Table I. Characteristics of cases and controls

	Cases (n = 718), n (%)	Controls (n = 1416), n (%)	OR (95% CI)	P^a
Age				
<40	20 (2.8)	40 (2.8)	—	
40–49	54 (7.5)	106 (7.5)	—	
50–59	196 (27.3)	390 (27.5)	—	
60–69	277 (38.6)	544 (38.4)	—	
70–79	171 (23.8)	336 (23.7)	—	1.000
Mean age ± SD	61.3 ± 10.0	61.8 ± 9.9		0.262
Sex				
Male	533 (74.2)	1054 (146.8)	—	
Female	185 (25.8)	362 (50.4)	—	0.920
Cumulative exposure to smoking (PY)				
0	176 (24.5)	575 (40.6)	1 (reference)	
<15	45 (6.3)	162 (11.4)	1.36 (0.91–2.04)	0.131
<30	75 (10.4)	204 (14.4)	2.07 (1.44–2.98)	8.6×10^{-5}
<45	131 (18.2)	205 (14.5)	3.82 (2.72–5.37)	1.36×10^{-14}
≥45	286 (39.8)	258 (18.2)	6.83 (4.98–9.36)	7.6×10^{-33}
Unknown	5 (0.7)	12 (0.8)		
Drinking habit				
Never	278 (38.7)	501 (35.4)	1 (reference)	
Former[b]	26 (3.6)	64 (4.5)	0.73 (0.45–1.19)	0.209
Current				
<5 g/day	60 (8.4)	174 (12.3)	0.63 (0.46–0.88)	0.007
<23 g/day	113 (15.7)	272 (19.2)	0.77 (0.58–1.01)	0.057
<46 g/day	94 (13.1)	192 (13.6)	0.91 (0.67–1.23)	0.528
≥46 g/day	132 (18.4)	191 (13.5)	1.29 (0.97–1.72)	0.080
Unknown	15 (2.1)	22 (1.6)		
Family history of lung cancer				
No	640 (89.1)	1289 (91.0)	1 (reference)	
Yes	78 (10.9)	127 (9.0)	1.23 (0.92–1.66)	0.169

CI, confidence interval.
[a]P-values were by chi-squared test or Mann–Whitney test for age and sex. Those for ORs were by Wald test.
[b]Former smokers and drinkers were defined as subjects who had quit smoking and drinking at least 1 year previously.

polymorphism (rs671) was based on TaqMan Assays (Applied Biosystems, Foster City, CA). In our laboratory, the quality of genotyping is routinely assessed statistically using the Hardy–Weinberg test and by retyping of a random sampling of 5% of subjects.

Assessment of alcohol intake and smoking exposure

Consumption of each type of beverage (Japanese 'sake', beer, 'shochu', whiskey and wine) was determined as the average number of drinks per day, which was then converted into a Japanese sake (rice wine) equivalent. One drink equates to one 'go' (180 ml) of Japanese sake, which contains 23 g of ethanol, equivalent to one large bottle (633 ml) of beer, two shots (57 ml) of whiskey and two and a half glasses of wine (200 ml). One drink of shochu (distilled spirit), which contains 25% ethanol, was rated as 108 ml. Total alcohol consumption was estimated as the summarized amount of pure alcohol consumption (g/day) of Japanese sake, beer, shochu, whiskey and wine among current regular drinkers. Cumulative smoking dose was evaluated as pack-years (PY), the product of the number of packs consumed per day and years of smoking.

Statistical analysis

To assess the strength of association between an *ALDH2* polymorphism and risk of lung cancer, odds ratios (ORs) with 95% confidence intervals were estimated using unconditional logistic models adjusted for potential confounders. Potential confounders considered in multivariate analysis were age, sex, smoking, drinking and family history of lung cancer with mutual adjustment of *ALDH2*. Smoking status was divided into five categories considering cumulative exposure to tobacco: 0, <15, <30, <45 or ≥45 PY. Alcohol exposure was also categorized into six levels: never-drinkers, former drinkers and current drinkers of <5, <23, <46 or ≥ 46 g/day. Differences in categorized demographic variables between cases and controls were tested by the chi-squared test. Mean values for age between cases and controls were compared by Student's t-test. Accordance with the Hardy–Weinberg equilibrium was checked for controls using the chi-squared test, and the exact P-value was used to assess any discrepancies between genotype and allele frequency. A P-value <0.05 was considered statistically significant. All analyses were performed using STATA version 10 (Stata Corp., College Station, TX).

Table II. Genotype distributions of *ALDH2* polymorphisms and their impact on the risk of lung cancer in recessive model

	ALDH2			P-value
	Glu/Glu	Glu/Lys	Lys/Lys	
Overall				
n (case–control)	322/688	326/605	70/123	
Model1[a]	1.00 (reference)		1.31 (0.95–1.81)	0.104
Model2[b]	1.00 (reference)		1.10 (0.77–1.57)	0.611

[a]Model 1 adjusted for age, sex and smoking (PY: 0, <15, <30, <45, ≥45 and unknown).
[b]Model 2 adjusted for model 1 with family history of lung cancer and drinking (never, former, current <5 g/d, current <23 g/d, current <46 g/d, current ≥46 g/d and unknown).

Results

Table I shows the distribution of cases and controls by background characteristics. Age and sex were balanced between cases and controls. Heavy smokers in terms of PY were significantly more prevalent among cases than controls (P < 0.001). ORs increased in dose-dependent manner and each of them showed high statistical significance. Drinking habit showed fluctuated association. Those who drank ≥46 g ethanol/day showed marginally increased risk of lung cancer, whereas those who drank <46 g ethanol per day or former drinker showed inverse association with variable statistical significance. No significant association was observed between positive family history and lung cancer risk.

Table II shows genotype distributions for *ALDH2* and its ORs and 95% confidence intervals for lung cancer risk. The frequencies of

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Table III. Adjusted OR[a] and 95% CI for cumulative exposure to smoking according to *ALDH2* genotype

	PY										
	Ca/co	0	Ca/co	<15	Ca/co	<30	Ca/co	<45	Ca/co	≥45	*P*-trend
ALDH2											
Glu/Glu	81/285	1.00 (reference)	23/85	1.31 (0.74–2.32)	31/100	1.78 (1.03–3.08)	64/102	3.89 (2.35–6.46)	110/119	6.72 (4.16–10.8)	1.6×10^{-16}
Glu/Lys	80/226	1.00 (reference)	20/62	1.41 (0.76–2.63)	34/93	1.66 (0.97–2.85)	49/84	2.83 (1.67–4.78)	142/134	5.36 (3.35–8.59)	4.5×10^{-14}
Lys/Lys	15/64	1.00 (reference)	2/15	1.01 (0.18–5.64)	10/11	10.2 (2.42–43.1)	18/19	11.4 (3.09–42.0)	25/14	23.2 (6.23–86.5)	2.6×10^{-7}

Ca/co, cases/controls; CI, confidence interval.
[a]ORs adjusted for age, sex, family history of lung cancer, smoking (PY: 0, <15, <30, <45, ≥45 and unknown) and drinking (never, former, current <5 g/d, current <23 g/d, current <46 g/d, current ≥46 g/d and unknown).

polymorphisms were in accordance with the Hardy–Weinberg equilibrium. On analysis of lung cancer overall, no significant elevation of risk was observed by *ALDH2* genotype in per allele model. As shown in Table II, although the association was rather clear between *ALDH2* polymorphism and lung cancer, it was not statistically significant in model 1 adjusted for smoking and matching factors. Association between *ALDH2* Lys/Lys became far from significant if drinking habit was included in the model, indicating strong confounding by drinking and Lys/Lys genotype. Among controls, 117 of 123 (95.1%) were never-drinkers in those with Lys/Lys, whereas 29.5% were never-drinkers among Glu/Glu or Glu/Lys subjects. In addition, heavier smokers were significantly common in those with *ALDH2* Glu/Glu or Glu/Lys subjects (19.1%) compared with Lys/Lys subjects (11.4%).

Table III shows the effects of cumulative exposure to smoking on lung cancer risk by *ALDH2* genotype as adjusted ORs. For *ALDH2*, adjusted ORs showed a marked difference by genotype. The ORs for Glu/Glu and Glu/Lys showed similar point estimates, at 6.72 and 5.36 for PY $\geq$ 45 compared with PY = 0, respectively, with statistical significance. Interestingly, individuals with *ALDH2* Lys/Lys showed a significantly greater risk of lung cancer with increased exposure to smoking. The ORs for those with PY $\leq$ 45 in *ALDH2* Lys/Lys was 23.2 compared with PY = 0 ($P = 2.8 \times 10^{-6}$), indicating possible interaction between cumulative exposure to smoking and the *ALDH2* Lys/Lys genotype. In contrast, we did not see any interaction between alcohol drinking and *ALDH2* genotype (data not shown). We explored effect of *ALDH2* Lys/Lys according to cumulative exposure, duration and intensity as shown in Table IV. It also supports that *ALDH2* Lys/Lys has greater impact in those with heavier exposure.

Table V shows stratified analyses according to histology and drinking status. Based on the results in Tables II, III and IV, we dichotomized the *ALDH2* genotype as Glu/Glu + Glu/Lys and Lys/Lys. Overall, adjusted ORs among those with Glu/Glu or Glu/Lys for PY <15, <30, <45 and ≥45 relative to never smokers were 1.39, 1.80, 3.44 and 6.25, respectively (*P*-trend = 1.4×10^{-30}), versus 1.01, 10.2, 11.4 and 23.2, respectively, for those with Lys/Lys (*P*-trend = 2.6×10^{-7}). We observed a statistically significant interaction between *ALDH2* genotype (Glu/Glu + Glu/Lys versus Lys/Lys) and cumulative dose of smoking (interaction $P = 0.036$). By histologic type, significant interaction was observed in adenocarcinoma (interaction $P = 0.009$), but others were not evaluable owing to the limited number of low-exposure subjects. Interestingly, a significant interaction between the *ALDH2* Lys/Lys genotype and cumulative smoking dose was consistently observed in never-drinkers (interaction $P = 0.041$), indicating that the interaction might exist independent of drinking (Table V).

Table IV. Adjusted OR and 95% CI for *ALDH2* Lys/Lys relative to *ALDH2* Glu/Glu and Glu/Lys according to smoking exposure[a]

Cumulative exposure to smoking	Ca/co Glu/Glu + Glu/Lys	Ca/co Lys/Lys	Odds ratio[b]	*P*-value
Cumulative exposure to smoking				
0	161/511	15/64	0.73 (0.40–1.35)	0.316
<15	43/147	2/15	0.41 (0.09–1.91)	0.258
<30	65/193	10/11	3.51 (1.37–8.97)	0.009
<45	113/186	18/19	1.77 (0.87–3.60)	0.113
≥45	261/244	25/14	1.82 (0.91–3.64)	0.09
Years of smoking				
0	161/11	15/64	0.73 (0.40–1.35)	0.316
<20	37/150	3/18	0.77 (0.21–2.84)	0.699
<40	198/381	28/21	2.81 (1.51–5.20)	0.001
≥40	247/242	24/20	1.29 (0.69–2.44)	0.427
Intensity of smoking (pieces per day)				
0	162/511	15/64	0.73 (0.40–1.35)	0.316
<20	99/233	10/18	1.33 (0.58–3.03)	0.498
<40	278/393	38/33	2.06 (1.23–3.45)	0.006
≥40	107/147	7/8	1.02 (0.33–3.14)	0.966

Ca/co, cases/controls; CI, confidence interval.
[a]Subjects who were unknown for cumulative smoking were excluded from analyses.
[b]ORs adjusted for age, sex, family history of lung cancer, smoking (PY: 0, <15, <30, <45, ≥45 and unknown) and drinking (never, former, current <5 g/d, current <23 g/d, current <46 g/d, current ≥ 46 g/d and unknown).

Discussion

In this study, we found a significant gene–environment interaction between cumulative exposure to smoking and *ALDH2* Lys/Lys for the risk of lung cancer among a Japanese population. A significant interaction among never-drinkers only strongly suggests that this interaction was independent of drinking behavior. In contrast, we did not find an association between lung cancer and *ALDH2* polymorphism alone.

Given the strong evidence for gene–environment interaction between alcohol drinking and *ALDH2* polymorphism in aerodigestive tract cancers in Japanese populations (17–19), we were interested to examine the possible role of the functional genetic polymorphisms involved in acetaldehyde metabolism, *ALDH2* Glu504Lys, in lung cancer. To our knowledge, only a few studies have investigated the association between lung cancer and *ALDH2* polymorphism (20,21). Yokoyama *et al.* (20) reported that the *ALDH2* Lys allele was associated with an increased risk of lung cancer among Japanese alcoholics, albeit in a study population of only seven cases. Minegishi *et al.* examined the impact of *ALDH2* in combination with drinking habit in 505 cases and 256 unmatched controls, who were extensively screened as non-cancer by chest computed tomography, bronchofibroscopy and video-assisted thoracoscopic biopsy under suspicion of lung cancer. Results showed a highly significant increase in the risk of lung cancer by alcohol consumption in those with the *ALDH2* Lys allele. When adjusted for age, sex and alcohol consumption, however, risk for individuals with the *ALDH2* Lys allele in these studies was not further increased by smoking. In contrast, we saw no evidence of interaction between *ALDH2* genotype and drinking behavior, which does not support the previous studies. Interaction between alcohol drinking and *ALDH2* polymorphism in the risk of lung cancer therefore remains to be determined.

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Table V. Adjusted OR[a] and 95% CI for the impact of smoking, *ALDH2* genotype and their interaction on lung cancer risk according to histrogical subtype and drinking status

	PY										
ALDH2	Ca/co	0	Ca/co	<15	Ca/co	<30	Ca/co	<45	Ca/co	≥45	*P*-interaction
Overall[b]											
Glu/Glu + Glu/Lys	161/511	1.00 (reference)	43/147	1.39 (0.92–1.05)	65/193	1.80 (1.23–2.12)	113/186	3.44 (2.41–4.97)	261/244	6.25 (4.49–8.70)	
Lys/Lys	15/64	1.00 (reference)	2/15	1.01 (0.18–5.64)	10/11	10.2 (2.42–43.1)	18/19	11.4 (3.09–42.0)	25/14	23.2 (6.23–86.5)	0.036
Histology											
Adenocarcinoma											
Glu/Glu + Glu/Lys	143/511	1.00 (reference)	27/147	0.95 (0.58–1.54)	42/193	1.36 (0.88–2.10)	55/186	1.95 (1.28–2.97)	107/244	3.04 (2.08–4.45)	
Lys/Lys	13/64	1.00 (reference)	2/15	1.19 (0.21–6.75)	7/11	7.71 (1.68–35.4)	10/19	7.00 (1.69–26.6)	13/14	13.6 (3.31–55.6)	0.009
Squamous/small cell carcinoma											
Glu/Glu + Glu/Lys	2/511	1.00 (reference)	7/147	14.9 (2.93–75.5)	18/193	27.5 (6.01–126.3)	40/186	63.9 (14.3–285.4)	111/244	129.8 (29.5–571.9)	
Lys/Lys	0/64	1.00 (reference)	0/15	NE	1/11	NE	4/19	NE	9/14	NE	NE
Drinking											
Never											
Glu/Glu + Glu/Lys	98/246	1.00 (reference)	13/17	2.77 (1.22–6.29)	19/39	2.14 (1.07–4.27)	21/31	3.15 (1.53–6.47)	57/47	6.00 (3.23–11.2)	
Lys/Lys	15/61	1.00 (reference)	2/14	0.96 (0.17–5.43)	10/11	9.17 (2.17–38.7)	18/19	10.1 (2.75–37.3)	23/12	22.2 (5.80–84.9)	0.041
Ever											
Glu/Glu + Glu/Lys	63/266	1.00 (reference)	30/130	1.21 (0.73–2.00)	46/154	1.71 (1.07–2.73)	92/155	3.44 (2.24–5.29)	204/197	6.20 (4.16–9.26)	
Lys/Lys	0/3	1.00 (reference)	0/1	NE	0/0	NE	0/0	NE	2/2	NE	NE

Ca/co, cases/controls; CI, confidence interval; NE, not estimated.

[a]Adjusted for age, sex and smoking (PY: 0, <15, <30, <45, ≥45 and unknown), family history of lung cancer and drinking (never, former, current <5 g/d, current <23 g/d, current <46 g/d, current ≥46 g/d and unknown).

[b]Five cases and 12 controls were excluded from analysis because of unknown PY status.

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In addition to being a metabolite of alcohol, acetaldehyde is also a constituent of tobacco smoke (10,11,22). Our present results show that the influence of exposure to acetaldehyde in cigarettes on lung cancer risk, which might be surrogated by cumulative smoking exposure, is remarkably stronger in individuals with Lys/Lys, who cannot metabolize acetaldehyde well. The possibility that this finding was confounded by alcohol consumption can be excluded since statistical significance was adequately reflected on the interaction in never-drinkers. The hypothesis that increased acetaldehyde concentrations contribute to the development of lung cancer is possible because the *ALDH2* Lys/Lys genotype almost completely lacks acetaldehyde oxidation activity. Nevertheless, we cannot deny the possible presence of an unknown gene that is both linked to *ALDH2* polymorphism and at the same time relevant to the metabolism and detoxification of carcinogens in tobacco smoke, albeit that no such gene has been reported to date. It is thought that *ALDH2* itself has no power to directly detoxify carcinogenic compounds in tobacco other than acetaldehyde and that detoxification ability in Lys/Lys individuals might be poor. In any case, confirmation of this association and clarification of its background mechanism are essential.

We note that distribution of histology was different between *ALDH2* Lys/Lys and others. Among ever smokers without history of drinking, adenocarcinoma was significantly more prevalent in those with *ALDH2* Lys/Lys (70.5%) compared with other genotypes (51.7%). This may suggest that possible involvement of acetaldehyde from either sources, smoking or drinking, in adenocarcinoma.

Our study had several methodological strengths and weaknesses. One strength is that it was conducted in a single region in central Japan with a substantial number of subjects and a high response rate. Although controls were selected from non-cancer patients at Aichi Cancer Center Hospital, it is reasonable to assume the same base population as that from which the cases were selected, warranting internal validity. In terms of controls, we previously confirmed that questionnaire-based lifestyle characteristics in this population were similar to those of the general population in Nagoya City in terms of a range of exposures of interest in HERPACC-I (16) and HERPACC-II (H. Ito, K. Matsuo, M. Inoue, K. Tajima, unpublished data), warranting the study's external validity. In addition, the equivalence of genotype distribution for the *ALDH2* polymorphism between our controls and those in public databases and former studies (21,23) for Japanese indicates a lack of bias in the selection of controls, justifying the external validity of our observation. A second strength was that potential confounding by age and sex was addressed by matching of these factors in cases and controls, and smoking and drinking were adjusted in the models.

One weakness of our study was that it was unclear whether the cumulative dose of smoking reflected cumulative exposure to acetaldehyde. A second potential weakness was residual confounding by known or unknown risk factors; in particular, the limited number of cases, particularly in stratified analyses by genotype, indicates the need to replicate our findings in a larger study. A third potential weakness was the information bias intrinsic to case–control studies. The HERPACC system is less prone to this bias than typical hospital-based studies, however, as the data for most if not all patients were collected before diagnosis. In particular, subjects and investigators had no information about *ALDH2* genotype, limiting the impact of information bias in the analysis.

In conclusion, our case–control study showed that the *ALDH2* Lys/Lys genotype, which results in null enzyme activity, modified the impact of smoking on the risk of lung cancer in a Japanese population. This result suggests the possible contribution of acetaldehyde to the pathogenesis of lung cancer. Further replication study is warranted.

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Simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma: application to a clinical pharmacokinetic study

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ABSTRACT: A simple and sensitive high-performance liquid chromatographic (HPLC) method was developed for determination of amrubicin and its metabolite amrubicinol in human plasma. After protein precipitation with methanol without evaporation procedure, large volume samples were injected and separated by two monolithic columns with a guard column. The mobile phase consisted of tetrahydrofuran–dioxane–water (containing 2.3 mM acetic acid and 4 mM sodium 1-octanesulfonate; 2:6:15, v/v/v). Wavelengths of fluorescence detection were set at 480 nm for excitation and 550 nm for detection. Under these conditions, linearity was confirmed in the 2.5–5000 ng/mL concentration range of both compounds. The intra- and inter-day precision and intra- and inter-day accuracy for both compounds were less than 10%. The method was successfully applied to a clinical pharmacokinetic study of amrubicin and amrubicinol in cancer patients. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: amrubicin; amrubicinol; cancer; protein precipitation; monolithic column

Introduction

Amrubicin, a completely synthetic 9-aminoanthracycline, is an active anticancer agent. Both amrubicin and amrubicinol, the C-13 hydroxy active metabolite of amrubicin, are inhibitors of the DNA topoisomerase II mediated cleavable complex. The antitumor activity of amrubicinol is 10–100 times greater than that of the parent compound *in vitro* (Yamaoka *et al.*, 1998). In phase I/II trials conducted in Japan the recommended dose of amrubicin was determined to be 45 mg/m^2 for three consecutive days every 3 or 4 weeks. In phase II trials of amrubicin monotherapy the response rate of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) was found to be 75.8% (Yana *et al.*, 2007) and 27.9% (Sawa *et al.*, 2006), respectively. Amrubicin was approved in Japan for the treatment of SCLC and NSCLC in December 2002. The major problem with amrubicin is hematological toxicity. An incidence of grade 3 or 4 toxicity of 76.8% has been found for neutropenia, 54.7% for leucopenia, 26.0% for anemia, 22.1% for thrombocytopenia and 35% for the more serious toxicity, febrile neutropenia (Kato *et al.*, 2006). The severity of these toxicities varies from individual to individual. Neutropenia has been reported to be associated with the area under the curve of the plasma amrubicinol concentration, which is one of the major pharmacokinetic (PK) parameters (Matsunaga *et al.*, 2006). A prospective PK and pharmacodynamic (PD) study was planned in our institution, the National Cancer Center Hospital (Tokyo, Japan), to evaluate the PK and PD parameters of amrubicin and amrubicinol and to develop an individualized dosing strategy for amrubicin.

Development of a simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma was required to conduct the PK/PD study. Four methods, including two HPLC methods (Noguchi *et al.*, 1998; Matsunaga *et al.*, 2006), an HPLC-MS-MS method (Yanaihara *et al.*, 2007) and a UPLC-MS-MS method (Li *et al.*, 2008), have already been reported. The two HPLC methods (Noguchi *et al.*, 1998; Matsunaga *et al.*, 2006) involve preparation by liquid–liquid extraction and solid-phase extraction, respectively, and they lack sensitivity because of low recovery and loss during processing. The HPLC-MS-MS method (Yanaihara *et al.*, 2007), on the other hand, involves preparation by solid-phase extraction, but the lower limits of quantification (LOQ) of amrubicin and amrubicinol is 20 ng/mL, which is higher than the plasma concentration of amrubicin 24 h after an intravenous bolus and higher than the concentration of amrubicinol, stated in the application for the approval of amrubicin. However,

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Abbreviations used: NSCLC, non-small cell lung cancer; PD, pharmacodynamic; PK, pharmacokinetic; SCLC, small cell lung cancer.

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although an UPLC-MS-MS method by protein precipitation (Li *et al.*, 2008) improved the LOQ and preparation procedure, it is not general measurement equipment in hospitals because the equipment is too expensive.

A monolithic column, which has applied for bioanalysis with in the past 10 years, has a lower back-pressure than packed beds (Nguyen *et al.*, 2006). Many applications of analysis using monolithic column have been investigated in μ-HPLC and capillary electrochromatography. Otherwise, few methods refer to the use of a monolithic column by HPLC. The advantage of using monolith columns is that sensitivity can be improved by a long column and large volume samples can be injected.

There are many methods of sample preparation, including protein precipitation, liquid–liquid extraction and solid-phase extraction. Protein-precipitation is the simplest method in terms of procedure and technique. Moreover, recovery with protein precipitation is higher than that with liquid–liquid extraction or solid-phase extraction because amrubicin and amrubicinol have both hydrophilic sites and hydrophobic sites.

The aim of this study was to use monolithic columns to develop a simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma.

Experimental

Chemicals

Amrubicin and amrubicinol were provided by Dainippon Sumitomo Pharmaceuticals Co. Ltd (Osaka, Japan). The chemical structure of amrubcin and amrubicinol are shown in Fig. 1. All other chemicals and reagents used were of analytical reagent grade or HPLC grade and were purchased from Wako (Osaka, Japan).

Chromatographic Instrumentation and Conditions

The chromatographic system consisted of an LC-20AD pump, a SIL-20AC auto sampler, an RF-10AXL fluorescence detector and C-R8A Chromatopac integrator with a CTO-10A oven (Shimadzu, Kyoto, Japan). Two connected Onyx Monolithic C_{18} (100 × 4.6 mm) columns were used with an Onyx Monolithic C_{18} Guard Cartridge (10 × 4.6 mm; Phenomenex, Torrance, CA, USA). Effluent was monitored with a fluorescence detector set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. The mobile phase consisted of tetrahydrofuran–dioxane–water (containing 2.3 mM acetic acid and 4 mM sodium 1-octanesulfonate; 2:6:15, v/v/v) pumped at flow rate of 0.9 mL/min at a column temperature of 35°C.

Preparation of Standards and Plasma Samples

Stock solutions of amrubicin and amrubicinol were stored in plastic microtubes to avoid adsorption to glassware, and stored at −80°C. Working solutions were obtained by diluting the stock solutions with acetonitrile. Blank plasma samples for use in validating the method were obtained from healthy volunteers. The patient plasma samples were obtained from the National Cancer Center Hospital. Both the blank plasma samples and patient plasma samples were stored at −80°C until analyzed. Heparin sodium salt was added to patient blood samples to prevent coagulation. Then the blood samples were centrifuged at 5000 rpm for 10 min.

Extraction Procedure

Plasma (100 μL) in a 1.5 mL screw-capped tube was diluted with 20 μL of 16 mM citric acid–16 mM Na_2HPO_4–0.9% NaCl solution, and after adding 480 μL of methanol the tube was shaken for 30 min. The mixture was then centrifuged for 10 min at 12,000 rpm. The supernatant was filtered through an UltraFree-MC filter (Millipore, Tokyo, Japan), and 250 μL of the solution was transferred into auto sampler vials and vortex-mixed with a 500 μL of 16 mM citric acid–16 mM Na_2HPO_4–0.9% NaCl solution. A 450 μL volume of the solution was injected into the HPLC system for analysis.

Validation

Specificity. The specificity of the method was evaluated by comparing different blank plasma samples and plasma samples spiked with amrubicin and amrubicinol. The blank plasma samples were collected from nine volunteers.

Accuracy, precision and recovery. Accuracy and precision were determined by replicate analysis ($n = 6$) of plasma samples spiked with three concentrations of amrubicin and amrubicinol: 10, 100 and 1000 ng/mL. Accuracy was evaluated as relative error (RE), and precision was evaluated as coefficient of variation (CV). Recovery was assessed by comparing the results of analyses of extracted plasma samples and unextracted standards containing the same concentrations.

Calibration curve. The LOQ was determined from the peak and the standard deviation of the noise level (SN). The LOQ was defined as the concentration of amrubicin and amrubicinol resulting in a peak height of 10 times SN. The calibration curve was generated by linear regression of the peak areas (y) of amrubicin and amrubicinol against the corresponding concentrations (x) of amrubicin and amrubicinol in plasma.

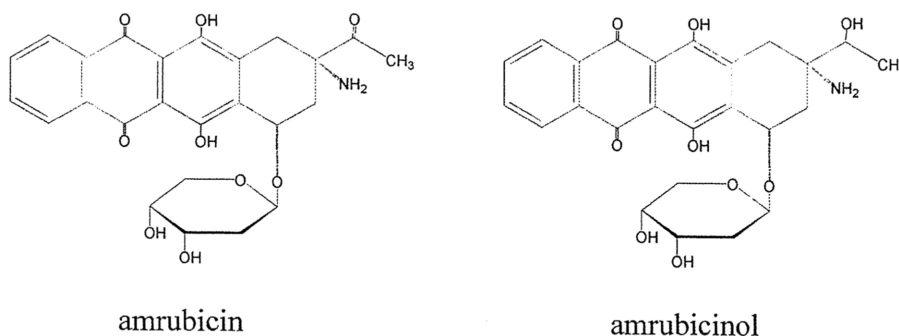

Figure 1. Chemical structures of amrubicin and amrubicinol.

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Analysis of Patient Samples

For the analysis of plasma concentration of amrubicin and amrubicinol, plasma samples were obtained from lung cancer patients treated with 40 mg/m^2 of amrubicin. All patients were enrolled in the prospective PK/PD study, which was aimed to evaluate the correlation between PK and PD of amrubicin and amrubicinol. Written informed consent was obtained from all patients. This study was approved by the Ethical Review Board of National Cancer Center Hospital and is ongoing. The plasma samples were obtained from blood samples collected immediately before injection, and immediately after the injection, and 5, 15 and 30 min, and 1, 2, 4, 8 and 24 h after the end of injection. Each sample was determined in triplicate.

Results

Specificity

No endogenous interference was observed at the retention times of amrubicin and amrubicinol. The retention time of amrubicin and amrubicinol was approximately 8.5 and 10.2 min, respectively. Representative chromatograms of the blank plasma sample, the plasma sample spiked and the patient plasma sample are shown in Fig. 2. The capacity factors (k') of amrubicin and amrubicinol were 1 and 1.4, respectively.

Accuracy, Precision and Recovery

The results for intra- and inter-day accuracy, precision and recovery are shown in Table 1. Intra-day accuracy ranged between −4.1 and 0.8% for amrubicin and between −9.8 and −2.1% for amrubicinol. Inter-day accuracy was between −3.1 and 3.0% for amrubicin and between −4.0 and 2.3% for amrubicinol. Intra-day precision was 1.4–8.8% for amrubicin and 1.3–4.2% for amrubicinol. Inter-day precision was 2.7–8.8% for amrubicin and 5.3–5.5% for amrubicinol. Recovery was greater than 95% at all concentrations (10, 100 and 1000 ng/mL) of amrubicin and amrubicinol.

Lower Limit of Quantitation

The LOQ was 2.5 ng/mL for both amrubicin and amrubicinol. At that level the coefficient of variation (CV) was 8.3% for amrubicin and 3.2% for amrubicinol ($n = 6$).

Linearity

Under the chromatographic conditions described, linearity and the appropriate correlation coefficient were achieved for amrubicin within the concentration range from 2.5 to 5000 ng/mL. The linear regression equation for amrubicin was $y = 526.3x + 6156.3$, and the correlation coefficient (r) was 0.999. Similar results were obtained for amrubicinol with the concentration range from 2.5 to 5000 ng/mL. The linear regression equation for amrubicinol was $y = 662.9x + 2947.7$, and the correlation coefficient (r) was 0.996.

Analysis of Patient Plasma Samples

The amrubicin and amrubicinol in the patient plasma samples were separated well under the optimal chromatographic conditions. Figure 2(C) shows a chromatogram of amrubicin and amrubicinol in a plasma sample from a patient who was treated at dose of 40 mg/m^2 of amrubicin. Figure 3 shows the concentration–time profiles for amrubicin and amrubicinol after an intravenous bolus. The amrubicin concentrations ranged between 4.3 and 2504 ng/mL, and the amrubicinol concentrations ranged between 3.0 and 18.5 ng/mL. These concentrations were similar to the concentrations stated in the application for approval of amrubicin.

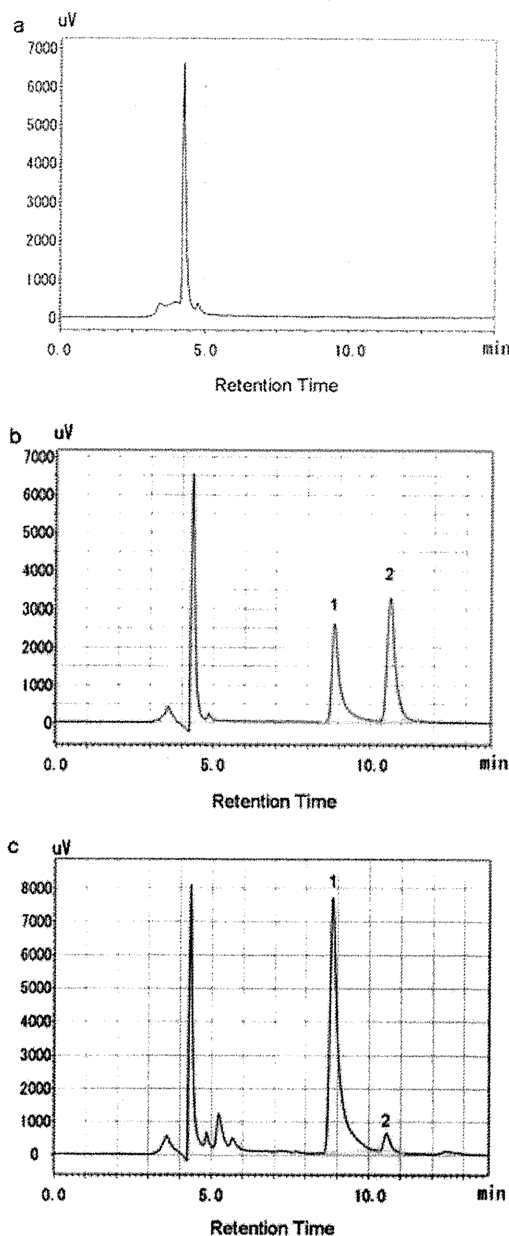

Figure 2. Representative HPLC chromatogram. (a) Blank plasma sample; (b) plasma sample spiked with 100 ng/mL amrubicin and amrubicinol; (c) patient plasma sample obtained 2 h after anintravenous bolus dose of 40 mg/m^2 of amrubicin, Peaks:1 = amrubicin; 2 = amrubicinol. For chromatographic condition see Experimental section.

Discussion

We developed a simple and sensitive method of determination for amrubicin and amrubicinol by HPLC. In our method protein

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Table 1. Intra-day and inter-day accuracy, precision and recovery of the HPLC method for amrubicin and amrubicinol

	Nominal concentration (ng/mL)	*n*	Experimental concentration (mean ± SD, ng/mL)	Precision (% CV)	Mean recovery (%)	Accuracy (%RE)
Intra-day						
Amrubicin	10	6	9.9 ± 0.6	5.9	98.9	−1.1
	100	6	95.9 ± 1.3	1.4	100.0	−4.1
	1000	6	1007.6 ± 88.2	8.8	100.7	0.8
Amrubicinol	10	6	9.0 ± 0.4	4.2	98.5	−9.8
	100	6	97.8 ± 1.3	1.3	97.8	−2.1
	1000	6	954.9 ± 14.6	1.5	95.5	−4.5
Inter-day						
Amrubicin	10	6	9.7 ± 0.3	2.7	98.0	−3.1
	100	6	102 ± 8.6	8.4	100.0	2.0
	1000	6	1029.8 ± 90.9	8.8	100.7	3.0
Amrubicinol	10	6	10.0 ± 0.5	5.3	99.3	0.1
	100	6	96.0 ± 5.5	5.7	99.3	−4.0
	1000	6	1023.2 ± 56.4	5.5	100.4	2.3

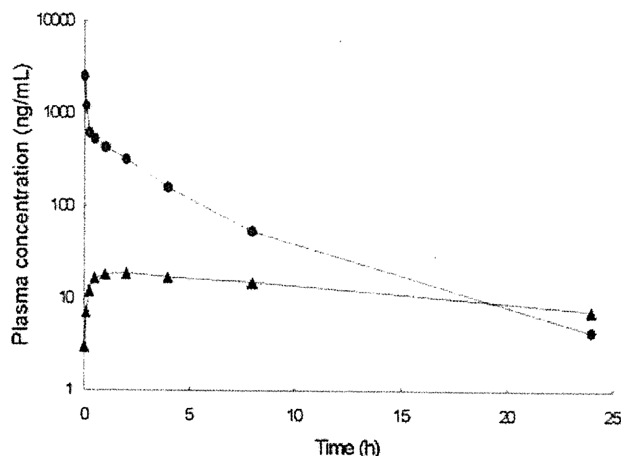

Figure 3. Plasma concentrations vs time curves of amrubicin (circles) and its metabolite amrubicinol (triangles) in a patient treated with 40 mg/m^2 of amrubicin.

precipitation is used to prepare the samples, and monolithic columns are used to make determination.

During the past decade monolithic columns have emerged as an alternative to traditional packed-bed columns. Monolithic columns are structurally very different from packed-bed columns. The most interesting characteristic of monoliths is their high external porosity resulting from the structure of the network of through-macropores. Another interesting characteristic is the structure of the stationary phase skeleton, which consists of a network of small, thin threads of porous silica. These structural characteristics allow the combination of the low hydraulic resistance of the column to the stream of mobile phase and an enhancement of the column of the rate of the mass transfer of the sample molecules through the column. In this way, the monolithic column improves back-pressure. Yunsheng *et al.* (2003) investigated the utility of monolithic column for direct HPLC-MS-MS analysis. Although access to the matrix in biological samples was prevented in analysis by packed-bed columns, the monolith column with high porosity could remove matrix macromolecules.

In this study, we evaluated packed-bed columns, such as Sumipax ODS A-212 (5 μm, 6 mm × 15 cm), Synergi Hydro-RP (4 μm, 4.6 mm × 15 cm), Luna C_{18} (4 μm, 4.6 mm × 15 cm) and Luna C_{18} (3 μm, 4.6 mm × 15 cm). The LOQ using Sumipax ODS A-212 was 5 ng/mL for both amrubicin and amrubicinol. Using the other three columns, we could not obtain sufficient result (data not shown). We considered the reason why small particles prevented the access of macromolecules in biological samples.

We connected two monolithic columns in tandem like a long column, which made it possible to determine low concentrations of amrubicin and amrubicinol without high pressure. As a result, the sensitivity of our method is equal or superior to that of other methods, including methods that use HPLC-MS-MS or UPLC-MS-MS, and its sensitivity is adequate for performance of the analyses in the PK study.

The sample preparation procedure in this method is based on protein precipitation, because it is simple. Moreover, recovery by protein precipitation is higher than by liquid–liquid extraction or solid-phase extraction, because amrubicin and amurbicinol have both hydrophilic sites and hydrophobic sites. Methanol was selected for protein precipitation, because methanol makes shaper peaks and enables better separation than other organic solvent (data not shown). We added an appropriate amount of buffer, which prevents broad peaks, to the samples after protein precipitation.

The fluorescence detector was set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. In the excitation wavelength, the highest energy should be obtained in the 480 nm region at the level of excitation lamps, according to the proposal for determination of anthracyclines (Sepaniak and Yeung, 1980). We set the excitation wavelength at 480 nm. Since the most sensitive detection wavelength for amrubicin and amrubicinol was 550 nm, we used it as the detection wavelength in our method. The mobile phase was a modification of a previous report (Noguchi *et al.*, 1998). 1-Octanesulfonate improved separation compared with other ion-pair agents including 1-heptanesulfonate, which used Noguchi's method (data not

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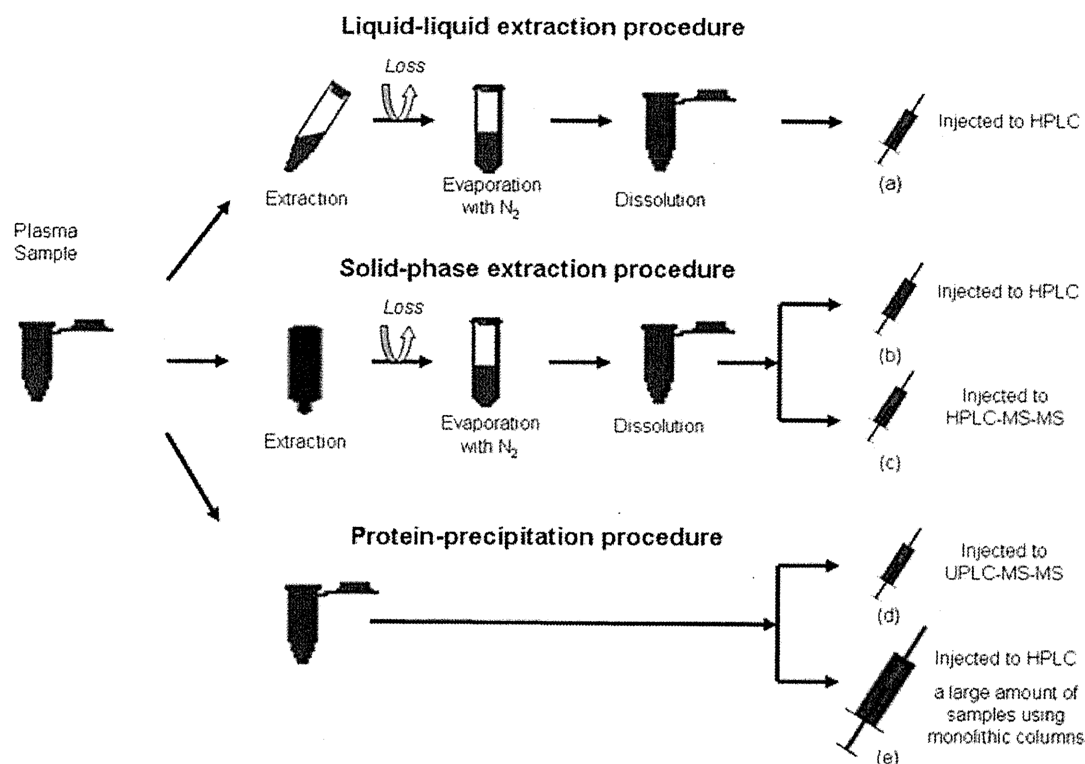

Figure 4. Four methods previously described methods for determination of amrubicin and amrubicinol in plasma; (a) HPLC method (Noguchi *et al.*, 1998) with sample preparation by liquid–liquid extraction; (b) HPLC method (Matsunaga *et al.*, 2006) with sample preparation by solid-phase extraction; (c) HPLC-MS-MS method (Yanaihara *et al.*, 2007) with sample preparation by solid-phase extraction; (d) UPLC-MS-MS method (Li *et al.*, 2008) with sample preparation by protein precipitation; (e) HPLC method with sample preparation by protein precipitation in this paper.

shown). We added 1-octanesulfonate to mobile phase as an ion-pair agent.

Previous reports have described four methods for determination of amrubicin and amrubicinol. The UPLC-MS-MS method (Li *et al.*, 2008) is the most sensitive of the four; however, UPLC-MS-MS is not widely available in hospitals. The other methods involve problems in relation to application to PK studies, such as low recovery or loss during processing (Fig. 4). A more simple and sensitive method that can be performed with equipment that is generally available was needed for analysis in hospitals.

We validated our method under Guidance for Industry of the Food and Drug Administration in Bioanalytical Method Validation, with regard to specificity, accuracy, precision, recovery and calibration curve for concentrations ranging from 2.5 to 5000 ng/mL, which were thought to be clinically relevant range for amrubicin and amrubicinol concentrations in plasma. Both the inter-day and intra-day accuracy and precision of the method were adequate. Our method provides good sensitivity, and was able to detect all points in our PK study.

Conclusion

A simple and sensitive HPLC method was developed for determination of amrubicin and amrubicinol in human plasma. In our method, we selected a monolithic column for determination and protein precipitation for preparation, and it was validated sufficiently. This method can be used clinically because the required equipment and technique are simple. The PK/PD study of amrubicin is ongoing, and a therapeutic drug monitoring study by this HPLC method is in the planning stage.

Acknowledgements

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Performing Phase I Clinical Trials of Anticancer Agents: Perspectives from within the European Union and Japan

Martin D. Forster[1], Nagahiro Saijo[2], Lesley Seymour[3], and Hilary Calvert[1]

Abstract

Drug discovery and early clinical development is an international endeavor, conducted in partnership between commercial entities such as biotechnology and pharmaceutical companies and academic investigators. Although once considered quite disparate, early clinical trials requirements and conduct are largely harmonized between the European Union, Japan, and the United States, increasing the opportunities for productive commercial-academic collaborations. *Clin Cancer Res; 16(6); 1737–44. ©2010 AACR.*

Cancer drug discovery and development is an international activity. Many of first-in-human studies of anticancer drugs are conducted in two sites in different countries, usually in North America (United States or Canada) and the other in Europe. Additional phase I studies including Japanese patients are often required for later marketing approvals in Japan. Phase I capabilities exist worldwide, including Australia, Asia, and South America. Although there are differences in attitudes between different countries in general, the concerns and approaches of phase I investigators are similar across the world. In particular there is emphasis on trial designs that minimize the number of patients treated with ineffective doses and maximize the possibility of eliciting therapeutic signals. We summarize here early clinical trial activities in Europe and Japan, highlighting key opportunities and challenges as part of this issue of *CCR Focus*, which examines the phase I clinical trial process (1).

European Union

In terms of organization, there is no overarching organization for European countries or even for one individual country. Rather, the tendency has been for potential sponsors to negotiate individually with individual centers. This has led to the focusing of many studies in relatively few centers where the expertise and the reputation for the conduct of these studies have been established. Of note, privately owned and commercially operated phase I clinical trials centers are emerging in Europe.

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Marketing approval for new drugs within the European Union is the responsibility of the European Medicines Agency, which is a decentralized body of the European Union with headquarters in London. The agency is responsible for the scientific evaluation of applications for European marketing authorization for medicinal products using a centralized procedure. Companies submit a single marketing authorization application to the agency. Once granted by the European Commission, this authorization is valid in all European Union (EU) and EEA-EFTA states (Iceland, Liechtenstein, and Norway). In contrast, approval for clinical trials of new agents is the responsibility of each member state, with each country providing its own regulatory authority.

First in human trials are clinical experiments and are therefore governed by a number of regulations and guidelines. Patients who participate are given an intervention that they would not normally receive, with an unknown efficacy and toxicity profile, and often undergo additional clinical assessments and tests. It is therefore essential to protect their safety and well being, which is the primary role of the regulatory guidelines. Adherence to these guidelines is particularly relevant in phase I studies, in which the investigational medicinal product may have been administered to few, if any, humans previously. The regulations also aim to ensure that the clinical trial data produced are robust and accurately represent the activities of the Investigational Medicinal Product (IMP).

The first internationally recognized guidelines were the Nuremberg Code, developed in 1948, following the inhumane experimentation on subjects without appropriate consent during the Second World War (2). The Nuremberg Code formed the basis for the Declaration of Helsinki, first declared by the World Health Authority in 1964, which has undergone a number of subsequent revisions (latest in 2008: ref. 3). All clinical studies need to follow its guidelines, although it is not legally binding in international law, and it has formed the backbone to the Code of Federal Regulations (title 45, volume 46) in the United States (4) and the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guidelines.

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Table 1. Europe: example of documents required for a clinical trial application

Clinical Trial Application required documents
EudraCT number
IB
IMPD
IND application (in the United States) if available
Information of investigators, recruiting sites, and analysis laboratories
Details of drug manufacturing and distribution
Trial production and sample consent form
Completed application form
Information from independent ethics committee
Fee

NOTE: From the Medicines and Healthcare products Regulatory Agency (9).

International conference on harmonization

In 1996, the ICH guidelines were developed, in an attempt to harmonize the requirements for registering medicines across the European Union, the United States, and Japan and to allow data produced from one country to be accepted by another (5). The tripartite guidelines expand on the Declaration of Helsinki to advise on quality, efficacy, patient safety, and other miscellaneous aspects of clinical research. They focus on the core principles of Good Clinical Practice (GCP), which cover trial design, conduct, and analyses, with strict pharmacovigilance and thorough informed subject consent (6). Quality topics describe IMP production and evaluation to Good Manufacturing Practice (GMP) and there are extensive guidelines on preclinical safety evaluation and the training and responsibilities of trial staff. ICH GCP provides international guidelines to standardize the conduct of clinical trials, but their implementation has been variable between different researchers and countries.

EU clinical trials directive

The variability in interpretation of ICH GCP led the European Union to develop the EU Clinical Trials and GCP Directives (2001/20/EC and 2005/28/EC, respectively), implemented from 2004. The directives formed a legal framework for clinical trial research, including phase I trials, and required incorporation into the legal systems of member states.

Directive (7) includes 24 articles required to be met, covering core areas including:

- The safety and well-being of clinical trial subjects,
- The procedures for independent ethical committee review and approval,
- The procedures to give regulatory approval before a trial starts recruitment,
- The procedures for authorities to inspect trial conduct to GCP standards,
- The standards for the manufacture and handling of IMPs,
- The procedures for reporting and processing adverse events.

In common with other EU directives, the Clinical Trials Directive (CTD) provides the principles, which are interpreted and converted to law separately by each member state. The interpretation and incorporation by individual states may be influenced by other laws already in place, such as the EU Data Protection Directive (95/46/EC). In addition subsequent rulings in individual states may also affect the implementation of the directive. An example would be the Human Tissue Act introduced to the United Kingdom in 2004 for studies involving clinical samples. This act was introduced following an event in which tissues and organs of children who had died were retained without parental consent, resulting in a very high-profile scandal featured in the media. The result is a series of stringent measures to control the acquisition, storage, and experimentation on human tissues of all kinds.

Differences in the existing national legislation and differing legal concerns within the different member states of the EU mean that cost, timelines, ease of setting up, and the conduct of new studies may vary significantly between different countries.

Trial set up and approval. ICH and EU CTD advise about preclinical evaluation and regulatory toxicology assessments following which a phase I protocol can be developed. An IMP dossier is also required, outlining the quality, safety, and use of IMPs in the study. This dossier forms the EU equivalent to the Investigational New Drug (IND) application required in the United States (requirements for the latter are discussed in this issue of *CCR Focus*; ref. 8). In parallel with the Investigational Medicinal Product Dossier (IMPD), an Investigator's Brochure (IB) is required, which outlines all available preclinical and clinical data (6). Both the U.S. Food and Drug Administration (FDA) and Committee for Human Medicinal Products and competent authorities from each individual European country are available for consultation to discuss development strategy and the pathway to registration for an IMP.

Regulatory authorization. As noted above, each country has its own regulatory authority, such as the Medicines and Healthcare products Regulatory Agency (MHRA) in the United Kingdom or the Ministry of Health in Italy and Germany, responsible for allowing a study to be conducted. Application requirements to gain regulatory approval differ a little between EU countries but the core documents for a Clinical Trial Application (equivalent to IND application in the United States) are listed in Table 1 (9), and regulatory approval is required for each country involved. EU regulatory authorities aim to assess applications within 30 days, extended up to 60 days if further details are required. The trial is then able to be registered onto an international clinical trials register, such as ClinicalTrials.gov.

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Although the authorization for clinical trials is done at a national level, all European Clinical IMP trials are required to be issued with a EU Drug Regulating Authorities Clinical Trials (EudraCT) number, which is issued by the European Medicines Agency (10).

EudraCT is the European Clinical Trials Database of all clinical trials commencing in the European Union from May 1, 2004 onwards. The EudraCT database was established in accordance with Directive 2001/20/EC. Each clinical trial with at least one site in the European Union receives a unique number for identification, the EudraCT number. The EudraCT number must be included on all clinical trial applications within the European Union and as needed on other documents relating to the trials (e.g., suspected unexpected serious adverse reaction reports).

Patient information and consent. There is strong emphasis on the protection of clinical trial patients in ICH GCP and EU CTD, leading to the generation of patient information sheets, which extensively explain the rationale of the study and possible risk of harm from the therapy. Although written in plain language these are often long and require careful explanation and time allowed to the subject for consideration, prior to informed consent being given. Patient information sheets may be required in multiple languages and within the European Union are submitted, along with the trial protocol, for independent ethical approval.

Independent ethical review. Ethical review is required to be done by an independent committee, but procedures depend on the number of sites involved and differ in different EU states. A single-center study may be reviewed by a local committee, whereas a national review may be possible in some countries (such as the United Kingdom or the Netherlands). A committee with particular expertise may be required for phase I trials and additional expert opinion may also be required if vulnerable adults and/or children are involved. This independent review differs from the Institutional Review Board (IRB) review required for trial registration within the United States. An ethics committee has a 60-day time limit to approve or decline a study, but applications may occur in parallel with the assessment of the scientific merit of the study and therefore need not add a time delay to trial initiation. It does, however, often allow easier subsequent amendments to be accepted either by chairman's review or formal committee reassessment.

In the United Kingdom, the Integrated Research Application System form has recently been introduced in an attempt to reduce the trial administrative burden. The regulatory applications are centralized to a single electronic form minimizing repetitive core data entry. Also in the United Kingdom, studies involving biomarkers on clinical samples are also required to comply with the Human Tissue Act. A Case Report Form is developed to gather data relevant to the study and individual site assessments, agreements, and approvals obtained prior to patient recruitment.

Trial conduct and completion. The ICH guidelines define GMP quality IMP production for clinical use and handling during trial evaluation (11). The procedures for drug labeling, supply, and distribution require implementation by sponsors, and differ between the European Union and United States. The EU regulations require a manufacturing authorization for the manufacturer or importers of an IMP, and one or more "qualified person(s)" to undertake responsibility for the quality assurance of each batch of unlicensed product. The qualified person needs to be based in the European Union and is therefore generally independent of the sponsor, who is able to do this role within the United States.

ICH GCP defines the documentation required to be collected in trial files, but there are several systems accepted for data storage, and the level of data monitoring and source data verification varies between studies in the European Union as it also does in the United States. Efforts to ensure data quality have been steadily increasing in all academic centers (12). Database systems validated in the European Union offer centralized statistical monitoring and automated data validation. These systems are often used in academic studies, thus requiring reduced source data verification thereby reducing monitor time and saving expense. Pharmaceutically sponsored studies often extensively monitor source data entry at added significant expense.

The EU CTD requires countries to have a system to independently inspect sponsor or participating trial institutions by regulatory bodies. Routine inspections can be preplanned or can be triggered by specific safety concerns. The EU CTD also allows for "urgent safety measures" to be taken by the sponsor before regulatory review if there are serious safety concerns.

A system for identifying and reporting adverse events is required by the EU CTD. Toxicity grades are usually standardized by the use of the CTCAE grading system and procedures, and reporting timelines are similar across the United States, the European Union, and Japan. The Eudra Vigilance Database contains safety data about all IMPs in EU clinical trials allowing information to be exchanged more easily between participating countries (13).

Acquisition of drugs

The advances in biology and in particular the advances in the understanding of the molecular biology of various different types of cancer have led to an exponential increase in the number of interesting anticancer targets for drug design. The concurrent advances in analytical technology, antibody development, and medicinal chemistry have enabled the discovery of agents with potential activity against these targets at an unprecedented rate. Potential new anticancer drugs are being developed by large pharmaceutical companies, smaller biotech companies, and academic groups, often supported by charitable funding. This increase in available agents has also led to efforts to improve clinical trial design to maximize efficiency and to

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understand whether the presumed drug target has been affected (14, 15).

Numerically by far the largest proportion of new drugs undergoing phase I trials are sponsored by the pharmaceutical industry, but some notable drugs have had their origins in academic institutions. Examples are carboplatin [Institute of Cancer Research (ICR) and Bristol-Myers Squibb], raltitrexed (ICR and AstraZeneca), and temozolomide (University of Aston and Schering Plough).

Phase I trials groups and networks

Working under the regulatory framework outlined above, a number of different organizations have been set up to conduct or promote the trials of innovative new cancer agents within the EU countries. The list below is not a comprehensive list, as many countries have phase I capabilities or networks, including Germany, Scandinavia, and Italy.

European Organization for Research on the Treatment of Cancer. Anticancer drugs of the 1970s and 1980s were usually toxic and expertise in the conduct of phase I studies with them was confined to a few centers. The Early Clinical Trials Group of the European Organization for Research on the Treatment of Cancer (EORTC) was very successful in bringing European expertise together and formed a valuable point of contact for the pharmaceutical industry. This group (which later became the Early Clinical Studies Group) conducted a large number of phase I trials on agents that are now in common use. However changes in the environment eventually led to the disbandment of this group in 2004. As oncology products became mainstream, the pharmaceutical industry acquired in-house expertise in conducting these trials. A number of centers developed a high level of specialization in phase I cancer trials and therefore could offer local expertise and a high throughput directly to sponsors. This evolution led to a gradual reduction in the number of new agents being available to the cooperative group, which eventually led the EORTC to focus on its highly successful phase III programs.

This pattern is now common in most European countries. There a large number of capable and active phase I centers in most European countries that are negotiating and conducting trials on a case-by-case basis with the sponsors, most of whom are pharmaceutical companies. Many of the previously Eastern European countries now have expanding and robust clinical trials activities and compete effectively with the longer established Western European operations. Some of the better known organizations are highlighted below and play a valuable role in coordinating activity and providing a collaborative and educational platform. Much of the actual phase I–II activity, however, takes place by direct negotiation of the sponsor (or Clinical Research Organization) with the centers.

Cancer Research UK (formerly Cancer Research Campaign – UK). In 1981 the Cancer Research Campaign set up a phase I committee of oncologists with an interest in early studies of investigational cancer agents with translational scientists, chemistry and formulation expertise. This group gradually transformed into the Cancer Research UK Drug Development Office and a funding committee called the New Agents Committee. The New Agents Committee is able to consider drug candidates at any stage of their development and can organize and fund bulk synthesis, formulation, and toxicology as well as phase I–II clinical trials. Phase I clinical trials are managed by the Drug Development Office, which can handle all the requirements for a GCP trial, including sponsorship, monitoring, and reporting. This is an unusual, if not unique facility, because it can take a drug from concept stage to clinical trial entirely within the charitable and/or academic arena. It has been instrumental in developing a number of

Table 2. Factors contributing to "Lag" for approval of new oncology therapeutics in Japan

Stage in approval process	Causes of delays
Delay in the start of development	Concerns about cost and delays
	Requests for additional data unique to Japan
	Slow review and/or consultation times
	Preference for positive phase II data prior to initiating
Prolonged trial conduct	Lack of protected time for clinical research, high clinical workload
	Lack of reimbursement and/or recognition for investigators
	Inexperience in clinical research, limited support staff
	Infrequent IRB meetings
	Additional paperwork and/or requirements unique to Japan (J-GCP)
	Patient reluctance (strong national healthcare system; concerns about safety; negative media releases; expectations about a positive outcome)
Prolonged J-NDA review	Limited number of reviewers
	Inconsistency in reviews and/or requirements

Abbreviations: J-GCP, Japan Good Clinical Practice; J-NDA, Japan New Drug Application.

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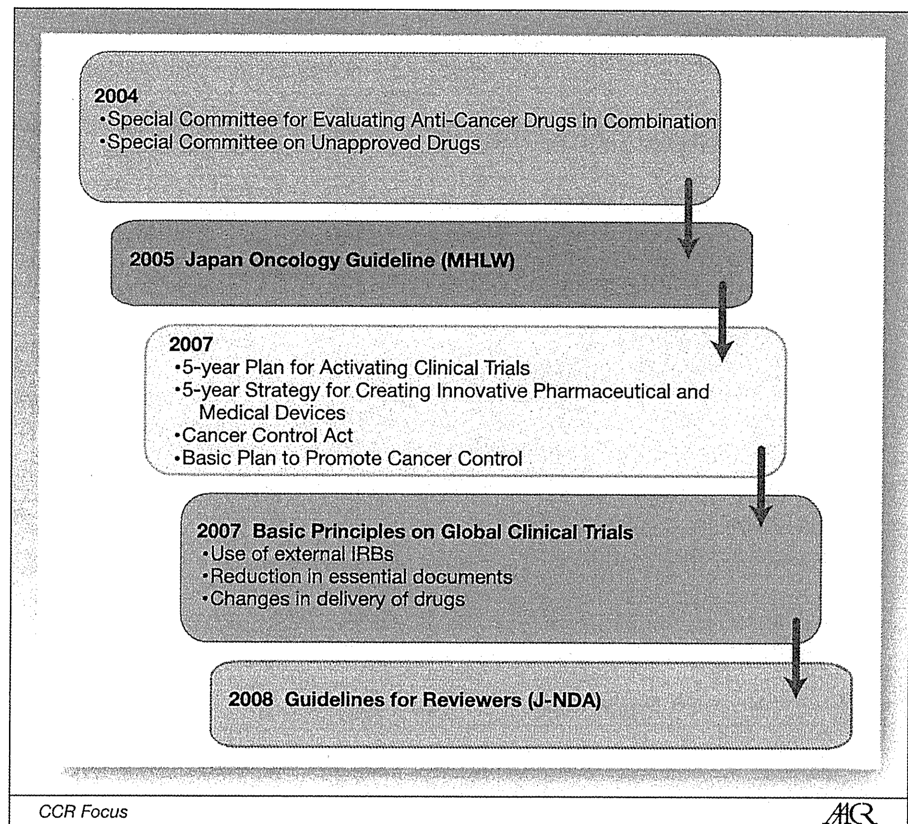

Fig. 1. Strategic changes initiated by the Ministry of Health, Labor and Welfare (MHLW). J-NDA, Japan New Drug Application.

licensed drugs, including temozolomide (16). The conduct of early clinical studies in cancer in the United Kingdom has also been greatly facilitated by the Experimental Cancer Medicine Network (17), which provides competitively allocated infrastructure funding to 19 centers around the United Kingdom.

However, it has become increasingly expensive to set up and conduct clinical trials, including early phase trials, largely owing to increased regulations and governance responsibilities. Phase I trial design has also evolved, expanding beyond simple toxicity evaluation to include multiple, often expensive, secondary and exploratory endpoints including predictive biomarkers, pharmacodynamic markers to show target inhibition in tumor or surrogate tissue, and preliminary efficacy outcomes. This means that the budget available to organizations such as CR UK will support far fewer trials than it would before the current regulations came into force. Similar budgetary considerations also apply to Clinical Development Partnerships (see below), which means that there are many interesting and potentially valuable new agents for which the clinical trials cannot be done.

The extraordinary success of modern drug development techniques, coupled with the increasing cost and complexity of clinical development, has resulted in many pharmaceutical companies having more promising agents in their pipeline than they have resources to develop. Acquisitions and mergers between these companies compound the situation often resulting in the pipelines of the merged company having several representatives of each class of drugs. The fact that many potentially useful drugs do not undergo clinical development is a potential loss to the oncology patient, because it is frequently not possible to make an accurate judgment of the clinical utility of a new agent without clinical data. Cancer Research UK Clinical Development Partnerships are designed to address this problem by offering to undertake early clinical development at the expense of CR UK. The collaborating company has an option to continue development with the drug if it looks successful in exchange for a revenue sharing agreement (18).

Southern Europe New Drug Organization. Southern Europe New Drug Organization (SENDO) was founded in 1997 to promote and coordinate transnational research and early clinical trials in Southern Europe with the aim of boosting research on new anticancer drugs using modern up-to-date methodology. It is a not-for-profit organization with centers in Switzerland, Italy, and Barcelona. It has all the expertise necessary for early clinical cancer drug development and a good network of collaborators for preclinical development (19).

Central European Society for Anticancer Drug Research. Central European Society for Anticancer Drug Research

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(CESAR) was founded in 2001 with a focus on research into identifying new anticancer agents, the development of new agents, and fostering the translation of laboratory research into the clinic. CESAR comprises scientists from basic research and preclinical and clinical oncology in Austria, Germany, and Switzerland. In addition, the CESAR has created a network of study centers experienced in oncology of solid tumors in a number of Central and Eastern European (CEE) countries with the aim to foster international cooperation between oncologists and study centers in this area. CESAR has a portfolio of phase I and translational studies open and organizes meetings to promote and coordinate research (20).

Phase I–II cancer trials in France. The French National Federation of Cancer Centers has established a group ("Essais précoces") coordinating early phase cancer trials in a number of French centers. The Institut National du Cancer (INCa) has also set up an agreement with U.S. National Cancer Institute Cancer Therapy Evaluation Program (CTEP) permitting a number of French centers to be selected for phase I–II trials sponsored by CTEP. The new French Cancer Plan has identified a need to establish a funded network of cancer centers for phase I–II trials.

Phase I–II cancer trials in Spain. There is substantial amount of phase I activity in Spain but no single overarching organization. A number of Spanish centers undertake a significant number of phase I trials and some of them receive governmental support as part of the Cooperative Network of Cancer Centers.

Phase I–II cancer trials in Switzerland. Within the SAKK group (the Swiss national group for clinical studies in cancer) there is an independent phase I group.

Japan

Despite Japan's leadership role in basic research and discovery, its prominence in early clinical research is less established, although separate trials have often been required because of the differing pharmacology of drugs in Asian patients. In recent years polymorphic variations in proteins involved in drug clearance have been identified that begin to explain these differences (21). Marketing

Table 3. Requirements for phase I studies in the European Union, Japan, and the United States

Study component	Requirements	Japan	EU	United States
Preclinical data and starting dose	ICH Guidelines for safety (S3, S4, S6, S7, S9, M3)	In Japan, ICH S9 is at Step 4; May use data from other phase I studies; level at which no DLT reported. If no ethic difference in metabolism and/or safety suspected, phase I data from other countries may be acceptable to support phase II studies	One species (rodent) acceptable	Two species (one nonrodent) required
Pharmacokinetics	ADME defined	Required	Desirable	Desirable
	Assays developed and available			
Facilities and Personnel	Adequate knowledge of preclinical data			
	Investigators knowledgeable in clinical pharmacology and oncology therapy			
	Number of centers	Single center preferred; if multicenter, good communication channels must be in place		Single center studies recommended
Patients	Usually cancer patients unless minimal toxicity (volunteers may be acceptable); Patients with poor performance status (ECOG 3 or 4) excluded			
	No standard options known to prolong life			
	Hospitalization?	Hospitalization may be required by PMDA		May be treated as inpatients or outpatients
	May include multiple tumor types or be tumor specific if appropriate			
	Criteria for organ function and/or eligibility	Additional criteria may be required		Standard
Design and conduct	Must evaluate more than a single dose			
	Pharmacokinetics	Especially for combination studies		Recommended
	Independent data monitoring committee	May be required		Not mandated

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; ECOG, Eastern Co-operative Group.

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Table 4. Summary of the regulatory and approval process for clinical trials in Japan

Process	Comments
PMDA-clinical trial notification	• Early consultation recommended • Follow ICH recommendations for preclinical data • Studies must address PK, PD, and PG considerations for Japanese patients, including ADME
IRB Approval Contract and budget	• Required • Standard method for cost calculation of clinical trials, but overhead costs varies by institutions and is negotiated individually with each institution on the basis of unique and complex requirements. • In general full cost retrieval required, including the cost of unplanned investigations • Interdepartmental coordination challenging (relevant when correlative studies are included)
IDMC	• Usually required even for phase I trials • If international IDMC in place, local IDMC may not be necessary

NOTE: ADME, absorption, distribution, metabolism, and excretion; PK, pharmacokinetic; PD, pharmacodynamic; PG, pharmacogenetic; IDMC, Independent Data Monitoring Committee.

approvals for Japan have lagged a number of years behind approvals in other jurisdictions (22), owing to a number of factors, summarized in Table 2 (23–25), including the requirement for data from Japanese patients, especially in later phase studies, as well as prolonged regulatory approval times, despite Japan being one of the largest markets for pharmaceuticals. Often, these considerations have resulted in development plans for Japan being implemented only after positive signals in phase II studies conducted in other regions, further delaying the availability of new agents for cancer patients in Japan and resulting in scientifically unattractive confirmatory trials. Other factors cited in the late inclusion of Japan in early clinical trials have included prolonged timelines (26) and high costs.

A number of major initiatives have been implemented since 2004 (Fig. 1) involving the Pharmaceuticals and Medical Devices Agency (PMDA; refs. 27–29), JMACCT (an organization of the Japan Medical Association; ref. 30), and Ministry of Health, Labor and Welfare (31), including initiatives to improve the infrastructure for clinical trials in Japan by supporting clinician researchers (investigators) and medical institutions in conducting clinical trials and developing clinical trials networks. Early data suggest that these initiatives have had an impact, with an increase in the numbers of clinical trials conducted in Japan and faster accrual times.

In addition to the changes noted, increasing understanding of ethnic differences in pharmacokinetic, pharmacodynamic, and pharmacogenetics has streamlined development plans. For example, phase I data for new therapeutics metabolized by CYP 1A2, 2E1, and 3A4/5, which are independent of ethnicity (32), may thus be derived from Japanese patients, include Japanese patients, or be used to allow the early initiation of phase II studies in Japan. Higher rates of mutations in the epidermal growth factor receptor have been documented in the east Asian population. In some instances, data from other Asian countries may be used to support early trials in Japan. Nonetheless, in some instances, phase I data from studies including Japanese patients may be mandatory.

Phase I studies in Japan. The requirements for the conduct of phase I studies are summarized in Tables 3 and 4, and, since the development and adoption of ICH S9 (Nonclinical Evaluation for Anticancer Pharmaceuticals), these requirements are now congruent with those in Europe and the United States. To date, relatively few first in human studies have been conducted in Japan, for the reasons described above with the majority using data from first in human studies from other countries to support starting doses. Although there are some cultural differences in the reporting of adverse event data (for example, the reporting of any laboratory changes >grade 1 as adverse events, or the attribution of causality), the conduct and design of phase I studies mirror those in Europe and the United States. When appropriate, and included as objectives of the study, patients and researchers are comfortable with biopsies or use of archival tissue for correlative studies.

Conclusions

Both the European Union and Japan are major markets for pharmaceuticals, and as such are important participants in early clinical trials. Although each jurisdiction was traditionally considered somewhat unique in terms of early drug development, major initiatives in the last decade have aligned drug development in Japan, Europe, and the United States. Although many new agents are developed in collaboration with the

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pharmaceutical industry, many academic drug discovery and development organizations exist, offering many opportunities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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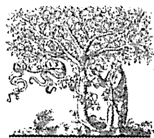

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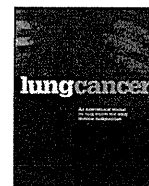

Comparison of chemotherapy for unresectable pulmonary high-grade non-small cell neuroendocrine carcinoma and small-cell lung cancer

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ABSTRACT

Background: Pulmonary large cell neuroendocrine carcinoma (LCNEC) shares several features with small cell lung carcinoma (SCLC). Most histologic diagnoses of LCNEC are currently obtained by surgical specimens. While the diagnosis of LCNEC by biopsy specimens is challenging, a definitive diagnosis of this highly malignant tumor is critical in unresectable cases to determine the optimal therapeutic strategy. The objective of this study was to assess the efficacy of chemotherapy for unresectable high-grade non-small cell neuroendocrine carcinoma (HNSCNEC) called by us, which likely includes most LCNECs except for combined types, and to compare the efficacy of chemotherapy for HNSCNEC, with that for extended disease SCLC (ED-SCLC).
Methods: Between September 2002 and October 2007, we reviewed 14 patients with HNSCNEC, which was defined using biopsy specimens according to histological and immunohistological criteria proposed by us. We simultaneously evaluated the clinical response to the chemotherapy and survival time of the 14 HNSCNEC and 77 ED-SCLC patients.
Results: The chemotherapy regimens in the 14 patients with unresectable HNSCNEC were platinum-based combination regimens or irinotecan or vinorelbine or docetaxel alone. The chemotherapy regimens in the 77 patients with ED-SCLC were platinum-based combination regimens. We assessed an objective response rate, a one-year survival rate, and median survival time as 50% (7/14), 34% and 10 months, respectively, in the 14 HNSCNEC patients, and as 53% (41/77), 48% and 12.3 months, respectively, in the 77 ED-SCLC patients.
Conclusion: The clinical efficacy of chemotherapy for unresectable HNSCNECs, including most LCNECs, is comparable to that for ED-SCLC.

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1. Introduction

In the 1970s, pulmonary neuroendocrine tumors were histologically classified into three categories, i.e., carcinoid, atypical carcinoid, and small cell lung carcinoma (SCLC). In 1991, Travis and colleagues proposed a fourth category: pulmonary neuroendocrine tumors. With this classification, large cell neuroendocrine carcinoma (LCNEC) was regarded as an entity distinct from typical carcinoid, atypical carcinoid, and small cell lung carcinoma (SCLC) [1]. In 1999, the World Health Organization (WHO) defined LCNEC as a variant of large cell carcinoma [2].

LCNEC has specific morphological features and a phenotype that identifies it as a neuroendocrine tumor. Several of these characteristics, such as organoid nesting, palisading, rosette-formation, and frequent mitotic figures, can be observed using light microscopy. The nuclei of LCNEC tumor cells can be differentiated from those of small cell carcinoma by the presence of vesicular or fine chromatin and/or frequent nucleoli. To confirm a neuroendocrine phenotype diagnosis, an immunohistochemical analysis using markers such as chromogranin A, synaptophysin, and neural cell adhesion molecule (NCAM) is required.

At present, most LCNECs are diagnosed using surgically resected specimens and rarely or never using biopsy or cytology specimens. Almost all publications concerning resected LCNECs are based on retrospective analyses of surgical specimens [3–7]. The incidence of the pre-therapeutic diagnosis of LCNEC in unresectable cases is unknown. Therefore, the overall clinico-pathological features of

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