imbalance.

Other potential factors included the smaller sample size and/or the early stopping of 9511, which may have overestimated the treatment effect.[10]

This common arm analysis demonstrates that the principal difference in OS was seen only in the cisplatin/irinotecan arms. The control etoposide/cisplatin arms in both 0124 and 9511 had identical OS results. In the context of irinotecan-based therapy, 1 hypothesis that has been posited is that there are inherent genetic differences related to genes involved in irinotecan drug disposition

**Table 4.** Common Arm Analysis of Efficacy

| Efficacy Measure | Cisplatin + Etoposide | | | Cisplatin + Irinotecan | | |
|---|---|---|---|---|---|---|
| | JCOG-9511 | SWOG-0124 | *P* | JCOG-9511 | SWOG-0124 | *P* |
| Response rate | 68% | 57% | .01 | 87% | 60% | <.001 |
| Median PFS, mo | 4.7 | 5.2 | .18 | 6.8 | 5.8 | .6 |
| Median OS, mo | 9.4 | 9.1 | .5 | 12.8 | 9.9 | <.001 |
| One-year survival rate | 38% | 34% | | 58% | 41% | |

JCOG-9511 indicates Japan Clinical Oncology Group 9511 trial; SWOG-0124, Southwest Oncology Group 0124 trial; PFS, progression-free survival; OS, overall survival.

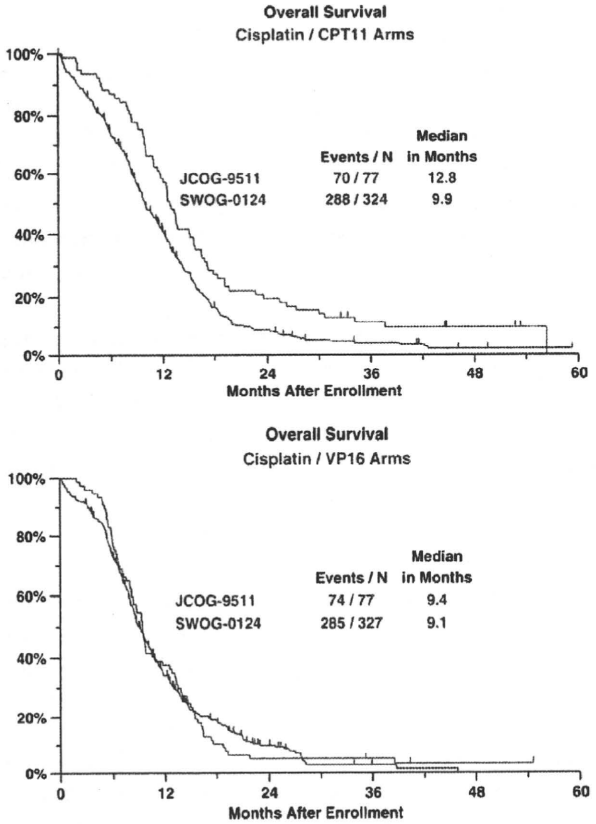


**Figure 1.** (*Top*) Overall survival analysis by trial is shown for the cisplatin and irinotecan (CPT-11) treatment arm. (*Bottom*) Overall survival analysis is shown for the cisplatin and etoposide (VP16) treatment arm. JCOG-9511 indicates Japan Clinical Oncology Group 9511 trial; SWOG-0124, Southwest Oncology Group 0124 trial.

between patient populations. Although a preliminary pharmacogenomic analysis of specimens from 0124 patients was performed to investigate some of these irinotecan-related genes, no specimens were available from the older 9511 trial for similar pharmacogenomic investigations. Hence, no direct comparison of relevant genotypes between trials is possible. However, insights on this issue can be derived from prior common arm joint collaborations between Southwest Oncology Group and Japanese investigators wherein patients with advanced nonsmall cell lung cancer were enrolled in Southwest Oncology Group and Japanese trials onto a common arm of paclitaxel and carboplatin.[11] In that experience, genes relevant to chemotherapy metabolism and transport were analyzed in both American and Japanese populations. Significant differences in toxicity, efficacy, and allelic distribution for genes involved in paclitaxel disposition or DNA repair were observed between Japanese and US patients, supporting the hypothesis that pharmacogenomics may in part be responsible for outcome divergence among patient populations. This may also partly explain the toxicity differences seen between the Japanese and North American populations, wherein Japanese patients apparently had increased hematologic toxicity (neutropenia, leucopenia, and anemia) in both treatment arms when compared with North Americans.

In addition, there appears to be some differences in the delivered DI in the cisplatin/irinotecan arms of both trials (as reported in the published papers). Specifically, more 9511 patients achieved a higher DI for both irinotecan and cisplatin as compared with 0124 patients. Enhanced DI for 9511 patients may potentially explain the differences in toxicity and efficacy between the trials. A more detailed and expansive analysis of dose delivery using individual patient data is required, but is beyond the scope of this article. Finally, it must be noted that other trials comparing similar chemotherapy regimens in SCLC have previously been published.[12,13] Some of us (P.N.L., R.N., and D.R.G.) have previously discussed these trials in the context of 0124 and 9511 in a recent editorial.[14] We refer readers to that editorial for additional details.

In conclusion, etoposide/cisplatin remains the reference treatment standard in North America. In Japan, cisplatin/irinotecan remains a standard treatment option. Significant differences in patient demographics, toxicity, and efficacy exist between Japanese and North American SCLC patients receiving identical treatment. These results, relevant in the current era of clinical trials globalization, warrant 1) consideration of differential patient characteristics and outcomes among patients receiving identical therapy, 2) utilization of the common arm model in prospective trials, and 3) inclusion of pharmacogenomic correlates in cancer trials where ethnic/racial differences in drug disposition are expected.

## CONFLICT OF INTEREST DISCLOSURES

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PRECLINICAL STUDIES

# Efficacy of RAD001 (everolimus) against advanced gastric cancer with peritoneal dissemination

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**Summary** Peritoneal dissemination occurs frequently in patients with unresectable advanced-stage gastric cancer. In this study, we tested the efficacy of the mTOR inhibitor RAD001 (everolimus) against advanced gastric cancer with peritoneal dissemination. Using the two cell lines, 58As1, a cell line exhibiting a high propensity for peritoneal metastasis, and its parental cell line, HSC-58, a human scirrhous gastric cancer cell line, we first examined the growth-inhibitory activity of everolimus in vitro. Methylene blue assay demonstrated a moderate inhibitory effect of the drug on both cell lines under normal culture conditions (maximal inhibitory effect: 50.5% at 1 μM, HSC-58, 65.3%, 58As1). However, under the hypoxic condition (1% $O_2$), while the growth-inhibitory activity of everolimus was greatly reduced in the HSC-58 cell line, the degree of reduction of the inhibitory activity was much smaller in the 58As1 cell line. Western blotting revealed that the degree of phosphorylation of mTOR and its downstream signaling molecules, p70S6K and 4E-BP1, was decreased under hypoxic conditions in HSC-58. On the other hand, phospho-p70S6K and phospho-4E-BP1 remained active under hypoxic conditions in 58As1, the molecular activity was suppressed by everolimus. Cell-cycle analysis showed that hypoxia-induced G1 arrest was not manifested in the 58As1 cells, unlike in the HSC-58 cells. Separately, an in vivo orthotopic mouse model of 58As1 revealed that everolimus significantly reduced peritoneal dissemination as evaluated by the quantitative photon counting method. Taken together, our results suggest that everolimus may have favorable activity against gastric cancer, particularly in cases with peritoneal dissemination.

**Keywords** Everolimus · Peritoneal dissemination · Gastric cancer · Hypoxia

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## Introduction

Gastric cancer is still one of the leading causes of cancer-related deaths in Japan as well as the rest of the world. Despite advances in the surgical techniques and establishment of various new chemotherapies over the past decades, the median survival of patients with unresectable advanced gastric cancer remains about 7 months with standard chemotherapy [1, 2]. Even after curative surgery for early-stage disease, the reported recurrence rate is 45–87% [3, 4]. In these cases, the most common type of recurrence is peritoneal dissemination, which represents a major factor in determining the patient prognosis [5–7]. To date, various treatments have been used for patients of gastric cancer with peritoneal dissemination, however, no effective therapy has been established. The 5-year survival rate of patients of advanced gastric cancer with peritoneal dissemination is only 2% with any treatments [8, 9]. Therefore, new

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treatment strategies are urgently needed for local and systemic cancer control in gastric cancer patients.

mTOR, a serine/threonine kinase, is involved in the control of translation in response to stress and is thought to play a critical role in regulating cell growth, cell cycle progression, and tumor genesis [10]. It has been reported that mTOR is frequently activated in human gastric cancer [11]. A recent study demonstrated that hypoxia-inducible factor-1α (HIF-1α), a protein whose activity is modulated by AKT/mTOR signaling, is an important regulator of cancer growth and angiogenesis in gastric cancer [11–13]. Furthermore, up-regulation of HIF-1α and VEGF mRNA has been observed in the peritoneal metastases, but not the primary tumor, in cases of gastric cancer [14]. From this report, it would appear that mTOR is a crucial factor in the development of peritoneal dissemination and that it might, therefore, be an attractive therapeutic target.

Everolimus is an orally bioavailable derivative of rapamycin that inhibits the ability of mTOR to phosphorylate p70S6K and 4E-BP1, thereby causing Go/G1 arrest and inhibiting cell cycle progression in cancer cells[15]. Numerous studies have demonstrated the ability of everolimus to suppress cancer growth both in vitro and in vivo [16, 17]. In an ovarian cancer model, everolimus was shown to inhibit ascites formation and peritoneal dissemination [18]. In March 2009, everolimus was approved by the FDA for the treatment of patients with advanced renal cell carcinoma (RCC) showing failure of treatment with sunitinib or sorafenib [19].

The peritoneal cavity is not adequately supplied by the systemic circulation, limiting the oxygen and nutrition supply to this regions; therefore, peritoneal metastatic tumor cells are thought to be exposed to unusual conditions, such as hypoxia and poor nutrition. Prolonged hypoxia could lead to inhibition of cell cycle progression and decrease in the number of actively growing cells, resulting in reduced sensitivity to anticancer drugs [20]. We hypothesized that cell lines with a high propensity for peritoneal dissemination might have the potential to adapt themselves to such environments and have a different mode of regulation of the mTOR activity.

## Materials and methods

### Reagents

Everolimus and placebo were obtained from Novartis Pharma AG (Basal, Switzerland) as dry powder for in vitro use, and as a microemulsion for in vivo use. The powder was dissolved in DMSO and 20 mM stock solution before being aliquoted and stored at −20°C until use. The microemulsion (2%, w/w) and placebo emulsion were also aliquoted and stored at −20°C.

### Cell cultures

58As1, a cell line that often causes ascites and has a high potential for peritoneal dissemination, was established from the human scirrhous gastric cancer cell line, HSC-58, by repeated orthotopic (gastric wall) implantation of cells that had metastasized to the abdominal cavity [21, 22]. The 58As1 cell line was transfected with luciferase and was named 58As1Luc [23]. The cell lines were maintained in RPMI-1640 medium (Sigma, St.Louis, MO) supplemented with 10 % heat-inactivated FBS.

### In vitro growth-inhibition assay

The cell-growth-inhibitory effect of everolimus was assessed by the methylene blue assay. The cell suspension was seeded at a density of 180 μl/well into 96-well microculture plates, followed by incubation of the plates for 24 hr. The cells were then treated with various concentrations of everolimus (0.1–1,000 nM) at 37°C under either a normoxic (21% $O_2$) or hypoxic (1% $O_2$) condition. After a 96-hr culture period, the cells were fixed with glutaraldehyde, stained with 0.05% methylene blue, and dissolved in 3% HCl. The absorbance of each well was measured at 650 nm.

### Immunoblotting

After incubation under a normoxic (21% $O_2$) or hypoxic (1% $O_2$) condition for 66 h, everolimus was added to each plate, and the plates were incubated for an additional 6 h in the same chamber. The cultured cells were washed and lysed in M-PER buffer with protease and phosphatase inhibitors. Protein samples (40 μg) were applied for western blotting on 10% SDS-PAGE. All the antibodies used were purchased from Cell Signaling technology (Beverly, MA). After standard SDS-PAGE and western blotting procedures, the proteins were visualized using the ECL system.

### Cell cycle analysis

HSC-58 and 58As1 cells ($1 \times 10^6$ cells) were seeded on to 100-mm Petri dishes 24 h prior to drug exposure. They were then exposed to 100 nM everolimus for 72 h under either a normoxic (21% $O_2$) or hypoxic (1% $O_2$) condition. Following drug exposure, the cells were harvested and suspended as single cells in phosphate-buffered saline (PBS). They were then washed with ice-cold PBS, fixed in 50% methanol/PBS and stored at −20°C until analysis. For the cell cycle analysis, $5 \times 10^6$ fixed cells were washed once in 30% methanol/PBS and twice in PBS. They were then re-suspended in 1 ml of boiled RNase A (1 mg/ml)

solution. After 30 min' incubation at 37°C, the cells were washed in PBS and stained with 2 ml ethidium bromide (50 μg/ml) for 20–30 min on ice under a light-protected condition. The cell cycle distribution was analyzed by flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA) and a cell cycle analysis software (Modfit, Verity, Topsham, ME).

### Peritoneal metastasis mouse model

Seven-week old female BALB/c nude mice were maintained under specific- pathogen-free conditions. A total of $1\times10^6$ 58As1Luc cells each were inoculated into the gastric wall of the mice after laparotomy. Four days after the inoculation, the mice were given everolimus (~5 mg/kg, daily, gavage) or placebo for 15 days. In vivo photon counting analysis was performed twice a week on a cryogenically cooled IVIS system using the Living Image acquisition and analysis software (Xenogen), as described previously [23]. Animal welfare was strictly monitored by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments at the National Cancer Center.

## Results

### In vitro growth-inhibition assay

We first performed a methylene blue assay in vitro to evaluate the growth-inhibition activity of everolimus on the HSC-58 and 58As1 cells. Both cell lines were found to be moderately sensitive to everolimus under the normoxic condition (maximal inhibitory effect: 50.5% at 1 μM, HSC-58, 65.3%, 58As1) (Fig. 1a). However, under the hypoxic condition, while the growth-inhibitory activity of everolimus was greatly reduced in the HSC-58 cell line, the degree of reduction of the inhibitory activity was much smaller in the 58As1 cell line (Fig. 1b). The 58As1 cell line has been established by repeated orthotopic implantation and cell collection from the peritoneal metastatic tumors. Due to the unstable supply of oxygen in the abdominal cavity, our results point to the potential of everolimus to suppress the growth of disseminated cells in cases of advanced gastric cancer.

### Effect of everolimus on the mTOR signaling pathway

To identify the molecular mechanisms underlying the growth-inhibitory activity of everolimus in these cell lines, we investigated the signal changes caused by everolimus under normoxic and hypoxic conditions. Under normoxic

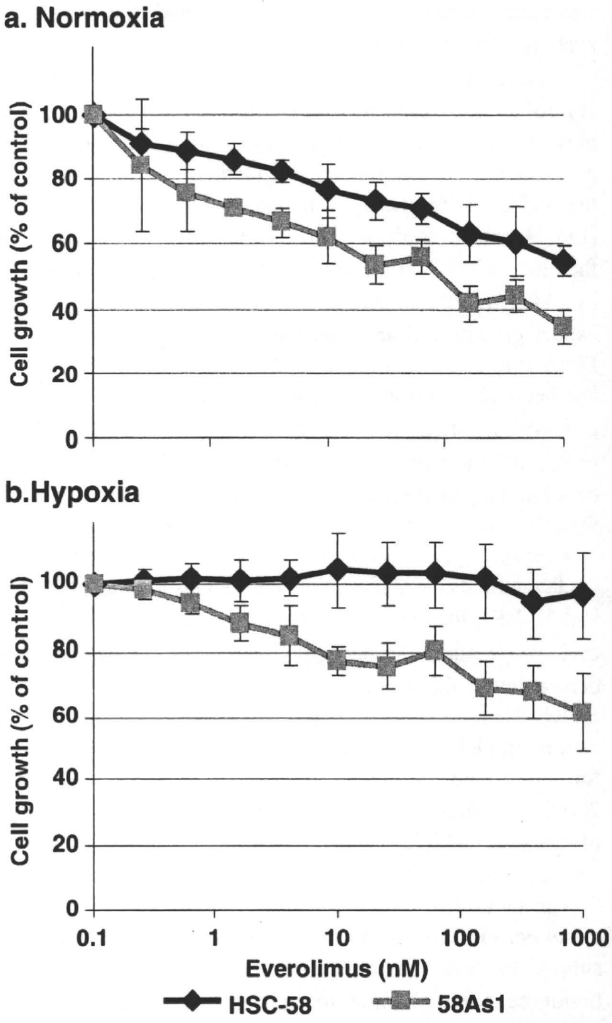


**Fig. 1** Effect of everolimus on the proliferation of HSC-58 and 58As1 cells. The cells were treated with the indicated concentrations of everolimus under normoxic or hypoxic conditions for 96 h. Cell viability was assessed by methylene blue assay. **a** Normoxic condition (20% O2 chamber), **b** Hypoxic condition (1%O2 chamber)

conditions, Akt and mTOR were phosphorylated in both the HSC-58 and 58As1 cell lines, indicating activation of Akt-mTOR signaling (Fig. 2a). No differences in the Akt protein and phosphorylation levels were observed in the cells between the normoxic and hypoxic conditions. Phosphorylation of mTOR at Ser2448 was marginally lower under the hypoxic condition as compared with that under the normoxic condition in the HSC-58 cells; however, no such difference was observed in the 58As1 cells.

Two downstream molecules in the mTOR pathway, p70S6K and 4E-BP1, were also examined. Under the normoxic condition, phosphorylation of p70S6K and 4E-BP1 was decreased by everolimus in both the cell lines, indicating inhibited downstream signaling of mTOR. Under

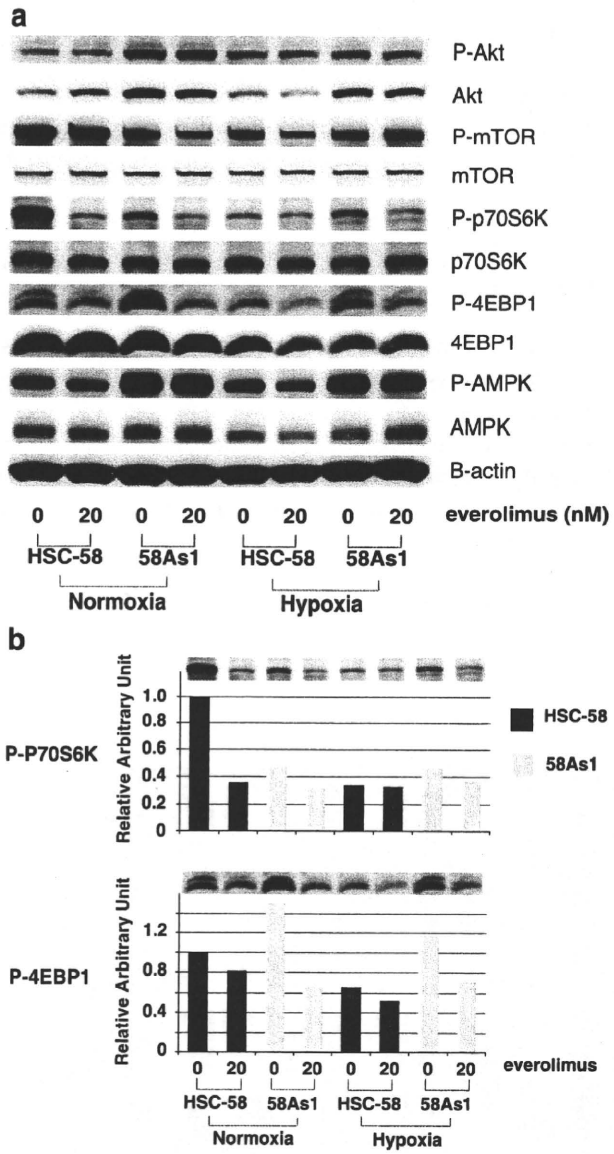


**Fig. 2** Effect of everolimus on mTOR signaling. **a** Cells were maintained in either a 20% or 1% O2 chamber for 66 h, then treated with everolimus for 6 h in the same chamber. Western blots were screened with antibodies against the indicated proteins. Under hypoxic conditions, the activities of two downstream molecules of mTOR, p70S6k and 4E-BP1, were significantly decreased in the HSC-58 cells. Conversely, these two molecules were observed at approximately the same levels under normoxic and hypoxic conditions in the 58As1cells. **b** Histogram indicating the relative densities of the bands on the western blot. The density of each band was measured using a bioimage analyzer (BAS4000, Fuji Film, Tokyo)

the hypoxic condition, phosphorylation of p70S6k and 4E-BP1 was significantly decreased in the HSC-58 cells, with the suppression by everolimus no longer remaining significant. Conversely, in the 58As1 cells, both phosphorylated P70S6K and 4E-BP1 were active under both normoxic and hypoxic conditions and they were suppressed by treatment with everolimus. AMPK, another modulator of protein synthesis and cell growth through the mTOR pathways, was also examined. AMPK activity was found to be the same under both hypoxic and normoxic conditions, and also unchanged by treatment with everolimus. The bands of phospho-p70S6K and phospho-4EBP1 were quantified by densitometry (Fig. 2b).

These results show that although the phosphorylation levels of upstream signaling molecules in the mTOR pathway were not remarkably suppressed under the hypoxic condition, phosphorylation of downstream molecules such as p70S6K and 4E-BP1 was clearly diminished in the HSC-58 cells. Such findings lend support to the argument that hypoxia suppresses mTOR signaling. Interestingly, this suppression of mTOR under the hypoxic condition was not observed in the 58As1 cells, and everolimus activity could be observed in these cells.

## Effect of hypoxia on the cell-cycle progression of HSC-58 and 58As1 cells

Flow-cytometric analyses showed that HSC-58 cells cultured under the hypoxic conditions accumulated in the G1 phase of the cell cycle (normoxia, 51.7%, hypoxia, 58.5%) (Table 1). Although everolimus-treated HSC58 cells also accumulated in the G1 phase under the normoxic condition (61.5%), under the hypoxic condition, no more increase of cells in the G1 phase was observed (60.6%). On the other hand, no remarkable accumulation in the G1 phase was observed for the 58As1 cells maintained under the hypoxic condition (normoxia, 38.3%, hypoxia, 38.8%). Furthermore, everolimus-treated 58As1 cells accumulated in the G1 phase under both normoxic (47.9%) and hypoxic conditions (53.5%). These findings indicate that the cellular proliferative activity of 58As1 cells is preserved even under severe hypoxic conditions, and that the effect of everolimus on the 58As1 cells is maintained even under the hypoxic condition.

**Table 1** Cell cycle analysis of HSC58 and 58As1 cells under hypoxia

| | Phase | HSC58 | HSC58 with everolimus | 58AS1 | 58AS1 with everolimus |
|---|---|---|---|---|---|
| Normoxia | G1 | 51.7 | 61.5 | 38.3 | 47.9 |
| | S | 40 | 30.9 | 45.9 | 40.6 |
| | G2/M | 8.3 | 7.7 | 15.8 | 11.6 |
| Hypoxia | G1 | 58.5 | 60.6 | 38.8 | 53.5 |
| | S | 33.5 | 31.4 | 44.3 | 36.0 |
| | G2/M | 8.0 | 8.0 | 16.9 | 10.5 |

### Effect of everolimus on the peritoneal metastasis mouse model

To estimate the potential growth-inhibitory effect of everolimus on peritoneal metastases, we subsequently implanted 58As1Luc into the gastric wall of each mouse after laparotomy. Oral administration of everolimus was then started on the fifth day after implantation. A daily regimen of 5 mg/kg of everolimus was proven to be efficacious and well-tolerated in previous studies by Homicsko et al., therefore, this regimen was also applied for our experiments [24]. Figure 3a shows typical examples of the photon counting analysis, while Fig. 3b shows the time-course of changes in the numbers of photons. In our previous study, we demonstrated a significant correlation between the luciferase emission level (photon number) and the tumor volume [23]. Approximately 12 days after implantation, the tumor cells started to significantly spread to the abdomen in the placebo group (Fig. 3a), and ascites associated with a clear increase in the waist size and body weight was observed. On the other hand, in the everolimus-treated group, the tumor proliferation was markedly suppressed. When the animals were sacrificed for autopsy, dissemination to the greater omentum, mesenterium, and parietal peritoneum was often observed in the placebo group (Fig. 3c); bloody ascites and liver metastasis were also seen.

## Discussion

No effective therapy has been established as yet against gastric cancer with peritoneal dissemination [8, 9]. The main purpose of this study was to examine the efficacy of everolimus in advanced gastric cancer cases with peritoneal dissemination. In our mouse model, everolimus exerted potent antitumor activity against peritoneal metastasis spontaneously disseminated from orthotopically transplanted scirrhous gastric cancer cells. Our data suggest that this activity is produced by the antitumor effect of everolimus on cancer cells under hypoxic conditions.

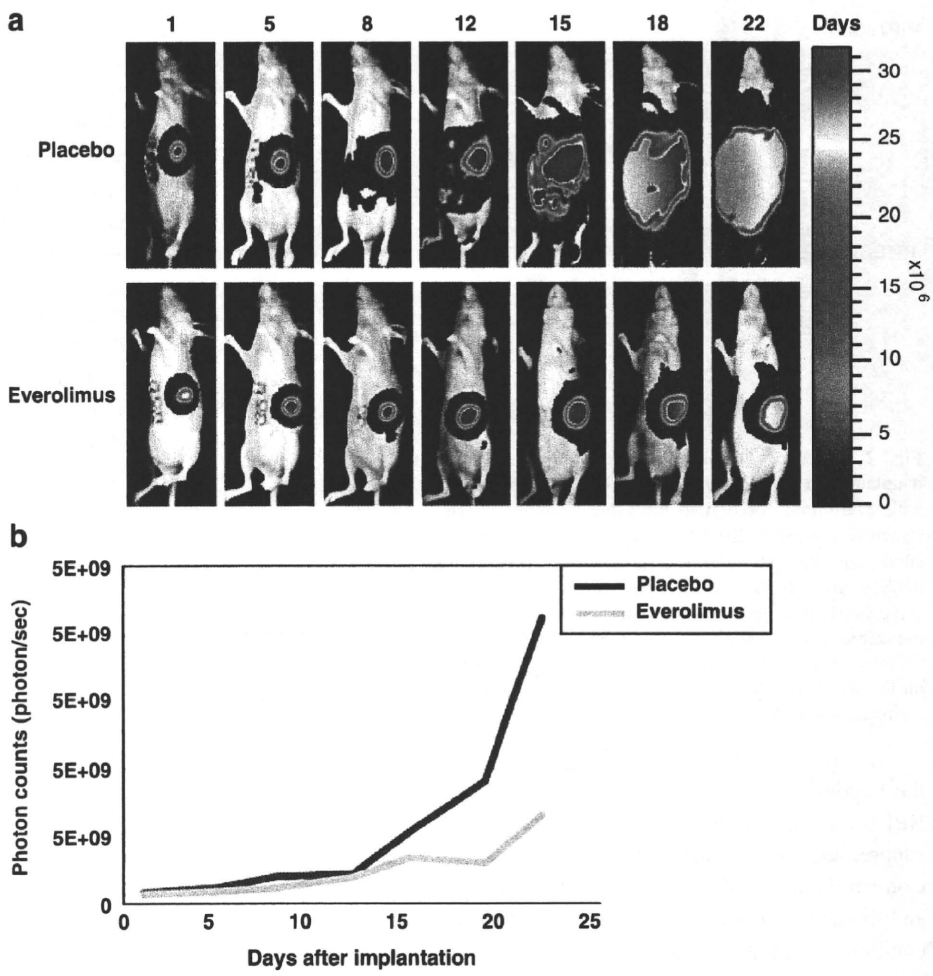


**Fig. 3** Nude mice implanted with 58As1Luc cells were visualized using an IVIS system. **a** Quantitative photon counting analysis to evaluate the progression of peritoneal dissemination. **b** Appearance of peritoneal dissemination. Abdominal distension due to ascites was observed in the mice in the placebo group. Metastasis to the greater omentum, mesenterium, and parietal peritoneum was evident in the placebo group. **c** Body weight change during treatment

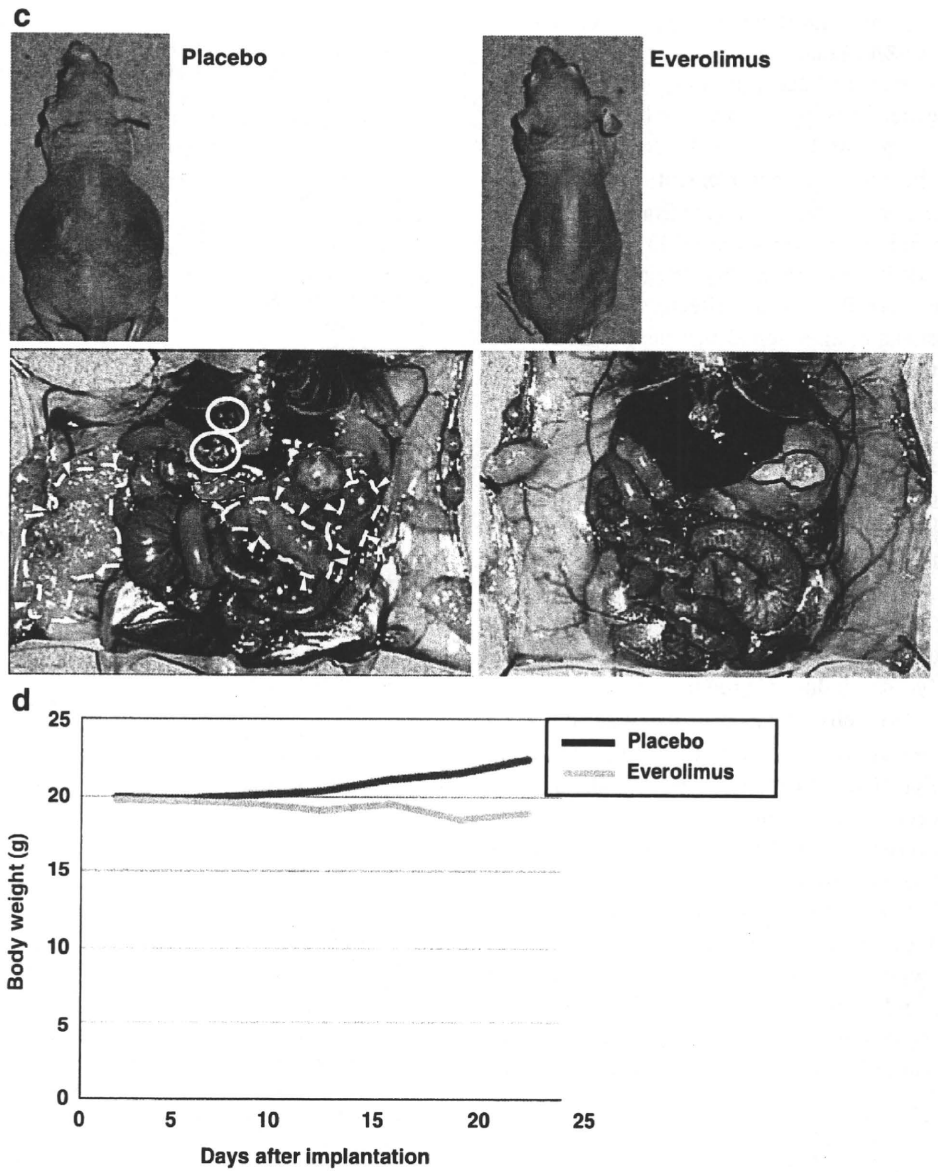


Fig. 3 (continued)

It has been reported previously that several cytokines, such as VEGF, EGF, HGF, CXCL12, and TNF-α, directly promote cancer cell growth in the development of peritoneal carcinomatosis [25–29]. However, therapies targeting such factors have not yet shown significant promise in treating advanced gastric cancer patients with peritoneal dissemination.

In this study, we focused on the environment of the peritoneal cavity. Peritoneal carcinomatosis secondary to gastric cancer is considered to be related to direct dissemination of the cells from the primary gastric tumor into the abdominal cavity. The disseminated cancer cells in the peritoneal cavity are not supplied with oxygen from the systemic circulation. Moreover, considering the distance from the functional micro vessels necessary for oxygen diffusion, cells that spread into the abdominal cavity may be exposed to hypoxic conditions. In fact, it has been reported that pancreatic cancer cells invading, proliferating, and disseminating into the abdominal cavity, create a severely hypoxic environment [30]. In our cell cycle distribution analysis, the proportion of HSC-58 cells in the G1 fraction was significantly increased in under the hypoxic condition, indicating that hypoxia negatively affects the tumor cell bioactivity. On the other hand, no remarkable change was observed in the DNA content of the 58As1 cells in hypoxic culture. These results suggest

that hypoxia does not significantly suppress the cellular activities in the 58As cells.

It is known that cellular activities, such as protein synthesis, are generally suppressed under hypoxia in order to conserve energy and avoid cell death. mTOR is considered to be an important element in sensing low oxygen levels and transforming cancers from an active state to a more dormant state. Arsham et al. [31] demonstrated that hypoxia rapidly and reversibly triggered hypophosphorylation of mTOR and its effectors, 4E-BP1 and p70S6K. Hamanaka et al. reported that neither LY294002, a PI3K inhibitor, nor rapamycin was toxic to LLC cells under hypoxic conditions. Furthermore, inhibition of the PI3K or mTOR pathway by LY294002 or rapamycin rescued cancer cells from death [32]. Hamanaka also reported that overexpression of the active form of S6K, which results in forced activation of mTOR, increased the rate of cell death in cancer cells maintained under hypoxia. In our study, we found that hypoxia did not significantly inhibit the mTOR signaling pathway in the highly metastatic 58As1 cells. and that these cells remained sensitive to everolimus. Hypoxia-induced growth inhibition of the HSC-58 and 58As1 cells occurred to the same degree in HSC-58 and 58As1 cells (data not shown). Therefore, 58As1 cells might have other strategies for adaptation to hypoxia. However, the detailed molecular mechanisms involved in the resistance to hypoxia, including suppression of mTOR activity, are still not clear.

In conclusion, the failure of mTOR suppression by hypoxia might be an important characteristic of gastric cancer cells with a high propensity for peritoneal dissemination, and everolimus may be effective against these cells. Further studies using various kinds of cancer cell lines and clinical samples will be needed for clinical application.

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**Original Articles**

# Lung Cancer Working Group Report

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Asia needs a guideline for non-small-cell lung cancer because of differences in medical care, medical care insurance, ethnic variation and drug approval lag within Asian countries and compared with Western countries. Due to ethnic differences, drug dosages are often higher in the USA than in Japan. EGFR mutation in non-small-cell lung cancer was detected in 32% of Asians but only 6% of non-Asians, while differences in irinotecan metabolism cause higher frequencies of toxicity (leukopenia, diarrhea) in Asians. Pharmacodynamic ethnic differences in relation to paclitaxel/carboplatin resulted in longer median survival and a higher 1-year survival rate for Japanese-advanced non-small-cell lung cancer patients compared with Americans. To solve the problem of drug lag, pharmaceutical companies must perform multinational Asian clinical trials with quick accrual of patients, while regulatory authorities must establish high-quality, efficient approval processes, and achieve regulatory harmonization. The National Comprehensive Cancer Network promotes creation of national clinical practice guidelines, and Korea, China and Thailand adapted the National Comprehensive Cancer Network guidelines. Many Asian countries still lack such guidelines, and there are no pan-Asian guidelines for non-small-cell lung cancer. Japan developed its own non-small-cell lung cancer guidelines and also a gefitinib guidance. The study group members concluded that immediate establishment of an Asian non-small-cell lung cancer guideline will be difficult because of the differences among the countries. Asian collaborative trials on treatment of non-small-cell lung cancer need to be started at an early date to generate Asian data.

*Key words: non-small-cell lung cancer – EGFR mutation – ethnic differences*



## GUIDELINES

Asia needs a guideline for non-small-cell lung cancer (NSCLC) (1,2). One reason is the differences in medical care for lung cancer within Asian countries (3–9), such as performance of systematic lymph node dissection versus sampling only. There are also differences in medical care insurance and the economic situations among Asian countries. Ethnic variation in pharmacogenomics is yet another reason for needing an Asian guideline (10–14). Differences exist in the selection of validated data, such as for histology, that is, non-squamous versus squamous, biomarkers such as ERCC1, RRM1 and MSH2 (15–23). The concept of consolidation/maintenance therapy also differs between Western and Asian countries. Drug lag in some Asian countries is another important factor affecting treatment of NSCLC (Table 1).

With regard to ethnic differences, the ICH-E5 guideline states that, 'Although ethnic differences among populations may cause differences in a medicine's safety, efficacy, dosage or dose regimen, many medicines have comparable characteristics and effects across regions.' However, comparison