TABLE 1. Patient Characteristics

Number of Patients	Acquired Resistance (n = 20)	Intrinsic Resistance (n = 44)	Sensitive (n = 29)	Total (n = 93)
Age				
Median	59.5	65.5	65	64
Range	32–85	34–76	42–86	32–86
Gender				
Male	6	26	10	42
Female	14	18	19	51
Smoking history				
Former/current Smoker	3	21	11	35
Never smoker	17	23	18	58
Histological type				
Adeno	19	39	29	87
Large cell	0	1	0	1
Squamous cell	0	2	0	2
Undifferentiated non-small cell carcinoma, or adenosquamous	1	2	0	3
EGFR-TKI treatment				
Gefitinib	19	36	27	82
Erlotinib	1	7	2	10
Vandetanib	0	1	0	1
Number of Tumors	**n = 23**	**n = 45**	**n = 29**	**n = 97**
EGFR mutation status				
Exon 19 deletion	12	14[a]	14[a]	40
L858R	11	30	16	57
G719X	0	2	0	2

[a] One patient's tumor had both exon 19 deletion and L858R point mutation.

absence of staining; 1+, weaker staining than normal bronchial epithelium; 2+, similar staining to normal bronchial epithelium; and 3+, clearly more intense staining than normal bronchial epithelium) (Supplementary Figure 1, http://links.lww.com/JTO/A197). The percentage and intensity were multiplied to give a scoring index (*H* score) ranging from 0 to 300, according to a previously reported method with minor modifications.[16] Turke et al.[16] reported that HGF expression was significantly higher in specimens with acquired resistance (mean ± SD: 205 ± 106) compared with pretreatment (126 ± 112). On additional evaluation with specimens showing acquired resistance from patients whose tumors were obtained only after acquiring EGFR-TKI resistance, HGF expression was similar (176 ± 126) to that of specimens with acquired resistance in patients with paired tumor specimens; they concluded that these findings with clinical specimens supported the suggestion that HGF mediated resistance to EGFR-TKIs. Therefore, we defined high-level HGF expression as *H* score ≥200 in this study. Evaluation was performed independently by two investigators (KT and MN) blinded to individual clinical information.

Cycleave Real-Time Polymerase Chain Reaction Assay for T790M Mutation

Details of the cycleave real-time polymerase chain reaction (PCR) assay have been described previously.[21] Briefly, tumor cell-rich areas in hematoxylin and eosin-stained sections were marked under a microscope, and tissues were scratched from the area of another deparaffinized unstained section. Pieces of the scratched tissue were incubated with 1× PCR buffer containing 100 μg/mL proteinase K for 1 hour at 54°C. After heat inactivation at 95°C for 3 minutes, the solution was used directly as the template DNA for the assay. Then, exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (Cepheid, Sunnyvale, CA) using Cycleave PCR Core kits (TaKaRa Co. Ltd., Ohtsu, Japan) with a T790M-specific cycling probe and a wild-type cycling probe. This assay detected as few as 5% cancer cells with T790M mutation in a background of cells with wild-type T790M in *EGFR*.

MET Amplification

Formalin-fixed, paraffin-embedded tissue sections (4 μm thick) were subjected to dual-color fluorescence in situ hybridization using a MET/CEP7 probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. Staining was evaluated as reported previously.[22,23]

Statistical Analysis

Statistical significance was determined by Student's *t* test. All statistical analyses were performed using GraphPad

TABLE 2. Expression of HGF, T790M Secondary Mutation, and *MET* Amplification in EGFR-TKI-Resistant Tumors Obtained from *EGFR* Mutant Lung Cancer Patients

	Acquired Resistance (n = 23)	Intrinsic Resistance (n = 45)	Sensitive (n = 29)
High-level HGF expression	14 (61%)	13[a] (29%)	3[b] (10%)
EGFR T790M secondary mutation	12 (52%)	0	0
MET amplification	2 (9%)	2 (4%)	0

[a] High-level HGF expression was detected in the stroma in two patients.
[b] High-level HGF expression was detected in the stroma in one patient.

Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). All tests were two sided, and $p < 0.05$ was taken to indicate statistical significance.

RESULTS

HGF Expression, T790M Secondary Mutation, and MET Amplification in Tumors with Acquired Resistance

Among 23 tumors with acquired resistance from 20 patients, *EGFR* T790M secondary mutation was detected in 12 tumors (52%) from 11 patients (60%) (Table 2). *MET* amplification was detected in two tumors (9%) from two patients (10%). As HGF is a soluble cytokine, evaluation of HGF is not as simple as that for genetically conferred T790M secondary mutation and *MET* amplification, which can be designated as plus or minus. As described in the Materials and Methods section, we defined high-level HGF expression as H score $\geq$200 in this study. High-level HGF expression was detected in 14 tumors (61%) from 13 patients (60%). In these 14 tumors, HGF was predominantly expressed in cancer cells.

The high HGF expression was simultaneously detected in 6 of 12 tumors positive for T790M secondary mutation (50%) (Table 3, Figure 1). High-level HGF expression was also detected simultaneously in one of two tumors positive for *MET* amplification (50%). These results suggested possible interactions among these three resistance factors, consistent with previous reports.[16,17]

Expression of HGF, T790M Secondary Mutation, and MET Amplification in Tumors with Intrinsic Resistance (Nonresponders)

T790M secondary mutation was not detected in 45 tumors with intrinsic resistance from 44 patients (nonresponders), but *MET* amplification was detected in two tumors (4%) (Table 2). *EGFR* D761Y secondary mutation was detected in two tumors (4%) from one patient[24](Supplementary Table 1, http://links.lww.com/JTO/A197). In contrast, high-level HGF expression in cancer cells was detected in 11 tumors (24%) from 11 patients. In addition, HGF was detected at high levels in stromal cells in two tumors (4%) from two patients (data not shown). In total, high-level HGF expression was detected in 13 tumors with intrinsic resistance (29%). Notably, high-level HGF expression was simultaneously detected in one of two *MET* amplification-positive tumors (50%) (Table 2). These results suggested the involvement of HGF in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients.

Expression of HGF, T790M Secondary Mutation, and MET Amplification in Sensitive Tumors

Neither *EGFR* T790M secondary mutation nor *MET* amplification was detected in 29 sensitive tumors from 29 patients. High-level HGF expression was detected in two tumors (7%) (Supplementary Table 2, http://links.lww.com/JTO/A197). High levels of HGF were detected in stromal cells in one tumor (3%). In total, a high level of HGF expression was detected in three sensitive tumors (10%). Thus, although high HGF expression level was detected even in sensitive tumors, the incidence of high HGF expression was much lower in sensitive tumors than in those with acquired or intrinsic resistance. In addition, mean H score of HGF in tumors with acquired resistance was significantly higher than that in sensitive tumors ($p < 0.001$, Student's t test) (Figure 2). There was no significant difference in mean H score of HGF between tumors with intrinsic resistance (nonresponders) and sensitive tumors.

DISCUSSION

Our previous studies[14,25,26] documented HGF-mediated resistance to EGFR-TKIs in *EGFR* mutant lung cancer, which was also confirmed by other groups.[16,27] Here, we demonstrated that a high level of HGF expression was detected most frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. Our data indicated that although T790M secondary mutation and *MET* amplification are predominantly responsible for acquired resistance, HGF may be responsible not only for acquired resistance but also for intrinsic resistance to EGFR-TKIs.

The mechanism of intrinsic resistance to EGFR-TKIs is not well understood. To our knowledge, this is the first study with more than 40 clinical specimens indicating the incidence of resistance factors in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer. Here, we found that a high level of HGF expression was most frequently (29%) detected in tumors with intrinsic resistance, compared with T790M secondary mutation (0%) and *MET* amplification (4%). It is noteworthy that although the high HGF expression level was detected in cancer cells in tumors with acquired resistance, HGF expression was detected in both cancer cells (10/12 tumors) and host stroma cells (2/12 tumors) in tumors with intrinsic resistance (nonresponders). HGF was reported to be produced by not only cancer cells but also stromal cells.[15] Our data clearly indicated that both cancer cells and stromal cells are sources of HGF, which induces intrinsic EGFR-TKI resistance in *EGFR* mutant lung cancer. As HGF-induced resistance could be reversed by anti-HGF antibody and the natural HGF inhibitor NK4,[25,27] highly produced HGF in

TABLE 3. Summary of Tumors with Acquired Resistance

ID	Gender	Histological Type	*EGFR* Mutation Status	Treatment	BOR	PFS	HGF	T790M	*MET* Amplification
KZ-1	M	Ad	Exon 19 del	Erlotinib	PR	254	60	–	+
KZ-2	F	Ad	L858R	Gefitinib	CR	1041	40	–	–
KZ-3	F	Ad	L858R	Gefitinib	PR	366	200	–	–
OK1—1	M	Ad	Exon 19 del	Gefitinib	PR	351	290	–	–
OK1—2							300	–	–
OK4—2	F	Ad	Exon 19 del	Gefitinib	PR	57	210	+	–
TS-1—3	F	Ad	L858R	Gefitinib	PR	180	90	–	–
TS-1—4							280	+	–
SG2	M	Ad	Exon 19 del	Gefitinib	PR	174	150	+	–
SG3	F	Ad	L858R	Gefitinib	SD	368	110	+	–
SG4	F	Ad	L858R	Gefitinib	PR	60	220	–	+
SG6	M	Ad	Exon 19 del	Gefitinib	PR	352	140	+	–
SG8	F	Ad	L858R	Gefitinib	SD	210	90	+	–
SG9	F	Ad	Exon 19 del	Gefitinib	SD	221	200	+	–
SG10	F	Ad	L858R	Gefitinib	CR	210	210	–	–
TB1—2	M	Ad	Exon 19 del	Gefitinib	PR	1770	230	+	–
TB2—2	F	AdSq	Exon 19 del	Gefitinib	PR	300	300	–	–
AC29—1	M	Ad	L858R	Gefitinib	PR	533	250	–	–
AC29—2							270	+	–
AC24	F	Ad	Exon 19 del	Gefitinib	PR	98	170	+	–
AC26	F	Ad	Exon 19 del	Gefitinib	SD	448	180	+	–
AC28	F	Ad	Exon 19 del	Gefitinib	PR	357	200	+	–
AC31	F	Ad	L858R	Gefitinib	PR	894	200	–	–

Ad, adeno; AdSq, adenosquamous; BOR, best overall response.

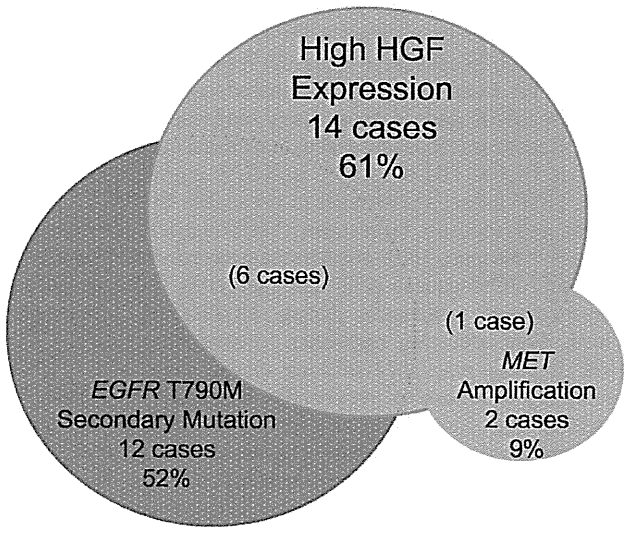

FIGURE 1. Incidences of high-level HGF expression, T790M secondary mutation, and *MET* amplification in 23 tumors with acquired resistance. Values in parentheses are the numbers of cases in which the tumors expressed two resistance factors simultaneously.

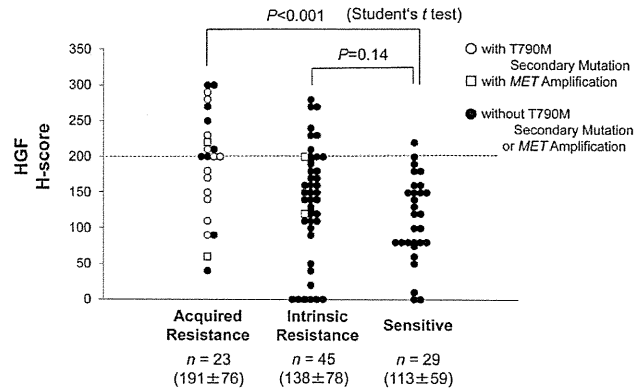

FIGURE 2. HGF expression score (*H* score) in EGFR-TKI-resistant tumors obtained from *EGFR* mutant lung cancer patients. Values in parentheses are mean ± SD of *H* score.

resistant tumors would be an ideal therapeutic target regardless of its origin.

It was of interest that a high level of HGF expression was detected in a small population of sensitive tumors. This was consistent with a previous report[16] indicating high-level HGF expression (*H* score ≥200) in several specimens from responders. Although the reason for the high level of HGF expression in tumors from responders is unclear at present, there are several possible explanations as follows. First, although HGF was expressed at high levels, natural inhibitors such as cleaved HGF and truncated MET, both of which inhibit binding of HGF to MET, may be generated in the tumors.[28,29] Second, negative regulators of MET tyrosine kinase activity such as protein kinase C may be activated and negate the effect of HGF on induction of EGFR-TKI resis-

tance in these tumors.[30] As the amounts of each clinical specimen were limited, we would like to perform further analyses in future studies should sufficient amounts of specimens become available.

Recent studies indicated that multiple resistance factors can be induced simultaneously in a single cancer. For example, Qi et al.[31] reported the simultaneous occurrence of *Met* mutation and activation of the EGFR pathway by ligand overexpression, similar to T790M mutation and HGF overexpression in EGFR mutant lung cancer, which caused resistance to Met-TKIs in gastric cancer. Katayama et al.[32] also reported that *ALK* gene amplification and gatekeeper mutation in *ALK* occurred simultaneously and conferred resistance to ALK inhibitors in EML4-ALK lung cancer. In this study, T790M secondary mutation and the high HGF expression level were simultaneously detected at high incidence (50%) in tumors with acquired resistance. Irreversible EGFR-TKIs were thought to have potential to control acquired resistance caused by T790M secondary mutation, but clinical responses were rarely observed in clinical trials.[33,34] We recently found that HGF induces resistance to not only reversible EGFR-TKIs but also irreversible EGFR-TKIs by activating the MET/PI3K/Akt pathway in *EGFR* mutant lung cancer cells with or without T790M secondary mutation.[26] Taken together, these observations suggest that HGF would be simultaneously expressed with T790M secondary mutation in tumors with acquired resistance and reduce the sensitivity to irreversible EGFR-TKIs in *EGFR* mutant lung cancer patients.

MET amplification has been detected in ~20% of tumors with acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer,[13,16,17] while the incidence reported in Japanese patients is rare.[14,18] Here, we detected *MET* amplification in two tumors (9%) with acquired resistance, suggesting that *MET* amplification can be detected in a significant proportion of tumors with acquired resistance even in Japanese patients. One case with high-level HGF expression and *MET* amplification (KZ-1) was treated with gefitinib and PFS was 254 days. The other case with low HGF and *MET* amplification (SG4) was treated with erlotinib and PFS was 60 days (Table 3). Although it is not possible to make definitive conclusions based on the data from only these two cases, the shorter PFS in the former case tentatively supports the observation that HGF accelerates expansion of preexisting clones with MET amplification.[16] Notably, simultaneous expression of these two factors was also detected in one tumor with intrinsic resistance (nonresponder). However, the mechanism by which HGF is induced in *EGFR* mutant lung cancer is still not well defined. Further examinations are warranted to elucidate the interaction between HGF expression and *MET* amplification in *EGFR* mutant lung cancer.

Among 68 resistant tumors, high-level HGF expression, T790M secondary mutation, and *MET* amplification were not detected in one tumor with acquired resistance and 31 tumors with intrinsic resistance, indicating the involvement of other mechanisms of resistance in these tumors. *EGFR* D761Y secondary mutation in exon 20 was detected in two tumors from the same patient.[24] *EGFR* D761Y mutation was originally identified in recurrent brain metastasis and was shown to induce intermediate-grade resistance to EGFR-TKIs.[35] In addition, rare secondary mutations (other than T790M and D761Y) or a preexisting resistance mutation in a minority of clones may also be involved in intrinsic resistance. Moreover, it was recently reported that a subpopulation of cancer cells that transiently exhibit a distinct phenotype characterized by engagement of IGF-1R activity, hypersensitivity to HDAC inhibition, and altered chromatin showed an intrinsic ability to tolerate exposure to EGFR-TKI.[36] Minor secondary mutations, a preexisting resistance mutation in a minority of clones, or chromatin-mediated drug resistance mechanisms may be involved in resistant tumors without high HGF expression, T790M secondary mutation, and *MET* amplification.

To overcome the HGF-induced resistance to EGFR-TKI in *EGFR* mutant lung cancer, double blockade of the EGFR pathway and HGF-MET pathway is therefore theoretically necessary.[14,16,27] To inhibit mutant EGFR with or without T790M secondary mutation, EGFR mutant-specific inhibitors were developed in addition to irreversible EGFR-TKIs.[37] To inhibit HGF-MET signaling, several inhibitors, including anti-HGF antibody, NK4 (natural antagonist of MET), and MET-TKIs, were developed.[16,25–27] Further studies are essential to determine optimal combined therapy with best efficacy and safety. In addition, a prospective study is required to determine whether immunohistochemical detection of HGF would be sufficiently reliable to identify patients with HGF-induced resistance to EGFR-TKIs. As levels of HGF in peripheral blood are correlated with clinical outcome to EGFR-TKIs in patients with non-small cell lung cancer,[38,39] such noninvasive methods may facilitate individual therapy for overcoming HGF-induced resistance to EGFR-TKIs in *EGFR* mutant lung cancer patients.

Recent studies indicated at least three important roles of HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to reversible EGFR-TKIs, gefitinib, and erlotinib, by restoring MET/Gab1/PI3K/Akt pathways.[14,16] Second, HGF accelerates expansion of preexisting *MET*-amplified cancer cells and facilitates MET amplification-mediated resistance during EGFR-TKI treatment.[16] Third, after acquiring resistance to reversible EGFR-TKIs, HGF induces resistance of lung cancer cells with T790M secondary mutation to irreversible EGFR-TKIs.[24] Here, we detected high-level HGF expression frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. These findings indicate the value of HGF as a therapeutic target for EGFR-TKI-resistant *EGFR* mutant lung cancer. Therefore, combined therapy with EGFR-TKIs and HGF-MET inhibitors in patients with HGF-induced resistance may improve the clinical outcome of *EGFR* mutant lung cancer.

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ORIGINAL ARTICLE

Pharmacokinetic and pharmacodynamic study on amrubicin and amrubicinol in Japanese patients with lung cancer

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Abstract
Purpose The pharmacokinetic (PK)–pharmacodynamic (PD) relationship of amrubicin and its active metabolite, amrubicinol, has only been evaluated using trough levels of these agents since the full PK profiles not yet been clarified so far. This study was performed to analyze the full PK profiles of amrubicin and amrubicinol and to evaluate their toxicity–PK relationships in Japanese patients.
Methods Amrubicin (35–40 mg/m^2) was administered to 21 lung cancer patients on days 1–3 every 3–4 weeks. Fourteen blood samples were obtained per patient over the course of 3 administration days. The plasma concentrations of amrubicin and amrubicinol were quantitated by HPLC, and the relationships between PK parameters of these compounds and hematological toxicities were evaluated.
Results The overall PK profiles of amrubicin and amrubicinol were well characterized using a 3-compartment model and a 1-compartment model with a first-order metabolic process, respectively. The major toxicities were hematological. The clearance of amrubicinol was significantly correlated with grade 4 neutropenia ($P = 0.01$). The percentage decreases in the neutrophil count, hemoglobin level and platelet count were well correlated with the amrubicinol AUC.
Conclusion The pharmacokinetic profiles of amrubicin and amrubicinol were clarified, and the subsequent PK–PD analyses indicate that the clearance of amrubicinol is the major determinant of neutropenia.

Keywords Amrubicin · Amrubicinol · PK–PD study · Myelosuppression · Blood cell destruction

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Introduction

Amrubicin (AMR) and its active metabolite, amrubicinol (AMR-OH), markedly inhibit topoisomerase II activity and are effective against lung cancer [1]. AMR is approved in Japan for the treatment of small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). In AMR monotherapy, AMR is administered at a dose of 35–45 mg/m^2/day on three consecutive days every 3–4 weeks. Six phase II studies for second-line or third-line AMR monotherapy for the treatment of SCLC have demonstrated overall response rates (ORRs) of 21–53% and a median survival period of 6–12 months [2–7]. Two phase II studies of previously treated NSCLC have been reported, with ORRs of 11.5 and 13.5%, respectively [5, 8]. In these phase II studies, the incidences of grade 3 or 4 myelosuppression were 82% (39–97%) [Median (Range)] for neutropenia, 28% (8–38%) for thrombocytopenia and 27% (5–41%) for anemia, respectively. Furthermore, the incidence of febrile neutropenia was 12% (2–35%).

The pharmacokinetic (PK)–pharmacodynamic (PD) profiles of AMR and AMR-OH have not yet been fully clarified so far. In a previous report by Matsunaga et al. [9],

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full-sampling data were obtained from only one subject treated with 30 mg/m^2 of amrubicin and other data were obtained spars-sampling points from 15 patients (30–45 mg/m^2). On the other hand, significant relationships were observed between the plasma trough level of AMR-OH on day 4 and neutropenia and anemia [10]. The trough level of AMR-OH was correlated with the percent change in the neutrophil count [10]. However, the previous studies did not obtain plasma sampling points capable of fully characterizing the PK profiles of AMR and AMR-OH for consecutive days. Generally, the use of plasma drug concentration at only one particular time point should cause some uncertainty for establishing efficacy–PK or toxicity–PK relationships, and full PK profiling of a drug and subsequent modeling approach are highly preferable.

We, therefore, conducted a PK–PD study on AMR and AMR-OH in which we determined the PK model parameters of these agents throughout 3 days of administration and evaluated the toxicity–PK relationships in Japanese lung cancer patients based on the full PK profiles.

Materials and methods

Patients and treatments

This study was conducted at the National Cancer Center Hospital, Tokyo, Japan. Patients were eligible for participation in this study if they were 20 years or older and had been diagnosed as having lung cancer and had received AMR monotherapy. Patients with hepatitis B or C virus or human immunodeficiency virus infections and those who were considered by their physician to be ineligible as a trial candidate were excluded. Written informed consent was provided by each patient before study enrollment. The study was approved by the ethical review boards of the National Cancer Center Hospital and Showa University. It was conducted in accordance with the Declaration of Helsinki and all applicable laws and regulations.

AMR (Calsed^{TM}; Dainippon Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan) was dissolved in 50 mL of physiological saline and was administered intravenously as a 5-min infusion at a dose of 35–40 mg/m^2/day on days 1–3 every 3–4 weeks. For second- or later-line treatment of small or non-small-cell lung cancer with AMR, the recommended dose of AMR is 40 mg/m^2, in general, with some dose reduction (e.g., 35 mg/m^2) as needed based on the judgment of the attending physician. Prophylactic antiemetics (granisetron and dexamethasone) were used only as required and according to the physician's discretion. Other medications for underlying diseases, complications and pain control were allowed.

Before treatment, all patients underwent a medical history survey, physical and hematological examinations and serum biochemistry tests. The physical examination and biochemistry tests were repeated as part of normal clinical practice. The toxicities were graded according to the National Cancer Institute Common Toxicity Criteria, Version 3.0. Response was assessed according to the Response Evaluation Criteria in Solid Tumors [11].

Pharmacokinetic sampling and drug assays

PK evaluations were performed in all patients during the initial cycle of treatment. Heparinized venous blood samples (4 mL) were taken before infusion, at the end of the AMR infusion (0 min), as well as at 5, 15 and 30 min and 1, 2, 4, 8 and 24 h after the end of the infusion and at 0 min and 8 h after the infusions on days 2 and 3.

The plasma samples were stored at −80°C until analysis. The plasma concentrations of AMR and AMR-OH were measured using a previously reported high-performance liquid chromatography (HPLC) method [12]. The components were separated using HPLC on two reverse-phase columns linked with a connector (Onyx Monolithic C18, 100 × 4.6 mm) using 4 mM sodium 1-octanesulfonate, 2.3 mM acetic acid:tetrahydrofuran:dioxane (15:2:6, v/v/v) as an eluent. AMR and AMR-OH were measured using a fluorescence detector set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. Standard AMR and AMR-OH powders with purities >99% were supplied by Dainippon Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). The assay was validated according to the guidelines recommended by the U.S. Food and Drug Administration. The limit of quantitation was 2.5 ng/mL for both AMR and AMR-OH. The percentage recovery from the plasma proved to be higher than 88.1%. Intraday accuracy ranged from −4.1 to 0.8% for AMR and −9.8 to −2.1% for AMR-OH. The interday accuracy ranged from −3.1 to 3.0% for AMR and −4.0 to 2.3% for AMR-OH. The intraday precision ranged from 1.4 to 8.8% for AMR and 1.3 to 4.2% for AMR-OH. The interday precision ranged from 2.7 to 8.8% for AMR and 5.3 to 5.5% for AMR-OH.

Pharmacokinetic analysis

The PK parameters were estimated using a non-linear least-squares regression analysis (WinNonlin, Version 5.0.1; Pharsight, Cary, NC, USA) with a weighting factor of $1/Y^2$, where Y represents the observed data. The individual plasma concentration–time data were fitted to one-, two- or three-exponential equations using a constant infusion input for AMR and a one- or two-compartment model with a first-order metabolic process from AMR to AMR-OH

(parameterized by k_{in}). The PK model was optimized on the basis of Akaike's information criteria (AIC). Fitted parameters were permitted in the computation of the following PK parameters: AUC, peak plasma concentration of day 1 (C_{max}), total body clearance (CL) and volume of distribution at steady state (Vd_{ss}).

Pharmacodynamic analysis

The relationships between PK parameters (AUC and C_{max}) of AMR-OH and the hematologic toxicity were evaluated. The percentage decrease in the hematologic count or level (neutrophils and hemoglobin) was calculated as follows:

$$\%\,\text{Decrease in hematologic count or level} = \frac{\text{pretreatment count or level} - \text{nadir count or level}}{\text{pretreatment count or level}} \times 100$$

The results were plotted as a function of the AUC and C_{max} of AMR-OH, respectively. Relationships between adverse effects and PK exposure (AUC) or C_{max} were fitted using a non-linear least-squares regression and a weighting factor of unity according to a sigmoid model, as follows:

$$\text{Adverse effect } (\%) = \frac{E_{max} \cdot \text{AUC}^{\gamma}}{\text{EC}_{50}{}^{\gamma} + \text{AUC}^{\gamma}} \times 100$$

or

$$\text{Adverse effect } (\%) = \frac{E_{max} \cdot C_{max}{}^{\gamma}}{\text{EC}_{50}{}^{\gamma} + C_{max}{}^{\gamma}} \times 100$$

where E_{max} represents the maximum effect and EC_{50} is the AUC (or C_{max}) of AMR-OH at which the effect was 50% of the maximum effect. A non-linear least-squares regression was conducted using WinNonlin to estimate E_{max}, EC_{50} and the sigmoidicity coefficient (γ). The strength of the relationship between the percent decrease in the hemoglobin level and the AUC (or C_{max}) of AMR-OH was assessed using a least-squares linear regression analysis.

In the PD analysis, the patient characteristics as well as the PK parameters were compared among patients who experienced grade 4 neutropenia (<500/μL). The patient characteristics that were evaluated for possible association with grade 4 neutropenia were age, sex, performance status (0 vs. ≥1), type of disease (SCLC vs. NSCLC), smoking index, prior surgery, prior thoracic or brain irradiation, the number of prior chemotherapy regimens (1 vs. ≥2), albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), serum α_1-acid glycoprotein (AGP) and pretreatment neutrophil counts. PK parameters, including C_{max}, AUC and CL of AMR and AMR-OH, were also compared between patients who experienced and those who did not experience grade 4 neutropenia.

Statistical analyses

The obtained data were presented as mean ± standard deviation (SD). To identify factors associated with grade 4 neutropenia, continuous variables were compared between patients with and those without grade 4 neutropenia using the Mann–Whitney *U*-test, and differences in the distribution of dichotomized variables were evaluated using the χ^2-test or the Fisher exact test, as appropriate. $P < 0.05$ was considered statistically significant, and all *P*-values were two-tailed. To identify variables significantly associated with grade 4 neutropenia, multivariate logistic regression analyses were performed. All statistical analyses were performed using the statistical software JMP 4.0 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

Twenty-one patients were enrolled in this study from May 2007 to June 2009. The patient characteristics are listed in Table 1. Seventeen patients had SCLC, and 4 patients had NSCLC. Seventeen patients were men, and 4 were women; all patients had a good performance status, and the median age was 64 years. All 21 patients had previously undergone at least one chemotherapy regimen. All patients had received a platinum agent (cisplatin or carboplatin), and 20 patients had received some form of topoisomerase inhibitor (irinotecan, etoposide or topotecan). Only one patient who had been diagnosed as having squamous cell carcinoma had not been treated with a topoisomerase inhibitor. All patients were included in the PK and toxicity evaluations. Eighteen patients were assessed for response and survival. Two patients were not assessed for response because of the occurrence of interstitial pneumonia or a cardiac event after the second cycle; chemotherapy was discontinued in these patients. Another patient developed disseminated intravascular coagulation (DIC), and chemotherapy was ceased because of the presence of grade 4 thrombocytopenia despite an insufficient response during the first cycle. Seven patients had received granulocyte colony-stimulating factor during the first cycle. All 21 patients had received prophylactic antiemetics. Among these patients, 8 were treated with granisetron and dexamethasone and 13 were treated with granisetron only prior to treatment with AMR on 3 consecutive days.

Pharmacokinetics

Patients received AMR at a dose of 40 mg/m^2 except for one patient with squamous cell carcinoma who received a

Table 1 Patient characteristics

$n = 21$	n	Median	Range
Sex			
Male/female	17/4		
Age (y.o.)		64	39–81
Disease			
SCLC (LD/ED)	5/12		
NSCLC (LCNEC/SQ)	3/1		
PS			
0/1/2	10/10/1		
Smoking history			
±	2/19		
Smoking index		1,165	0–3,200
Pretreatment			
Surgery			
±	15/6		
Radiation			
±	11/10		
Thoracic	5		
Whole brain	6		
Other	1		
Chemotherapy			
0/1/2≤	0/16/5		
CDDP/CPT	11		
CBDCA/ETOP	8		
Others	12		
Characteristics			
Height (cm)		163.8	155–177.5
Body weight (kg)		57	36–74.95
Body surface area (m^2)		1.62	1.28–1.89
Serum creatinine (mg/dL)		0.8	0.6–1.5
Aspartate amino transferase, AST (IU/L)		24	15–111
Alanine transaminase, ALT (IU/L)		18	7–61
Total bilirubin (mg/dL)		0.4	0.3–1.5
Lactate dehydrogenase, LDH (U/L)		233	133–1,286
Serum albumin (g/dL)		3.9	2.5–4.6
α_1-Acid glycoprotein, AGP (mg/dL)[a]		103.5	50–292
White blood cell (×1,000/μL)		5.4	2.5–15.2
Hemoglobin (g/dL)		11.9	7.2–15.3
Platelet (×10,000/μL)		22.2	12.2–37.3
Absolute neutrophil count, ANC (×1,000/μL)		3.6	1.4–11.9

SCLC small-cell lung cancer, *LD* limited disease, *ED* extensive disease, *NSCLC* non-small-cell lung cancer, *LCNEC* large-cell neuroendocrine carcinoma, *SQ* squamous cell carcinoma, *PS* performance status, *CDDP* cisplatin, *CPT* irinotecan, *CBDCA* carboplatin, *ETOP* etoposide

[a] α_1-Acid glycoprotein (AGP) data were obtained from 18 patients

dose of 35 mg/m^2 based on the judgment of the attending physician. Together, the patients received a total of 71 cycles (median of 4 cycles [range, 1–7]) of therapy. A total of 294 plasma samples were obtained for the PK analyses. The PK profiles for AMR and AMR-OH were well characterized using a 3-compartment model and a 1-compartment model with a first-order metabolic process from AMR to AMR-OH, respectively (Fig. 1). The plasma dispositions of AMR and AMR-OH, to which the PK models were fitted, are shown in Fig. 2, and the pharmacokinetic parameters are listed in Table 2.

The plasma concentrations of AMR decreased sharply shortly after the drug infusion and then slowly declined as a result of drug elimination and distribution into the peripheral and blood compartments. The infusion of AMR was followed 2 h later (t_{max}) by the peak plasma concentration of AMR-OH (C_{max}: 23 ± 7 μg/L; Fig. 2; Table 2). In two patients, AMR was metabolized rapidly to AMR-OH, leading to the generation of a large variation in the metabolic rate constant (k_{in}).

Toxicities

Grade 3/4 hematological toxicities consisted of neutropenia (81%), thrombocytopenia (33%) and anemia (19%). The most frequent grade 4 hematological toxicity, observed in 13 patients (62%), was neutropenia. The mean percentages of the decrease in the white blood cell count, absolute neutrophil count, platelet count and hemoglobin level for all 21 patients were 71 ± 20%, 85 ± 21%, 56 ± 29% and 16 ± 7%, respectively. All non-hematological toxicities were mild (≤grade 2). Three patients (14%) experienced febrile neutropenia. Dose reduction was required in 32% (6/19) of the patients, and a treatment delay (4 weeks or more) was needed in 12 patients because of prolonged hematological toxicity during the second cycle.

Responses

One patient with SCLC had a complete response, 8 patients had partial responses (SCLC 6, large-cell neuroendocrine carcinoma 2), 4 patients had stable disease (SCLC) and 5 patients had progressive disease (SCLC 4, squamous 1).

PK–PD relationship for hematologic toxicity

The present PK–PD analyses demonstrated that the percentage decrease in the absolute neutrophil count was related to the AUC and C_{max} of AMR-OH, as described by the sigmoid maximum effect (E_{max}) model. The plot in Fig. 3a and b depicts the E_{max} relationship using the data obtained from all patients. On the basis of the E_{max} model

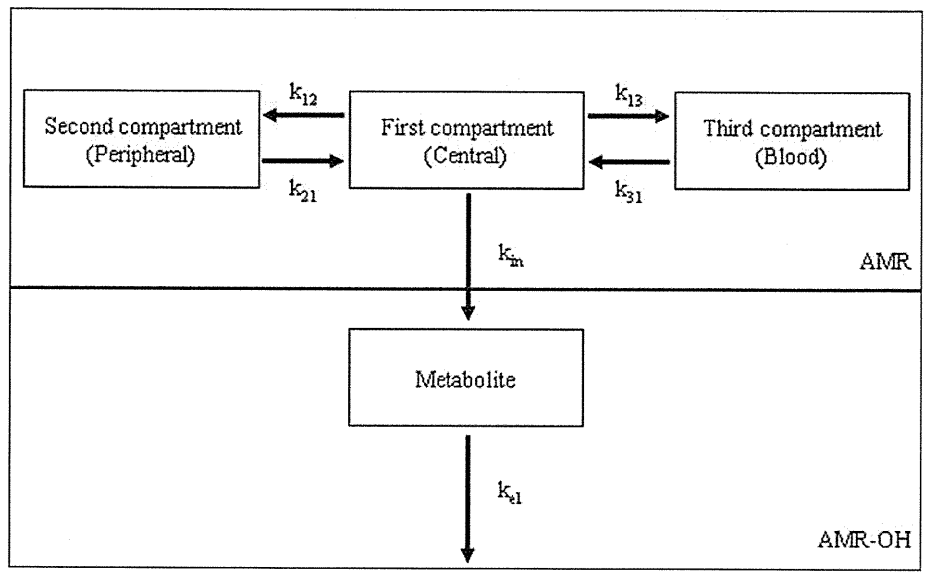

Fig. 1 Schematic illustration of pharmacokinetic model for amrubicin and amrubicinol. The model includes three compartments for amrubicin and one for amrubicinol: k_{12}, k_{21}, k_{13} and k_{31} represent the rate constants for the intercompartmental transfers of AMR. k_{in} represents the metabolic conversion rate constant from AMR to AMR-OH. k_{el} represents the elimination rate constant for AMR-OH

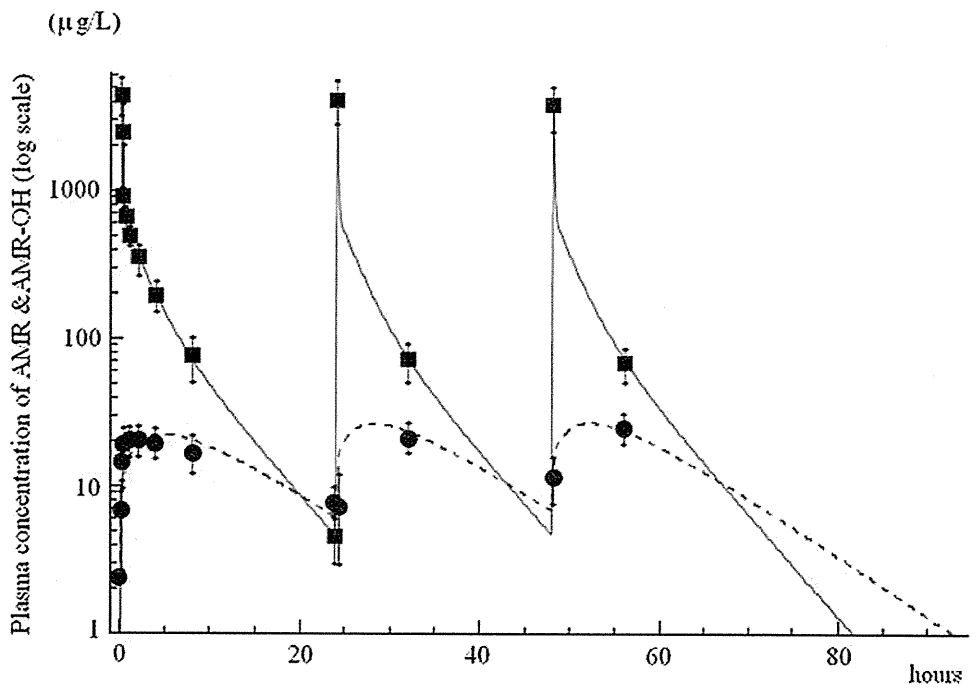

Fig. 2 Concentrations in plasma versus time curves of AMR and AMR-OH. Plasma concentration–time profiles for AMR (*filled square*) and AMR-OH (*filled circle*). *Squares* or *circles* and vertical bars, mean measured concentration ± SD; lines, best-fit lines from the pharmacokinetic analysis (*solid lines* for AMR and *dashed lines* for AMR-OH)

fitting, the AUC and C_{max} that yielded a 50% decrease in the absolute neutrophil count were predicted to be 306.1 h μg/L and 13.2 μg/L, respectively. The shapes of the curves were steep ($\gamma = 8.4$ for AUC; $\gamma = 7.7$ for C_{max}), and these models provided a correlation between the PK of AMR-OH and neutropenia ($r = 0.8296$ for AUC; $r = 0.8035$ for C_{max}). A least-squares linear regression analysis showed that the percent decrease in the hemoglobin level could be estimated by the AUC and C_{max} of AMR-OH ($r = 0.6554$ for AUC; $r = 0.7267$ for C_{max}) (Fig. 3c, d). The AUC and C_{max} that yielded a 50% decrease in the platelet count were predicted to be 549.4 h μg/L and 22.1 μg/L, respectively. The shapes of the curves were gentle ($\gamma = 2.0$ for AUC; $\gamma = 1.9$ for C_{max}), and these models provided the correlation between the PK of AMR-OH and thrombocytopenia ($r = 0.679$ for AUC; $r = 0.6301$ for C_{max}) (Fig. 3e, f). When the characteristics of patients who experienced or did not experience grade 4 neutropenia were compared, the distribution of the performance status was significantly different, and the pretreatment body weight in patients with grade 4 neutropenia was significantly lower than in those without

Table 2 Pharmacokinetic parameters of AMR and AMR-OH

PK parameter	NCA		Model (single dose)[a]		Model (multiple dose)[b]	
	Mean	SD	Mean	SD	Mean	SD
AMR (NCA; constant infusion, Model; 3-compartment infusion)						
AUC (h μg/L)	3,218	684.6	3,175	730.7	3091	579
CL (L/h)	20.6	5.1	20.1	5	21.0	4.4
C_{max} (μg/L)	4,355	1,308	5,500	3,734	4,200	1,149
Vd_{ss} (L)	71.5	15.9	73	17.1	74.0	16.8
k_{12} (1/hr)	–	–	8.0	3.0	6.4	3.5
k_{13} (1/hr)	–	–	2.0	2.7	1.0	2.1
k_{21} (1/hr)	–	–	2.4	1.8	2.4	2.5
k_{31} (1/h)	–	–	0.4	0.2	0.4	0.2
AMR-OH (NCA; extravascular, model; 1-compartment with first-order metabolism)						
k_{in} (1/h)	–	–	12.7	32.3	2.3	2.0
AUC (h μg/L)	515	146	506.9	140.1	459.5	122.3
CL (L/h)	134.3	48.2	135.3	44.9	150.0	53.9
C_{max} (μg/L)	22.5	6.7	22.2	6.6	23.1	6.7
Vd_{ss} (L)	2,899	1,083	2,935	1,223	2,708	1,042

NCA non-compartmental analysis

[a] Model analysis using 9 plasma sampling points (0, 5, 15 and 30 min, and 1, 2, 4, 8 and 24 h after the end of infusion) after AMR infusion on day 1

[b] Model analysis using all plasma sampling points (0, 5, 15 and 30 min and 1, 2, 4, 8 and 24 h after the end of infusion on day 1 and 0 min and 8 h after the end of infusion on days 2 and 3) after AMR infusion on days 1–3

(Table 3). Among the PK parameters, the CL of AMR-OH and the Vd_{ss} of AMR were significantly lower in patients with grade 4 neutropenia.

On the other hand, in the present multivariate analysis, none of the parameters were identified as significant variables of grade 4 neutropenia.

Discussion

The intrinsic activity of the metabolite of AMR, AMR-OH, has been known to be 10–100 times higher than that of AMR [13].

The aims of the present study were to determine the PK parameters of AMR and AMR-OH and to clarify the relationships between PK parameters and toxicity associated with AMR therapy. For this purpose, we carried out an extensive PK–PD study on AMR and AMR-OH in 21 patients with lung cancer, where a total of 14 blood sampling per patient was accomplished over the course of 3 intravenous AMR administration days.

The PK profiles of AMR and AMR-OH were well characterized using a 3-compartment model with a short infusion and a 1-compartment model with a first-order metabolic process from AMR to AMR-OH, respectively. All PK model parameters of AMR and AMR-OH over the 3 administration days could be well extrapolated using the compartment model parameters obtained from a 24-h single-dose and non-compartmental analysis. The PK profiles for AMR and AMR-OH did not show non-linearity or accumulation. Therefore, the 3-day PK profile can be simulated using the plasma trough level observed on the first administration day, enabling the doses on days 2 and 3 to be adjusted, if necessary.

Using the compartment analysis, we were able to perform a kinetic approach to identifying the mechanisms responsible for the metabolism of AMR to AMR-OH and the subsequent metabolic pathway, thereby enabling a quantitative correlation between the PK and the hematological toxicities arising from AMR therapy. The AMR-OH clearance is an apparent clearance, since the percentage of AMR metabolized into AMR-OH is unknown and subject to interindividual variability.

In the present PK–PD study, it was found that a higher C_{max} and AUC of AMR-OH in the plasma was associated with a risk of grade 4 neutropenia and the percentage decrease in the absolute neutrophil count, as well as with the decrease in the platelet count. On the other hand, both parameters were well correlated with a linear model of the percentage decrease in the hemoglobin level. AMR is a quinone-containing anthracycline agent. Doxorubicin has a similar quinone structure and is known to reduce its respective semiquinone-free radicals in the presence of flavoenzymes. Free radicals can also be formed from the

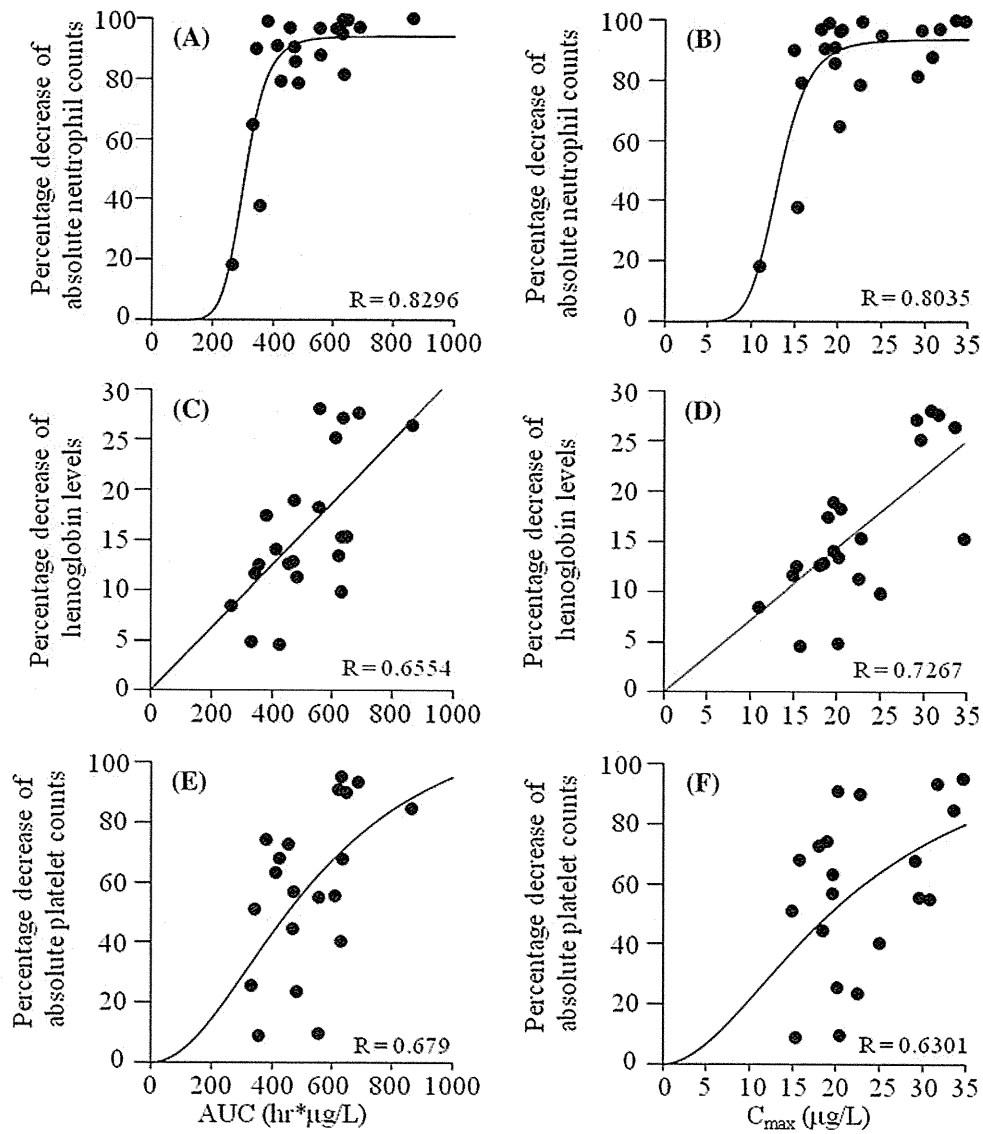

Fig. 3 PK–PD correlation between hematological toxicity and AMR-OH PK parameters. Relationship between the percent decrease in the neutrophil, hemoglobin or platelet count and the AUC or C_{max} of AMR-OH; The solid lines indicate the best fit of a sigmoid E_{max} pharmacodynamic model to the data [neutrophil, AUC: (**a**), C_{max}: (**b**), platelet, AUC: (**e**), C_{max}: (**f**)]. The *solid line* is the linear regression line, and the *dashed line* is the 95% CI for individual estimates [hemoglobin, AUC: (**c**), C_{max}: (**d**)]

interaction of doxorubicin with iron to form a doxorubicin-iron III complex. The in vitro data indicated that dexrazoxane, which prevents anthracycline-mediated cardiotoxicity, inhibited the binding of doxorubicin to red blood cells but had no effect on the association of doxorubicin with erythrocyte ghosts [14]. These findings and a previous report describing an interaction between anthracyclines and iron or hemoglobin support our notion that the third compartment of the parental compound corresponds to the blood cells and that AMR-OH was converted from AMR in the blood, forming an AMR-OH-iron III complex that may directly destroy blood cells. Therefore, the cause of the severe hematological toxicities of AMR may be related not only to myelosuppression but also to the destruction of blood cells.

In multivariate analysis, we could not find the pretreatment factors or PK parameters to predict the severe neutropenia, possibly because the sample size was too small. On the other hand, body weight was found to be positively correlated with the Vd_{ss} of AMR. Moreover, the low Vd_{ss} of AMR was significantly correlated with the high AUC of AMR-OH, suggesting that patients with the low Vd_{ss} of AMR rapidly metabolized AMR to AMR-OH; as a result, hematological toxicities tended to be serious to such patients.

AMR was metabolized rapidly to AMR-OH in 2 patients, leading to the generation of large variations in the metabolic rate constant (k_{in}). The AMR-OH concentrations at the end of infusion in these patients were 29.9 and 11.0 ng/mL, far above the average AMR-OH concentration at the corresponding time point. Other PK parameters and their variations were similar between the single-dose and multiple-dose studies. When these data were excluded, the k_{in} in the single-dose study was determined to be 2.8 ± 0.5 (mean ± SD), which is close to the k_{in} value determined in the multiple-dose study.

The major pathway of AMR metabolism involves the reduction in the C-13 carbonyl group to a hydroxyl group

Table 3 Characteristics and pharmacokinetics of AMR and AMR-OH in patients with or without grade 4 neutropenia

Neutropenia	Neutrophils ≥ 500/μL	Neutrophils < 500/μL	*P* value
Patients (*n*)	8	13	
Sex (*n*)			0.13
Female	0	4	
Male	8	9	
Performance status (n)			0.001
0	8	3	
≥1	0	10	
Number of prior chemotherapy regimens (*n*)			0.33
1	5	11	
≥2	3	2	
Age (years)			1.00
Median	65	64	
Range	39–76	42–81	
Body weight			0.02
Median	65	53	
Range	55–75	36–72	
Serum creatinine			0.11
Median	0.9	0.8	
Range	0.7–1.5	0.0–1.1	
Total bilirubin			0.19
Median	0.4	0.6	
Range	0.3–0.6	0.3–1.5	
Aspartate amino transferase, AST			0.25
Median	23	26	
Range	15–32	16–111	
Serum albumin			0.08
Median	4.1	3.8	
Range	3.5–4.4	2.5–4.6	
AMR clearance, AMR-CL (L/h)			0.06
Median	21.9	18.5	
Range	18.8–33.8	11.7–24.4	
AMR distribution, Volume at steady state, Vd_{ss} (L)			0.02
Median	79.2	63.2	
Range	64.5–116.9	45.7–87.6	
AMR-OH clearance, AMR-OH-CL (L/h)			0.01
Median	167.0	105.9	
Range	86.8–278.4	77.6–149.8	

Sex, performance status and number of prior chemotherapy regimens were analyzed using the χ^2-test or the Fisher exact test. Others were analyzed using the Mann–Whitney *U*-test

by carbonyl reductase (CBR). Then, AMR and AMR-OH are inactivated by NAD (P) H: quinone oxide reductase (NQO) and NADPH P-450 reductase [15].

Genetic polymorphisms of these metabolic enzymes are reportedly related to the PK of several anticancer agents [16–18]. CBR-1 D2 diplotypes tagged by at least one variant allele at the CBR-1 c.627C > T and +967G > A loci are correlated with significantly higher exposure levels of doxorubicin, suggesting the possibility that the intracellular conversion to doxorubicinol is reduced in Asian patients with breast cancer [15]. In another investigation, the NQO-1 609 C > T polymorphism resulted in a significantly reduced tumor NQO-1 activity and a reduced survival in subsets of patients receiving intraperitoneal hyperthermic mitomycin C [18]. However, the exact reason for interindividual variations in the PK of AMR remains unknown; thus, the relationship between genetic polymorphisms of metabolic enzymes and transporters for AMR and AMR-OH should be evaluated in the future.

In this study, several types of lung cancer patients were enrolled (including 17 patients with SCLC, 3 with large-cell neuroendocrine carcinomas and 1 with squamous cell carcinoma). Furthermore, among 17 patients with SCLC, five subjects had limited disease and 12 had extensive disease. These variations of the tumor properties inevitably led to a limitation of this study, i.e., the difficulty in clarifying the PK–PD relationship between the AUC of AMR/AMR-OH and the tumor response.

In conclusion, we clarified the full PK profiles of AMR and AMR-OH and found that the CL of AMR-OH is the major determinant of neutropenia. PK–PD evidence has not yet been reported for this compound on a global basis so far, since AMR is approved only in Japan. Thus, the present findings should be of great importance for avoiding or reducing severe hematological toxicities associated with AMR therapy. In order to confirm our findings and to identify factors influencing the interindividual variabilities in the PK–PD parameters for AMR, further population PK studies and pharmacogenetic studies on a larger number of patients are highly warranted.

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Clinical features of unresectable high-grade lung neuroendocrine carcinoma diagnosed using biopsy specimens☆

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ABSTRACT

Background: The overall clinicopathological features or the optimal therapy for large cell neuroendocrine carcinoma (LCNEC) have yet to be defined, because LCNEC has not been studied in the same depth as had small cell lung carcinoma (SCLC) in both clinical and biological standpoints. The aim of this study was to elucidate the clinical features of high-grade neuroendocrine carcinoma (HGNEC)-probable LCNEC diagnosed by biopsy, and compare therapeutic efficacy with patients with SCLC.
Methods: We retrospectively examined the chart of total of 25 patients who underwent chemotherapy or chemoradiotherapy as initial therapy for a histologic diagnosis of HGNEC-probable LCNEC, using biopsy samples and compared their data with those of 180 patients with SCLC. We analyzed their responses to chemotherapy and/or radiation therapy and survival outcomes.
Results: In 25 patients with HGNEC-probable LCNEC, 18 patients initially received chemotherapy (17 (94%) of whom received platinum-based chemotherapy) with an overall response rate (ORR) of 61%. The remaining 7 patients received chemoradiotherapy with an ORR of 86%, and 12 of the 25 patients who received second-line chemotherapy had an ORR of 17%. A total of 101 patients with SCLC who initially received chemotherapy had an ORR of 63%, and 79 patients who initially received chemoradiotherapy had an ORR of 98%, and 102 of the 180 patients who received second-line chemotherapy had an ORR of 45%. The 1-year overall survival rate for patients with stage IV HGNEC-probable LCNEC ($n = 13$) and those with ED-SCLC ($n = 80$) was 34% and 49%, respectively ($p = 0.84$).
Conclusion: The overall response rate to initial treatment and the survival outcomes of HGNEC-probable LCNEC were comparable to those of SCLC, but the effectiveness of second-line chemotherapy appeared to differ between the 2 groups.

1. Introduction

Large cell neuroendocrine carcinoma (LCNEC) of the lung and small cell lung carcinoma (SCLC) are both now considered to be high-grade neuroendocrine carcinomas arising in the lung. Travis et al. [1] were the first to propose the term LCNEC in 1991, to describe cancer which exhibits neuroendocrine morphologic features such as rosette formation, organoid nesting, and palisading, large tumor cells (typically 3 times larger in diameter than a small resting lymphocyte) with a low nuclear/cytoplasmic ratio, numerous nucleoli, a high mitotic rate (>10 in 10 high-power fields), a large degree of necrosis, and immunohistochemical positive staining findings for 1 or more neuroendocrine markers [2]. The tumor cells of SCLC are round, oval, or spindle-shaped; usually less than the size of three small resting lymphocytes, and have scant cytoplasm, finely granular chromatin, and absent or inconspicuous nucleoli [2]. The morphologic features of LCNEC differ distinctly from those of SCLC by definition, however, distinguishing LCNEC from SCLC based on the tumor cell size and chromatin morphology may be difficult in some cases.

SCLC has poorer outcome, despite its marked chemosensitivity, enabling temporary remission in most SCLC patients because most tumors relapse after chemotherapy or chemoradiotherapy. The standard therapeutic strategy for SCLC has already been

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Table 1
Proposed criteria for diagnosis of pulmonary HGNEC-probable LCNEC using biopsy specimens.

1.	Solid tumor nesting without either acinar or squamous differentiation
2.	Moderate or marked cellular atypia
3.	Large cell size with low nuclear to cytoplasmic ratio or moderate to abundant eosinophilic cytoplasm
4.	Vesicular and/or coarsely granular nuclear chromatin
5.	Prominent nucleoli
6.	Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A, and synaptophysin)
7	Ki-67/MIB1 labeling index >40%

NCAM, neural cell adhesion molecule; HGNEC, high-grade neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma.

established, and second-line chemotherapy has been recognized to be well-tolerated and effective in patients with chemotherapy-sensitive SCLC [3–7]. In contrast, the overall clinicopathological features or the standard treatment for LCNEC have yet to be defined, because LCNEC has not been studied in the same depth as had SCLC in both clinical and biological standpoints. Moreover, the incidence of the pre-therapeutic diagnosis of LCNEC in unresectable cases is unknown. Although obtaining a definitive diagnosis of LCNEC using small biopsy specimen is difficult, there is an urgent need to establish the diagnostic criteria for LCNEC. Therefore, instead of diagnosing LCNEC, we usually use the term "high-grade neuroendocrine carcinoma (HGNEC)-probable LCNEC" based on the proposed criteria (Table 1).

The aim of this study was to elucidate the clinical features of unresectable HGNEC-probable LCNEC (HG-pLCNEC) with those of SCLC, and compare their outcomes.

2. Patients and methods

2.1. Patient enrollment

From January 2002 through December 2009, we retrospectively examined the charts of total of 25 patients with a histologic diagnosis of HG-pLCNEC, using biopsy specimens. Diagnoses of HG-pLCNEC were all confirmed by pathological examination on biopsy specimens according to the modified criteria for the diagnosis of high-grade non-small cell neuroendocrine carcinoma using biopsy specimens proposed by Igawa et al. [8] (Table 1). All patients had undergone a minimum of 1 course of chemotherapy or chemoradiotherapy as initial therapy. Furthermore, the data of a total of 180 patients with histologically confirmed SCLC who had completed a minimum of 1 course of chemotherapy or chemoradiotherapy were examined as a control group. We used these criteria because the diagnostic criteria for LCNEC in the third edition of the World Health Organization (WHO) guidelines, which have been mainly established for cases of surgical specimens, and fulfilling the diagnostic criteria for LCNEC according to the WHO classification system is often difficult with biopsy specimens. We extracted the clinical data of patients from their medical records, all of whom had been given diagnoses of unresectable HG-pLCNEC or SCLC based on the results of pre-therapeutic evaluation including physical examination, chest radiography, computed tomography (CT) of the chest and abdomen, magnetic resonance imaging (MRI) of the brain, isotopic bone scan, and positron emission tomography (PET) or combined PET-CT. Their clinical disease staging was then reassessed according to the 7th edition of the International Union Against Cancer TNM classification system [9]. Data collection and analyses were approved by the institutional review board in December 2010, and the need to obtain informed consent from patients was waived due to the retrospective nature of the study.

2.2. Histopathology

We reviewed all the available pathology slides of biopsy specimens in this study. After fixing the specimens with 10% formalin and embedding them in paraffin, serial 4 μm sections were stained with hematoxylin–eosin (HE). The sections were reviewed by 2 observers (Y.S. and G.I.) and we classified HG-pLCNEC if they fulfilled all the relevant criteria as described above (Table 1). Immunohistochemical analysis was performed to confirm the neuroendocrine features of the specimens. Formalin-fixed paraffin sections were stained for a panel of neuroendocrine markers, including a polyclonal anti-chromogranin A antibody (Ventana, Arizona), anti-neural cell adhesion molecule (NCAM) antibody (Nippon Kayaku, Tokyo, Japan), and monoclonal anti-synaptophysin antibody (DAKO, Glostrup, Denmark). Immunohistochemically, neuroendocrine differentiation was considered to be positive if the tumor cells exhibited focal, patchy, or diffuse staining in the intracellular areas for one or more of these 3 antibodies. The anti-human Ki-67 antigen was identified by use of a monoclonal mouse anti-human Ki-67 (clone MIB1) antigen (DAKO, Glostrup, Denmark). Only nuclear immunostaining was considered to be positive. The labeling index of Ki-67/MIB1 in each tumor was estimated as a percentage of positive cells by counting from 100 to 1000 tumor cells.

2.3. Evaluation

Response criteria were evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST) guidelines [10]. Patients were evaluated to confirm disease progression or relapse by physical examination, chest radiography, and CT of the chest and abdomen. In some patients, we used PET-CT, MRI or bone scintigraphy to detect the extent of disease progression.

2.4. Statistical analysis

Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. Overall survival (OS) was measured from the first day of treatment to the date of death from any cause or the date on which the patient was last known to be alive. All tests were two-sided, and p-values less than 0.05 were considered to be represent statistically significant difference. We used Statview 5.0 software (SAS Institute Inc., Cary, NC) to perform statistical analysis.

3. Results

Overall, 25 patients were recognized to have tumors with histological characteristics consistent with HG-pLCNEC based on biopsy specimens. The typical microscopic appearances of the transbronchial biopsy specimens in the current study are shown in Fig. 1. The tumor cells showed a proliferation of polygonal cells, and a low nuclear–cytoplasmic ratio, with no differentiation of acinar or squamoid features (A). Positive immunostaining findings for NCAM antibody were observed (B), but findings for chromogranin A and synaptophysin were negative (data not shown). The diagnoses of 17 of 25 patients were obtained by transbronchial lung biopsy, and the diagnoses of the remaining 8 patients were obtained by CT-guided needle biopsy.

The characteristics of all the patients examined in this study are shown in Table 2. Among the 25 patients with HG-pLCNEC, the median age was 67 years (range 48–83 years), and 22 patients (88%) were men. Of the 25 patients, all (100%) were current or former smokers. Stage III B was noted in 7 patients (28%), and 13 patients (52%) had stage IV. In the patients with HG-pLCNEC, 18

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Table 2
Patient characteristics.

Characteristics	Category	HGpL	%	SCLC	%
No. of patients		25		180	
Age	Median (range)	67 (48–83)		68 (28–84)	
Gender	Male	22	88	148	82
	Female	3	12	32	18
Smoking status	Ever	25	100	172	96
	Never	0	0	8	4
Clinical stage	I	0	0	2	1
	II	1	4	12	7
	IIIA	4	16	37	21
	IIIB	7	28	39	22
	IV	13	52	90	50
Initial therapy	CT	18	72	101	56
	CRT	7	28	79	44
Tumor marker					
NSE	Median (NL: <16 ng/ml) (range)	30 (10–273)		29 (3–585)	
ProGRP	median (NL: <46 pg/ml) (range)	234 (10–20,000)		488 (7–18,000)	

HGpL, high-grade neuroendocrine carcinoma probable large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; CT, chemotherapy; CRT, chemoradiotherapy; NL, normal level; NSE, neuron-specific enolase; ProGRP, pro-gastrin-releasing peptide.

(72%) received chemotherapy, and 7 (28%) received chemoradiotherapy as initial treatment.

Among the 180 patients with SCLC, there were 99 patients with limited disease SCLC (LD-SCLC), and the number with extensive disease SCLC (ED-SCLC) was 81. The median age was 68 years (range 28–84 years), and 148 patients (82%) were men. Of 180 patients, 172 (96%) were current or former smokers. In the SCLC patients, 101 (56%) patients initially received chemotherapy, and 79 (44%) patients received chemoradiotherapy.

Of the 25 patients with HG-pLCNEC, 12 patients (48%) received second-line chemotherapy. The remaining 13 patients did not receive chemotherapy due to death from disease, adverse events caused by initial treatment, or no active treatment determination. Of the SCLC patients, 104 (58%) received second-line chemotherapy. A diagram of the tumor types and management in patients in this study is shown in Fig. 2.

Treatments and clinical response are summarized in Table 3. The regimens of initial treatment chemotherapies are listed in Table 3(a). Of 18 patients with HG-pLCNEC who initially received chemotherapy, 17 (94%) received platinum-based chemotherapy, and the 7 patients who had chemoradiotherapy received platinum-based chemotherapy and concurrent radiation of 45–60 Gy. Of the 101 patients with SCLC who underwent chemotherapy, the most frequently administered chemotherapy regimen was carboplatin and etoposide ($n=42$), and the second most frequent was cisplatin and etoposide ($n=23$).

Among the 18 patients with HG-pLCNEC initially receiving chemotherapy, 1 achieved a complete response (CR) and 10 achieved a partial response (PR), with an overall response rate (ORR) of 61% (Table 3(b)). One patient with CR and 4 patients with PR received cisplatin and irinotecan. There were 2 PRs observed in the patients treated with carboplatin and paclitaxel, and 1 PR was observed in each group of patients treated with either cisplatin and vinorelbine, cisplatin and docetaxel, cisplatin and amrubicin, or irinotecan alone. Among the 7 patients with HG-pLCNEC initially receiving initially chemoradiotherapy, 6 achieved PR, with an ORR of 86%. In the patients treated with cisplatin and vinorelbine, 3 PRs were observed while 2 PRs were observed in the patients treated with cisplatin and etoposide, and 1 patient achieved PR with carboplatin and etoposide.

Among the 101 patients with SCLC initially receiving chemotherapy, 2 achieved CR and 62 patients achieved PR, with an ORR of 63%. Among the 79 patients with SCLC initially receiving chemoradiotherapy 21 achieved CR and 56 patients achieved PR, with an ORR of 98%.

The regimens of second-line chemotherapies are listed in Table 3(c). The following chemotherapy regimens were used in 12 patients with HG-pLCNEC: amrubicin alone ($n=4$), docetaxel alone ($n=3$), cisplatin and irinotecan ($n=3$), carboplatin and etoposide ($n=1$), and cisplatin and irinotecan and etoposide ($n=1$). In 102 patients with SCLC who received second-line chemotherapy, the frequent administered chemotherapy regimen was cisplatin and irinotecan ($n=34$), and the second most frequently administered was amrubicin alone ($n=18$).

Among the 12 patients with HG-pLCNEC receiving second-line chemotherapy, 2 patients achieved PR, with an ORR of 17% (Table 3(d)). One patients achieved PR in each group of patients treated with cisplatin and irinotecan, and carboplatin and paclitaxel. Among the 102 patients with SCLC receiving second-line chemotherapy, 4 achieved CR and 41 patients achieved PR, with an ORR of 45%. These results indicate that the effectiveness of second-line chemotherapy appeared to differ between HG-pLCNEC and SCLC patients in the present study, but the difference was statistically not significant ($p=0.12$).

Fig. 3 shows the OS curves for the stage IV HG-pLCNEC and ED-SCLC groups. The 1-year OS rate for patients with stage IV HG-pLCNEC was 34%, and that for patients with ED-SCLC was 49%, with no statistically significant difference ($p=0.84$).

4. Discussion

We set out to determine the clinical features of HG-pLCNEC and other related tumors diagnosed by biopsy specimens and compare these with those of SCLC. We also examined the efficacy of chemotherapy or chemoradiotherapy between HG-pLCNEC and that of SCLC. Little is known about the optimal treatment strategy of LCNEC because most publications concerning LCNEC are based on surgical materials, with limited cohort data [11–13]. From a treatment point of view, it is imperative to establish the appropriate definitive diagnostic criteria based on the examination of biopsy or cytologic specimens, and then evaluate the efficacy of chemotherapy or chemoradiotherapy for those patients with unresectable tumors.

It is often difficult to diagnose LCNEC with small biopsy specimens, because of the possibility of crushed remnants of tissue artifacts due to insufficient specimen size, and some morphological overlap regarding cell size or nucleus size between SCLC and LCNEC [14]. In order to resolve this histological ambiguity in neuroendocrine carcinoma cases with regard to a diagnosis of LCNEC

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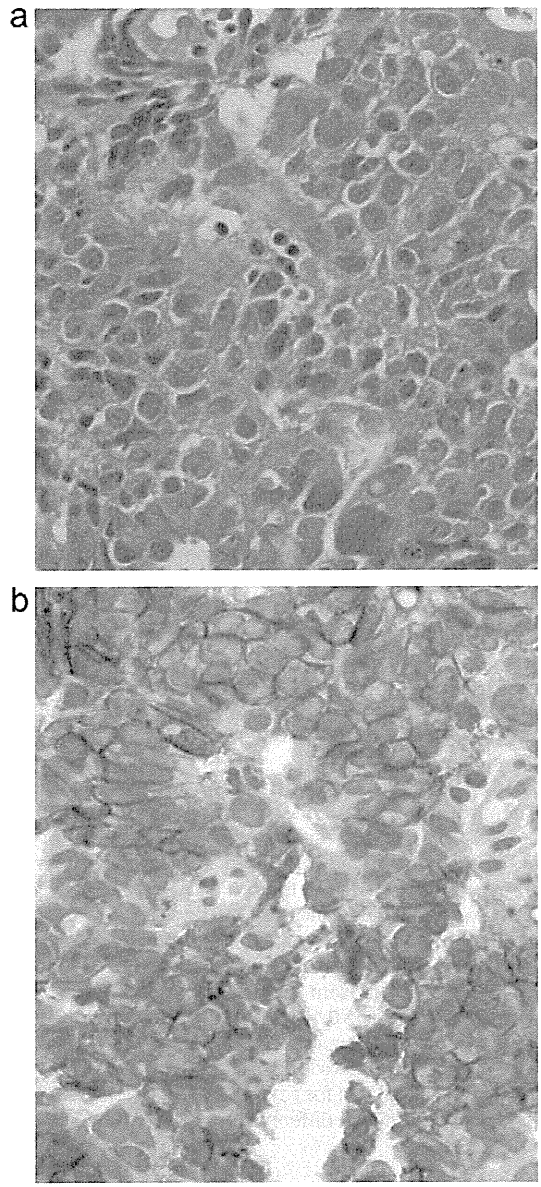
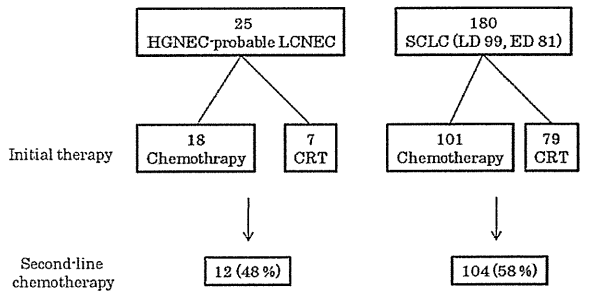


Fig. 1. A biopsy specimen diagnosed as HGNEC-probable LCNEC. (A) The histological features of HGNEC-probable LCNEC are shown with hematoxylin–eosin (HE) staining. The tumor cells are large, with a proliferation of polygonal cells, and have a low nuclear–cytoplasmic ratio, with no differentiation of acinar and squamoid features (400×). (B) Positive staining for neural cell adhesion molecule (NCAM).



HGNEC: high-grade neuroendocrine carcinoma, LCNEC: large cell neuroendocrine carcinoma, SCLC: small cell lung carcinoma, LD: limited disease, ED: extensive disease, CRT: chemoradiotherapy

Fig. 2. The characteristics of patients enrolled in this study.

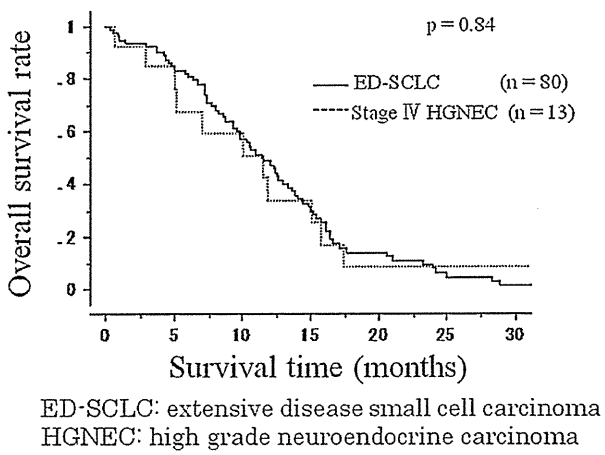


Fig. 3. Overall survival (OS) curve for stage IV HGNEC-probable LCNEC and ED-SCLC groups. The 1-year overall survival rate for patients with stage IV HGNEC-probable LCNEC was 34%, and that for patients with ED-SCLC was 49% ($p = 0.84$).

or SCLC, many researchers have performed immunohistochemistry or molecular analysis [15–18]. Hiroshima et al. reported that the frequencies of the expression of CD56, mASH1, TTF-1, and p16 were higher and that of NeuroD was lower in SCLC than in LCNEC in immunohistochemical analysis. The authors stated that LCNEC and SCLC are different morphologically, phenotypically, and genetically, although there are some overlapping features [15]. Nitadori et al. performed tissue microarray analysis of surgically resected LCNEC and SCLC specimens using 48 antibodies, and demonstrated that significant expression of CK7, CK18, E-cadherin, and β-catenin is more characteristic of LCNEC than of SCLC, suggesting that LCNEC and SCLC have a different biologic phenotype [17]. Ullmann et al. examined comparative genomic hybridization for LCNEC and SCLC, and reported that there were differences in the expression at 3q, 6p, 10q, 16q, and 17p [19]. On the other hand, Jones et al. demonstrated that cDNA microarrays gene expression profiles showed LCNEC was not differently clustered from SCLC, but different from large cell carcinoma or other NSCLC histology [20]. Although the clinicopathological features of LCNEC were similar to those of SCLC, and there is a histological ambiguity with regard to a diagnosis of LCNEC or SCLC, some biological behaviors of LCNEC were different from those of SCLC. Because there is actually the difficulty regarding the use of kinds of imuunohistochemical antibodies in daily practice, we have used the unique diagnostic criteria for HG-pLCNEC developed specifically for biopsy specimens by Igawa et al. [8]. However, lung cancer including LCNEC diagnosed by biopsy materials might not be representative of the whole tumor characteristics, particularly in heterogeneous cancers. Combinations with SCLC do occur, but such tumors are classified as combined variants of SCLC. Therefore, when using only biopsy materials for diagnosis, misdiagnosis may be unavoidable. The HG-pLCNECs examined in the present study might be mostly LCNECs and other related tumors, which included combined subtypes or other histological types, and excluded pure SCLC. This is one of the potential limitations of the present study.

To the best of our knowledge, there are few retrospective studies on the therapeutic efficacy of chemotherapy and/or radiation therapy for LCNEC [8,21,22], and this is the first study to examine

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Table 3
Treatments and clinical response.

(a) Initial therapy and chemotherapy regimens

	HGpL	SCLC
No. of patients	25	180
Initial therapy		
Chemotherapy (%)	18 (72)	101 (56)
CDDP + CPT-11	8	21
CDDP + VNR	4	0
CBDCA + PTX	2	0
CBDCA + ETP	1	42
CDDP + DTX	1	0
CDDP + AMR	1	2
CPT-11	1	0
CDDP + ETP	0	23
Others	0	13
Chemoradiotherapy (%)	7 (28)	79 (44)
CDDP + VNR	3	0
CBDCA + ETP	2	5
CDDP + ETP	2	72
Others	0	2

(b) Clinical response after initial therapy

	HGpL (n = 25)		SCLC (n = 180)	
Initial therapy	Chemotherapy only	CRT	Chemotherapy only	CRT
No. of patients	18	7	101	79
CR	1	0	2	21
PR	10	6	62	56
SD	5	1	19	2
PD	2	0	12	0
NE	0	0	6	0
Response rate (%)	11/18 (61)	6/7 (86)	64/101 (63)	77/79 (98)

(c) Second-line chemotherapy regimens

	HGpL	SCLC
No. of patients	12	102
AMR	4	18
DTX	3	0
CDDP + CPT-11	3	34
CBDCA + ETP	1	12
CDDP + ETP + CPT-11	1	11
CDDP + ETP	0	10
Others	0	17

(d) Clinical response after second-line chemotherapy

Response	HGpL (n = 12)	SCLC (n = 102)	p-Value
CR	0	4	
PR	2	41	
SD	4	16	
PD	6	42	
NE	0	1	
Response rate (%)	2/12 (17)	45/102 (43)	0.12

HGpL, high-grade neuroendocrine carcinoma probable large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; CDDP, cisplatin; CPT-11, irinotecan; VNR, vinorelbine; CBDCA, carboplatin; PTX, paclitaxel; ETP, etoposide; DTX, docetaxel; AMR, amrubicin; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; CRT, chemoradiotherapy.

second-line chemotherapeutic efficacy. In the current study, the majority of patients with HG-pLCNEC were predominantly men, smokers, with elevated NSE and ProGRP values. These resemble the clinical features of those of SCLC, similarly to several previous reports regarding the clinicopathological characteristics of LCNEC [11,12]. We obtained a response rate to initial chemotherapy of 61% and that to chemoradiotherapy of 86% in patients with HG-pLCNEC, which was similar to those of SCLC. The survival of patients with stage IV HG-pLCNEC was also similar to that of ED-SCLC patients. Considered together, these results suggest that there was no statistically significant difference in the initial treatment efficacy between the HG-pLCNEC and SCLC groups. Some authors have reported no statistically significant difference in survival outcome between LCNEC and SCLC [11,13], whereas the survival of patients with surgically resected LCNEC is reported to be intermediate between that of atypical carcinoid and SCLC [23]. Many authors reported that survival in LCNEC was poorer than that in stage-matched NSCLC, and adjuvant therapy might be effective in cases of early stage LCNEC [24–27].

The present study showed that the ORRs of second-line chemotherapy were 17% and 45% for patients with LCNEC and SCLC, respectively. In patients with SCLC, the prognosis at relapse is poor, and response to second-line chemotherapy correlates with response to first-line therapy and also to the interval between first-line chemotherapy and disease progression. Second-line chemotherapy has been recognized to be well-tolerated and effective, with an ORR of 15–88% in patients with chemotherapy-sensitive SCLC [3–7]. The present study suggested that chemotherapeutic efficacy in patients with HG-pLCNEC might be lower than in those with SCLC, even though the chemotherapeutic regimens were heterogeneous. The number of patients with HG-pLCNEC in this study was too small to draw any definite conclusion in terms of differing benefits of chemotherapy regimens for NSCLC and SCLC, or a possible difference in second-line chemotherapeutic sensitivity between LCNEC and SCLC. However, although LCNEC is categorized as a NSCLC, molecular findings in SCLC and LCNEC showed some differences but much overlap, and overall clinicopathological features and the initial treatment response of LCNEC in our study or several published articles suggest that these tumors would be better classified as a high-grade neuroendocrine tumor comparable with SCLC, suggesting that chemotherapies using an SCLC-based standard protocol might be effective and significantly improves the survival of patients with LCNEC compared with those using a NSCLC-based protocol [12,24,26].

In conclusion, these results, although limited, that the clinical efficacy of initial chemotherapy and/or radiation therapy for patients with HG-pLCNEC is similar to that of SCLC, and there might be a different sensitivity to second-line chemotherapy between HG-pLCNEC and SCLC. Improved diagnostic criteria, specifically developed for biopsy specimens, are needed to analyze the biological behavior of LCNEC. Moreover, prospective additional studies in a larger series are clearly mandatory to confirm our data, and the role of a therapy strategy with SCLC-based regimens deserves sensitivity to chemotherapeutic agents and the optimal treatment protocol.

Conflict of interest statement

The authors declare no potential conflicts of interest regarding this study.

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