controlled trial'. We also examined reference lists of original articles, review articles, relevant books and the Physician Data Query registry of clinical trials.

Selection of trials

If at least one platinum-based doublet and one single new agent therapy were included in a randomised trial, it was considered to be eligible. A platinum-based doublet included one platinum agent and one new agent (paclitaxel, docetaxel, irinotecan, gemcitabine, or vinorelbine), and the single agent had to be one of these new agents. Trials were excluded from our analysis if the new agents used in the platinum-based doublet were different from the single-agent therapies. Patients with pathologically confirmed advanced NSCLC who had not previously received chemotherapy were enrolled in these trials.

Validity assessment

We carried out an open assessment of the trials and used the instrument reported by Jadad et al. [17]. However, no evident differences were observed among the trials. Therefore, the result of the validity assessment was not considered in the meta-analysis.

Data abstraction

To avoid bias in the data abstraction process, two observers (K.H. and H.U.) independently abstracted the data from the trials and subsequently compared the results. All data were checked for internal consistency, and disagreements were resolved by discussion among the investigators. We tried to contact principal investigators of the trials to confirm or update both published and unpublished data.

Quantitative data synthesis

We applied odds ratios (ORs) to assess objective response rate and toxic events. We constructed 2×2 tables from abstracted data for responses and for each toxic event. ORs and their variances for the subjects who received a platinum-based doublet relative to those receiving single new agent therapy alone were calculated from the tables. For OR calculations, we excluded ineligible subjects from each evaluation.

Hazard ratios (HR) were applied to assess the survival advantage of platinum-based doublets compared with that of single-agent therapy alone. Crude log HR and its variance for each trial were calculated using the abstracted survival probabilities at each time point according to the methods proposed by Parmar et al. [18]. Minimum and maximum follow-up times were used to estimate censored subjects under the assumption that censoring happens constantly throughout follow-up. If the minimum follow-up time was not available, time zero was substituted for it. As we assumed a constant hazard for the two types of therapy within an individual trial, all of the survival probabilities available in each trial were used to obtain a representative HR for each trial instead of limiting time points to specified times. HRs were calculated to represent how many times

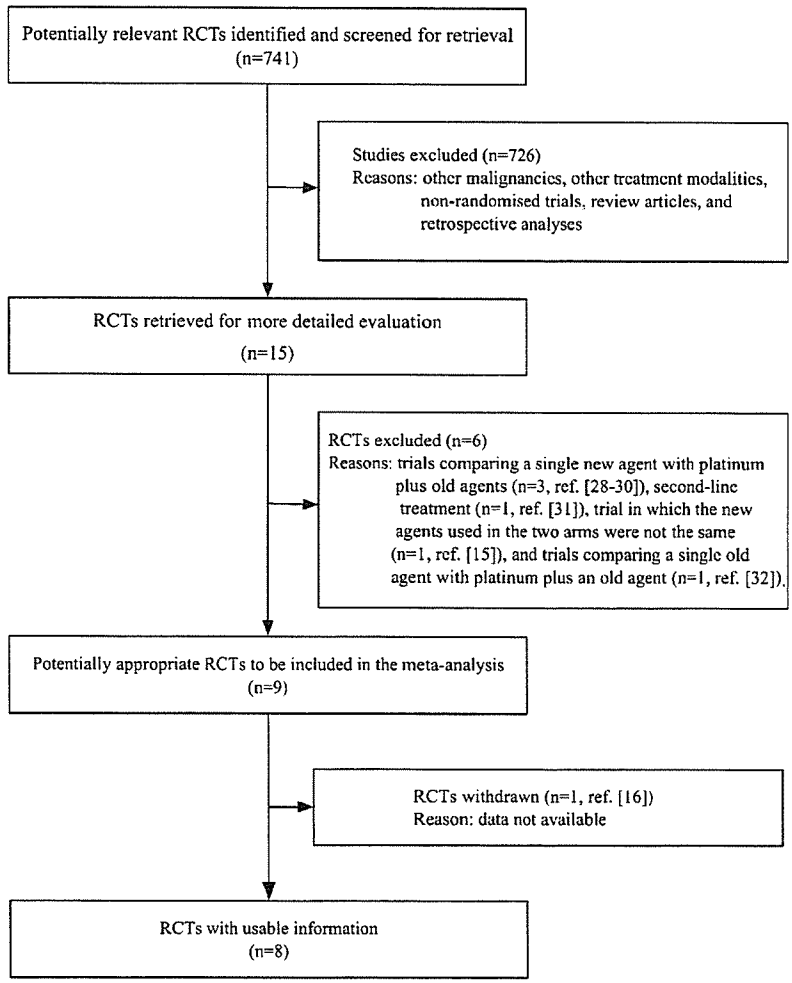

Figure 1. A flow chart showing the progress of trials through the review. RCT, randomised controlled trials.

higher the probability of death was from any cause in patients receiving a platinum-based doublet compared with those receiving single-agent therapy. Therefore, a HR below unity indicates that the platinum-based doublet is better than single-agent therapy.

A general variance-based method was used to estimate the summary HR, ORs and their 95% confidence intervals (CIs). We looked for heterogeneity among the trials based on standard methods [19]. We also calculated the between-study variation (τ^2) from the Q statistic according to the method described by DerSimonian and Laird [20]. Regardless of the statistical significance of the Q test, we applied a random effect model which allows meta-analyses to take between-study variation into consideration. We also used Begg's funnel plots [21] and Egger's test [22] to detect possible publication bias. Meta-regression analysis was applied to detect the source of heterogeneity in the survival analysis. The factors examined in meta-regression analysis were study quality score [17], starting year of the trial, proportion of patients with performance status (PS) 0–1, using the World Health Organization criteria or others proportion of stage IV patients, proportion of male patients, and inclusion of carboplatin. A cumulative meta-analysis was planned in order to take trial quality into consideration when the trial showed quality score heterogeneity [17].

All statistical analyses were conducted with STATA ver. 8 software (College Station, TX, USA). We defined a statistical test with a P value less than 0.05 as significant.

Results

Trial flow

The flow chart of our study is shown in Figure 1. Ultimately, eight trials involving 2374 patients with advanced NSCLC were analysed in this meta-analysis [7–14]. There were no trials that were excluded from our study only because they were published in languages other than English. One of the remaining 10 potentially appropriate trials that compared CDDP and gemcitabine with gemcitabine alone in 72 patients was excluded from our analysis because we were unable to obtain the relevant data [16].

Characteristics of the eight trials

Baseline characteristics of the eight trials are listed in Table 1. In total, 2351 patients were randomised to a platinum-based doublet (1191 patients) or a single new agent therapy (1160 patients); 23 patients enrolled in one trial were excluded before randomisation [12]. Further information about one published trial [8] and three unpublished trials [11–13] were obtained by contacting the principal authors. Other potential sources of heterogeneity, including platinum type (CDDP versus carboplatin), were examined by meta-regression analysis. However, none of these factors were associated with significant differences in outcomes with a few exceptions as described below.

Response rate and overall survival

Data on objective response rates were available for all eight trials (Table 2). Based on intention-to-treat analysis using all randomised patients, the objective response rate to a platinum-based doublet was more than two-fold higher than to single-agent therapy (OR = 2.32; 95% CI = 1.68–3.20 Figure 2). Neither a funnel plot nor a rank correlation test regarding response rate indicated the existence of publication bias ($Z = 1.24$, $P = 0.22$). The heterogeneity test yielded a significant result ($P = 0.017$). Meta-regression analysis showed

Table 1. Characteristics of the eight trials included in this meta-analysis

Author	Year	Publication form	No. of randomised patients	Chemotherapy regimen	Sex (male, %)	PS 0–1 (%)	Stage IV (%)
Depierre	1994	F	121	P 80 mg/m^2, d1, q3ws + V 30 mg/m^2, weekly	91	72	53
			119	V 30 mg/m^2, weekly	82	70	56
Le Chevalier	1994	F	206	P 120 mg/m^2, d1, q4ws + V 30 mg/m^2, weekly	88	80	50
			206	V 30 mg/m^2, weekly	91	77	47
Lorusso	1995	F	34	P 80 mg/m^2, d1 + V 25 mg/m^2, d1,8, q3–4ws	94	52	32
			35	V 25 mg/m^2, weekly	100	55	39
Deza	1996	A	89	P 100 mg/m^2, d1 + V 30 mg/m^2, d1,8,15, q4ws	82	78	75
			73	V 30 mg/m^2 weekly	90	81	71
Georgoulias	2002	A	160	P 80 mg/m^2, d2 + D 100 mg/m^2, d1, q3ws	94	93	64
			148	D 100 mg/m^2, d1, q3ws	90	89	65
Lilenbaum	2002	A	284	C AUC = 6, d1 + PTX 225 mg/m^2, d1, 3 h, q3ws	68	83	71
			277	PTX 225 mg/m^2, d1, 3 h, q3ws	69	82	73
Sederholm	2002	A	164	C AUC = 5, d1 + G 1250 mg/m^2, d1,8, q3ws	60	82	54
			170	G 1250 mg/m^2, d1,8, q3ws	52	88	63
Negoro	2003	F	133	P 80 mg/m^2, d1 + I 60 mg/m^2, d1,8,15, q4ws	76	94	62
			132	I 100 mg/m^2, d1,8,15, q4ws	74	94	66

All trials were randomised phase III trials except for Lorusso's trial that was designed as a randomised phase II trial.
PS, performance status; F, full text; A, abstract form; P, cisplatin; V, vinorelbine; D, docetaxel; C, carboplatin; AUC, area under the plasma concentration–time curve; PTX, paclitaxel; G, gemcitabine; I, irinotecan; d, day; ws, weeks.

that a higher percentage of stage IV patients had a reduced response ($P=0.01$).

Data on overall survival were available for all eight trials (2331 patients, Table 3). Survival analyses were carried out based on intention-to-treat analysis in five trials and seven, six and seven patients in the trials reported by Lorusso et al. [9], Deza et al. [11] and Negoro et al. [10], respectively, were excluded from the survival analysis after randomisation. Since none of the trials gave the HR required for meta-analysis, we applied HRs calculated from KM curves in all trials, based on

Table 2. Responses in the eight trials

Author	Chemotherapy regimen	No. of responding patients	No. of randomised patients	Objective response (%)
Depierre	CDDP + VNR	50	121	41
	VNR	18	119	15
Le Chevalier	CDDP + VNR	57	206	28
	VNR	28	206	14
Lorusso	CDDP + VNR	13	34	38
	VNR	4	35	11
Deza	CDDP + VNR	31	89	35
	VNR	30	73	41
Georgoulias	CDDP + DOC	57	160	36
	DOC	29	148	20
Lilenbaum	CBDCA + PTX	82	284	29
	PTX	47	277	17
Sederholm	CBDCA + GEM	40	164	24
	GEM	18	170	11
Negoro	CDDP + CPT	55	133	41
	CPT	26	132	20

CDDP, cisplatin; VNR, vinorelbine; DOC, docetaxel; CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; CPT, irinotecan.

Table 3. Survival in the eight trials

Author	Chemotherapy regimen	Intention-to-treat analysis	No. of assessable patients	Median survival time (weeks)	1-year survival (%)	*P* value
Depierre	CDDP + VNR	Yes	121	33	28	0.48
	VNR		119	32	21	
Le Chevalier	CDDP + VNR	Yes	206	40	38	0.05
	VNR		206	31	34	
Lorusso	CDDP + VNR	No	31	38	NR	NS
	VNR		31	30	NR	
Deza	CDDP + VNR	No	83	41	NR	0.23
	VNR		73	33	NR	
Georgoulias	CDDP + DOC	Yes	160	43	45	NS
	DOC		148	34	40	
Lilenbaum	CBDCA + PTX	Yes	284	38	37	0.20
	PTX		277	29	33	
Sederholm	CBDCA + GEM	Yes	164	43	41	0.02
	GEM		170	39	32	
Negoro	CDDP + CPT	No	129	50	47	NR
	CPT		129	46	42	

All *P* values were extracted from original papers.
CDDP, cisplatin; VNR, vinorelbine; DOC, docetaxel; CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; CPT, irinotecan; NR, not recorded; NS, not significant.

the method of Parmar et al. [18]. Platinum-based doublet therapy was associated with a 13% improvement in overall survival compared with single-agent therapy (HR = 0.87; 95% CI = 0.80–0.94, $P<0.001$, Figure 3). Similarly, a funnel plot and rank correlation test regarding survival confirmed the absence of publication bias ($Z=0.83$, $P=0.40$).

Toxicity

Eight trials encompassing 2251 patients provided toxicity profile results (Table 4). Complete data for neutropenia were not obtained in three trials [9, 11, 13]; data for thrombocytopenia and nausea/vomiting were not available in two trials each [9, 11]; and data for nephrotoxicity were not available in one trial [11]. The heterogeneity test was statistically significant for neutropenia and nausea/vomiting. Further meta-regression analysis failed to show any significant source of heterogeneity from examined factors for neutropenia. Regarding nausea/vomiting, male subjects had an increased chance of experiencing a nausea/vomiting event and use of carboplatin reduced it. Platinum-based doublet therapy significantly increased the frequency of all toxic effects over single-agent therapy, whereas no significant difference in treatment-related mortality was observed between the two treatment modalities (1.4% versus 1.2%, OR = 0.97; 95% CI = 0.41–2.26, $P=0.94$).

Discussion

Lilenbaum et al. [12] previously carried out a meta-analysis to compare the effect of combination chemotherapy with that of single-agent chemotherapy on overall survival in patients with advanced NSCLC [23]. They concluded that overall survival was modestly improved with combination chemotherapy. However, in the majority of trials analysed, outdated chemotherapy regimens were used, and only small numbers of patients were included. Thus, the impact of platinum plus one of the new agents on the survival of advanced NSCLC patients compared with that of single new agent therapy remained undetermined.

In this study, we used data from trials comparing platinum plus one of the new agents with the new agent alone; three trials evaluated in our study were also included in Lilenbaum's study [7, 8, 11]. Our results indicate that the addition of platinum to single new agents is important, if they have adequate organ function and good performance status. However, it remains unclear which new drug should be combined with platinum in a platinum-based doublet and further investigation is necessary.

Two meta-analyses on the addition of a second drug for recently advanced NSCLC have been presented [24, 25]. Both were based on literature data. Using 33 trials with 7872 patients, Delbaldo et al. [24] demonstrated a significant increase in response rate and survival in favour of two drugs (OR = 0.39; 95% CI = 0.35–0.45, $P<0.001$ and HR = 0.79; 95%CI = 0.75–0.83, $P<0.001$). Baggstrom et al. [25] identified 17 trials randomising 4421 patients with advanced NSCLC to one drug or two drugs, and demonstrated that the doublet chemotherapy is superior in terms of overall survival as well as response. Although the eligible trials and patient populations were different among the studies, all three studies including our study indicate that a one-drug regimen is inferior in terms of response and survival.

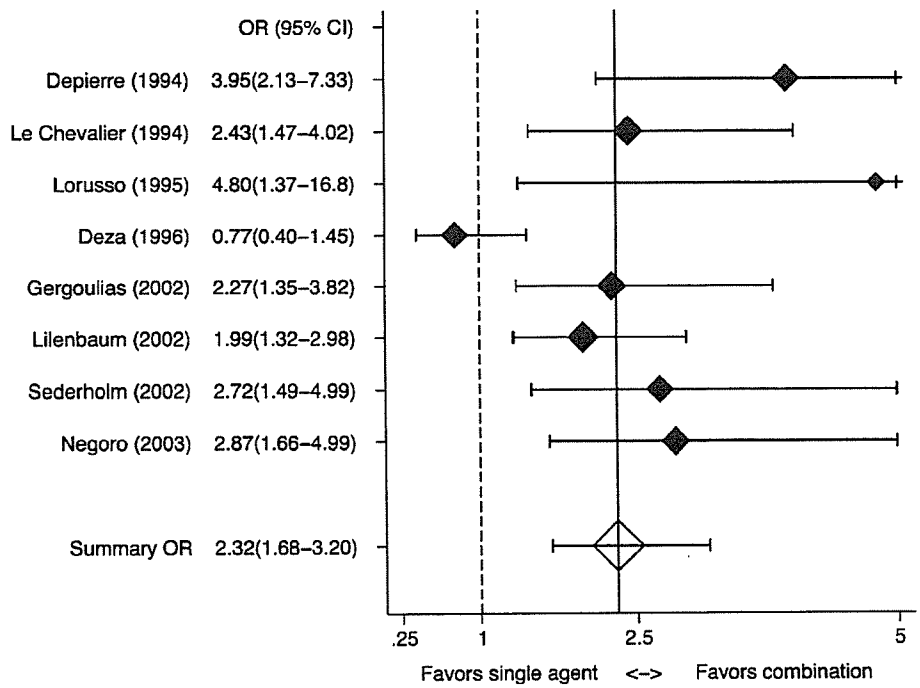

Figure 2. Response to a platinum-based doublet compared with a single new agent alone. The heterogeneity test yielded a significant result ($P=0.017$). Meta-regression analysis showed that an increased percentage of stage IV patients had a reduced response ($P=0.01$).

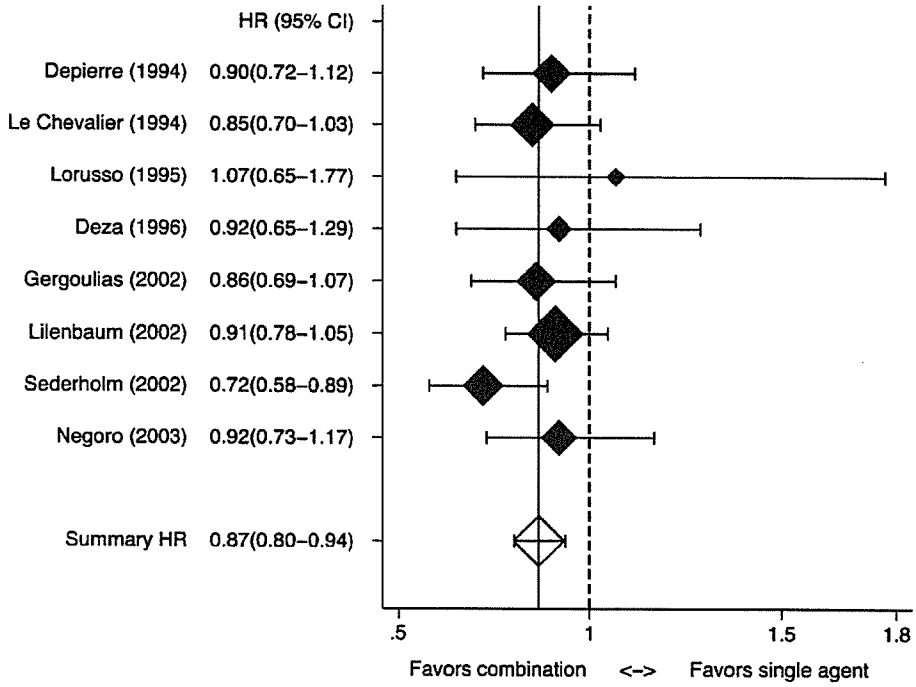

Figure 3. Overall survival with a platinum-based doublet compared with a single new agent alone. The summary hazard range indicated a 13% hazard event reduction in those receiving a platinum-containing regimen compared to those given single new agent therapy. The heterogeneity test yielded no significant result ($P=0.704$).

Table 4. Toxic effects in trials comparing platinum-containing regimen with single new agent alone (grades 3 and 4)

Toxicity	No. of evaluable trials	Combination chemotherapy		Single-agent therapy		OR (95% CI)	*P* value for *Q* test
		No. of patients with toxicity (%)	No. of evaluable patients	No. of patients with toxicity (%)	No. of evaluable patients		
Neutropenia	5	507 (56.5)	897	281 (32.2)	872	3.12 (1.98–4.91)	0.002
Thrombocytopenia	6	90 (8.5)	1054	6 (0.6)	1041	14.4 (6.75–30.5)	0.992
Nephrotoxicity	7	62 (5.7)	1085	9 (0.8)	1072	6.44 (2.95–14.0)	0.376
Nausea/vomiting	6	223 (21.2)	1054	60 (5.8)	1041	4.01 (1.94–8.30)	0.001

Heterogeneity tests showed significant results for neutropenia and nausea/vomiting. We failed to find any source of heterogeneity in the factors using the meta-regression analysis. Regarding nausea/vomiting, male subjects had an increased chance of experiencing a nausea/vomiting event and use of carboplatin reduced it.

OR, odds ratio; CI, confidence interval.

We included the two trials in which the effect of carboplatin was investigated [12, 13]. Recently, we carried out another meta-analysis of the trials that compared CDDP-based chemotherapy with carboplatin-based chemotherapy, which revealed that combination chemotherapy consisting of CDDP plus a new agent yields a substantial survival advantage compared with carboplatin plus a new agent in patients with advanced NSCLC [26]. Thus, inclusion of trials using carboplatin will give conservative *P* values for summary statistics. However, the highly significant association found in the current analysis indicates that CDDP is important in the treatment of advanced NSCLC. In addition, meta-regression analysis in this study showed that inclusion of trials using carboplatin did not change the results in our study, which suggests that the inclusion of the two trials using carboplatin did not alter our main conclusion. Further investigation will be needed to clarify the role of carboplatin in the treatment of advanced NSCLC.

Our study has several limitations. First, one major problem is that our analyses were based on abstracted data, since an individual patient data based meta-analysis would give a more robust estimate of the association [27]. Therefore, physicians should interpret our results carefully. Second, some of the trials we identified were reported in abstract form only, which made it difficult to extract complete data for our meta-analysis. However, additional updated data fully adequate for this meta-analysis were obtained in several cases by contacting the principal investigators. Third, as is often the case with

meta-analysis, one must still be cautious in interpreting our results because of the substantial effect of heterogeneity, although we applied a random-effect model to obtain summary statistics. The significant results from the heterogeneity tests for response rate, neutropenia and nausea/vomiting represent potential heterogeneity and may modify the association we found in our study. Possible publication bias is also a potential harm in our study, though we did not detect it statistically. Finally, the dose of new agent used in the platinum-based doublet was different from that in the single-agent therapy in Negoro's trial [10]. Irinotecan was administered at a dose of 100 mg/m^2 in the single-agent therapy, whereas 60 mg/m^2 of irinotecan was combined with cisplatin in the combination arm. It might be problematic to analyse the importance of platinum in our study. However, we considered that the effect would be very small, if any, because it occurred in only one of the eight trials and because the same administration schedule for irinotecan was used in both arms.

In conclusion, this is the first published meta-analysis, to our knowledge, of randomised trials of platinum-based doublet versus single new agent therapy alone. Although modest, the survival improvement obtained with the platinum-based doublet in comparison to a single new agent therapy indicates the importance of platinum in the treatment of advanced NSCLC.

Acknowledgements

We wish to thank Dr Alain Depierre, Dr Ernesto Gil Deza, Dr Rogerio C Lilenbaum and Dr Christer Sederholm for their valuable comments and for providing data.

References

1. Smith W, Khuri FR. The care of the lung cancer patient in the 21st century: a new age. Semin Oncol 2004; 31 (2 Suppl 4): 11–15.
2. Non-small cell lung cancer collaborative group. Chemotherapy in non-small cell lung cancer: A meta-analysis using updated data on individual patients from 52 randomized clinical trials. BMJ 1995; 311: 899–909.
3. Bunn PA. Chemotherapy for advanced non-small-cell lung cancer: who, what, when, why? J Clin Oncol 2002; 20: 23s–33s.
4. Ranson M, Davidson N, Nicolson M et al. Randomized trial of paclitaxel plus supportive care versus supportive care for patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2000; 92: 1074–1080.
5. Baggstrom MQ, Socinski MA, Hensing TA, Poole C. Third generation chemotherapy regimens (3GR) improve survival over second generation regimens (2GR) in stage IIIB/IV non-small cell lung cancer (NSCLC): a meta-analysis of the published literature. Proc Am Soc Clin Oncol 2002; 21: 306a (Abstr 1222).
6. Yana T, Takada M, Origasa H et al. New chemotherapy agent plus platinum for advanced non-small cell lung cancer: a meta-analysis. Proc Am Soc Clin Oncol 2002; 21: 328a (Abstr 1309).
7. Le Chevalier T, Brisgand D, Douillard J-Y et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: Results of a European multicenter trial including 612 patients. J Clin Oncol 1994; 12: 360–367.
8. Depierre A, Chastang C, Quoix E et al. Vinorelbine versus vinorelbine plus cisplatin in advanced non-small cell lung cancer: a randomized trial. Ann Oncol 1994; 5: 37–42.
9. Lorusso V, Pezzella G, Catino AM et al. Results of a clinical multicentric randomized phase II study of non-small cell lung cancer treated with vinorelbine–cisplatin versus vinorelbine alone. Int Oncol 1995; 6: 65–68.
10. Negoro S, Masuda N, Takada Y et al. Randomized phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. Br J Cancer 2003; 88: 335–341.
11. Deza EG, Balbiani L, Coppola F et al. Phase III study of navelbine (NVB) vs NVB plus cisplatin in non small cell lung cancer (NSCLC) stage IIIB or IV. Proc Am Soc Clin Oncol 1996; 15: 394 (Abstr 1193).
12. Lilenbaum RC, Herndon J, List M et al. Single-agent versus combination chemotherapy in advanced NSCLC: A CALGB trial of efficacy, quality of life, and cost-effectiveness. Proc Am Soc Clin Oncol 2002; 21: 1a (Abstr 2).
13. Sederholm C. Gemcitabine compared with gemcitabine plus carboplatin in advanced non-small cell lung cancer (NSCLC): a phase III study by Swedish Lung Cancer Study Group (SLUSG). Proc Am Soc Clin Oncol 2002; 21: 291a (Abstr 1162).
14. Georgoulias V, Ardavanis A, Agelidou M et al. Preliminary analysis of a multicenter phase III trial comparing docetaxel versus docetaxel/cisplatin in patients with inoperable advanced and inoperable advanced and metastatic non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2002; 21: 291a (Abstr 1163).
15. Monnier A, Zatloukal P, Siffnerova H et al. Docetaxel (D) versus vinorelbine and cisplatin (VC) in chemotherapy-naïve patients with advanced non-small cell lung cancer (NSCLC) and with a normal level of serum alpha 1 acid glycoprotein (AAG): Interim results of a multinational, randomised phase II. Ann Oncol 2002; 13 (Suppl 5): 133 (Abstr 487PD).
16. Berardi R, Porfiri E, Massidda B et al. Gemcitabine (GEM) and cisplatin (PL) versus gemcitabine alone in stage IV non small cell lung cancer: Preliminary results of a randomized multicenter phase III study. Proc Am Soc Clin Oncol 2001; 20: 347a (Abstr 1385).
17. Jadad AR, Moore RA, Carroll D et al. Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin Trials 1996; 17: 1–12.
18. Parmar MB, Torri V, Stewart L. Extracting summary statistics to perform meta-analyses of the published literature for survival end points. Stat Med 1998; 17: 2815–2834.
19. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002; 21: 1539–1558.
20. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986; 7: 177–188.
21. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics 1994; 50: 1088–1101.
22. Egger M, Smith GD, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ 1997; 315: 629–634.
23. Lilenbaum RC, Langenberg P, Dickersin K. Single agent versus combination chemotherapy in patients with advanced nonsmall cell lung carcinoma. A meta-analysis of response, toxicity, and survival. Cancer 1998; 82: 116–126.
24. Delbaldo C, Syz N, Michiels S, Le Chevalier T et al. Adding a second or a third drug to a chemotherapy regimen in patients with advanced non-small-cell lung carcinoma (NSCLC): A meta-analysis of the literature. Proc Am Soc Clin Oncol 2003; 22: 623 (Abstr 2507).
25. Baggstrom MQ, Socinski MA, Hensing TA, Poole C. Addressing the optimal number of cytotoxic agents in stage IIIB/IV non-small cell lung cancer (NSCLC): A meta-analysis of the published literature. Proc Am Soc Clin Oncol 2003; 22: 624 (Abstr 2510).

26. Hotta K, Matsuo M, Ueoka H et al. Meta-analysis of randomized clinical trials comparing cisplatin to carboplatin in patients with advanced non-small-cell lung cancer. J Clin Oncol 2004; 22: 3852–3859.
27. Clarke MJ, Stewart LA. Obtaining individual patient data from randomized controlled trials. In Egger M, Smith GD, Altman DG (eds): Systematic Reviews in Health Care. London, UK: BMJ Publishing Group 2001; 109–121.
28. Ten Bokkel HWW, Bergman B, Chemaissani A et al. Single-agent gemcitabine: an active and better tolerated alternative to standard cisplatin-based chemotherapy in locally advanced or metastatic non-small cell lung caner. Lung Cancer 1999; 26: 85–94.
29. Vansteenkiste JF, Vandebroek JE, Nackaerts KL et al. Clinical–benefit response in advanced non-small-cell lung cancer: A multicentre prospective randomised phase III study of single agent gemcitabine versus cisplatin-vindesine. Ann Oncol 2001; 12: 1191–1193.
30. Perng RP, Chen YM, Ming-Liu J et al. Gemcitabine versus the combination of cisplatin and etoposide in patients with inoperable non-small-cell lung cancer in a phase II randomized study. J Clin Oncol 1997; 15: 2097–2102.
31. Takenaka M, Tanio Y, Okishio K et al. A randomized phase II of weekly carboplatin and CPT-11 versus docetaxel for relapsed non-small cell lung cancer. Proc Am Soc Clin Oncol 2001; 20: 263b (Abstr 2805).
32. Splinter TAW, Sahmoud T, Festen J et al. Two schedules of teniposide with or without cisplatin in advanced non-small cell lung cancer: randomized study of the European Organization for Research and Treatment of Cancer Lung Cancer Cooperative Group. J Clin Oncol 1996; 14: 127–134.

J Cancer Res Clin Oncol (2005) 131: 147–151
DOI 10.1007/s00432-004-0626-z

ORIGINAL PAPER

Toshiyuki Kozuki · Nobukazu Fujimoto · Hiroshi Ueoka
Katsuyuki Kiura · Keiichi Fujiwara · Katsuhiko Shiomi
Koichi Mizobuchi · Masahiro Tabata · Shuji Hamazaki
Mitsune Tanimoto

Complexity in the treatment of pulmonary large cell neuroendocrine carcinoma

Received: 20 April 2004 / Accepted: 25 August 2004 / Published online: 5 November 2004

Abstract *Purpose*: According to the World Health Organization (WHO) classification of pulmonary large cell neuroendocrine carcinoma (LCNEC), one of the neuroendocrine tumors of the lung, is considered as a variant of non-small cell lung carcinoma. The objective of this study was to investigate the treatment strategy for LCNEC. *Methods*: We retrospectively reviewed the clinical information of 12 patients with LCNEC. *Results*: Three patients with stage I disease underwent curative resection but all relapsed within 20 months. One with stage IIA disease underwent non-curative resection received adjuvant chemoradiotherapy (cisplatin plus etoposide) and is well with no evidence of recurrence. Two with stage IIIB disease received concurrent chemoradiotherapy. Both achieved partial response (PR) but relapsed within 2 months. One elderly patient with stage IIIA disease received vinorelbine alone and did not respond. Of five patients with stage IV disease, three received platinum-based chemotherapy but no patient achieved PR. Of five patients with gefitinib as salvage therapy, one achieved PR. *Conclusions*: The prognosis of LCNEC is poor. To improve the outcome, we must evaluate the effectiveness of adjuvant or neoadjuvant therapy in patients with resectable disease. In addition, the evaluation of systemic and multimodality treatment strategies similar as in small cell lung cancer is worthy of consideration.

Keywords Large cell neuroendocrine carcinoma (LCNEC) · Chemotherapy · Chemoradiotherapy · Gefitinib · Serum tumor marker

K. Kiura (✉) · M. Tabata
Department of Respiratory Medicine,
Okayama University Hospital, 2-5-1 Shikata-cho, 700-8558
Okayama, Japan
E-mail: kkiura@md.okayama-u.ac.jp
Tel.: +81-86-2357227
Fax: +81-86-2328226

T. Kozuki · N. Fujimoto · H. Ueoka
K. Fujiwara · M. Tanimoto
Department of Hematology, Oncology and Respiratory Medicine
(Department of Internal Medicine II), Okayama University
Graduate School of Medicine and Density, Okayama, Japan

S. Hamazaki
Department of Pathology,
Okayama University Hospital, Okayama, Japan

K. Shiomi · K. Mizobuchi
Kagawa Rosai Hospital, Kagawa, Japan

Introduction

In 1991, Travis et al. proposed pulmonary large cell neuroendocrine carcinoma (LCNEC) as the fourth category of neuroendocrine tumors in addition to typical carcinoid, atypical carcinoid, and small cell lung carcinoma (SCLC) (Travis et al. 1991). In the revised World Health Organization (WHO) classification published in 1999 (Travis et al. 1999), LCNEC is recognized as one of the variants of large cell carcinoma. LCNEC has a characteristic morphology with features of both non-small cell lung carcinoma (NSCLC) and neuroendocrine carcinoma.

The frequency of LCNEC is reported to be 1.6–3.1% of total lung cancers (Takei et al. 2002; Jiang et al. 1998; Mazieres et al. 2002). Most patients have been treated using the strategy for NSCLC, but there are some reports indicating that the clinical outcome of LCNEC is poorer than that of NSCLC even in early stage disease (Jiang et al. 1998; Dresler et al. 1997). Since the majority of the previous reports focused on surgically treated cases because of the difficulty in obtaining a preoperative pathological diagnosis, there are only a few reports that have evaluated the treatment of advanced LCNEC including the efficacy of systemic chemotherapy and/or radiotherapy. The number of patients in these reports, however, was too small to assess the effectiveness of systemic treatment. The standard treatment strategy for advanced LCNEC has not yet been clearly established.

In this study, we summarize the clinical features and treatment outcome of 12 patients with LCNEC. We

investigated the clinical efficacy of chemotherapy or chemoradiotherapy for LCNEC and discuss the treatment strategy for the advanced LCNEC.

Patients and methods

Patients

Between January 1998 and May 2003, 12 patients were given a diagnosis of LCNEC in two hospitals (Okayama University Hospital and Kagawa Rosai Hospital). We retrospectively obtained the clinical information including gender, age, smoking status, Eastern Cooperative Oncology Group (ECOG) performance status (PS), stage, serum tumor markers, and treatment outcome by reviewing the medical records. Survival data was determined from the day of histological diagnosis to the day of last follow-up. Clinical stage was evaluated by the standard staging procedure including chest radiographs, chest computed tomographic (CT) scans, brain magnetic resonance images or CT scans, technetium-99m hydroxymethylene diphosphonate bone scans, and abdominal CT scans. We assessed objective tumor response according to the WHO response criteria (Miller et al. 1981).

Pathological diagnosis of LCNEC

The diagnosis of LCNEC was based on the following WHO 1999 criteria (Travis et al. 1999): 1) neuroendocrine morphologic features (organoid, trabecular, palisading and rosettes); 2) neuroendocrine features by immunohistochemisry such as expression of chromogranin, synaptophysin, neural cell adhesion molecule, and neuron specific enolase (NSE); 3) non-small cell features (i.e., cell size, nuclear / cytoplasmic ratio, nucleoli); 4) necrosis and high mitotic ratio of greater than ten per ten high-power fields. Histological samples obtained by surgery, transbronchial or needle biopsies and/or autopsy were reviewed independently by two pathologists (K.M. and S.H.) and disagreements were resolved by discussion.

Serum tumor marker evaluation

Several serum tumor markers were measured routinely as part of the pretreatment examination. The serum levels of carcinoembryonic antigen (CEA), squamous cell carcinoma-related antigen (SCC), cytokeratin 19-fragments (CYFRA), carbohydrate antigen 19–9 (CA 19–9), sialyl Lewis X-i (SLX), NSE and pro-gastrin releasing peptide (ProGRP) were measured using radioimmunoassay, enzyme immunoassay, and/or electrochemiluminescence immunoassay. The cut-off levels of these markers are 240 IU/l for lactate dehydrogenase (LDH), 5 ng/ml for CEA, 1.5 ng/ml for SCC, 2.8 ng/ml for CYFRA, 40 U/ml for CA 19–9, 38 U/ml for SLX, 10 ng/ml for NSE and 46 pg/ml for ProGRP.

Results

Patient characteristics

The characteristics of the 12 patients, 11 men and one woman, investigated in this study are summarized in Table 1. The median age of patients was 60 years ranging from 43 years to 77 years. Two patients with a preoperative diagnosis of NSCLC were re-classified as LCNEC following pathological examination of the surgically resected specimens. Three patients were diagnosed by needle biopsy, although one of these patients had been diagnosed as poorly differentiated adenocarcinoma at the initial operation. Four patients were diagnosed by transbronchial biopsy, two by supraclavicular lymph node biopsy, and one at autopsy. Pretreatment ECOG PS was 0 in four patients, 1 in five, 2 in

Table 1 Summary of patient characteristics and clinical outcome (*M* male, *F* female, *BSC* best supportive care, *CDDP* cisplatin, *ETP* etoposide, *TRT* thoracic radiation therapy, *VNR* vinorelbine, *DCT* docetaxel, *CBDCA* carboplatin, *GEM* gemcitabine, *PD* progressive disease, *NC* no change, *PR* partial response)

Case	Age	Sex	Smoking (Pack-years)	PS	TNM	Stage	Treatment	Regimen	Response	Survival (month)
1	52	M	24	0	T1N0M0	IA	Lobectomy	-	-	12.5
2	61	M	80	0	T1N0M0	IA	Lobectomy	-	-	24.1 +
3	56	M	53	1	T2N0M0	IB	Lobectomy	-	-	19.0
4	72	M	78	1	T1N1M0	IIA	Non-curative lobectomy Chemoradiotherapy	CDDP + ETP + TRT 44 Gy	PR	50.3 +
5	77	M	30	1	T2N2M0	IIIA	Chemotherapy	VNR	PD	6.4 +
6	59	M	85	1	T1N3M0	IIIB	Chemoradiotherapy	CDDP + DCT + TRT 56 Gy	PR	12.9 +
7	58	F	60	2	T4N3M0	IIIB	Chemoradiotherapy	CDDP + ETP + TRT 45 Gy	PR	9.5
8	43	M	46	1	T4N0M1	IV	Chemotherapy	CDDP + ETP	PD	2.8
9	68	M	20	0	T1N2M1	IV	Chemotherapy	CBDCA + GEM	PD	2.0 +
10	71	M	114	0	T1N0M1	IV	Chemotherapy	CDDP + DCT + GEM	NC	8.2 +
11	59	M	0	3	T1N1M1	IV	BSC	-	-	0.6
12	70	M	50	3	T3N2M1	IV	BSC	-	-	2.6

one and 3 in two. Eight patients were current smokers, three were former smokers, and one patient had never smoked. The median pack-years of cigarette smoking was 52.5 pack-years ranging from 20 pack-years to 114 pack-years, and was more than 40 pack-years in eight patients. Stage according to the Union Internationale Contre le Cancer (UICC) staging system was stage IA in two patients, IB, IIA, and IIIA in one patient, IIIB in two patients and IV in five patients (Sobin and Wittekind 1997).

Serum tumor markers

Serum levels of CYFRA were elevated in four of six patients examined (67%, 22–96%), NSE in five of eight (63%, 95% C.I. 24–91%), LDH in six of eleven (55%, 23–83%), SLX in one of two (50%, 1–98%), CEA in five of twelve (42%, 15–72%), ProGRP in two of six (33%, 4–78%), CA19–9 in none of four (0%, 0–60%) and SCC in none of nine patients (0%, 0–34%) (Table 2). Serum levels of CYFRA ($n=4$), NSE ($n=3$) and ProGRP ($n=2$) increased in all five patients with stage IV disease and CEA and LDH levels were elevated in four (80%, 28–95%) and three (60%, 15–95%) of five patients, respectively.

Treatment outcome of patients with stage I-II disease

Four patients (stage IA: 2, IB: 1 and IIA: 1) underwent lobectomy. Curative surgery was performed in three patients with stage I (cases 1, 2 and 3), but all patients developed recurrent disease in 7 months, 10 months, and 20 months. The initial recurrence sites were mediastinal lymph nodes and disseminated multiple bone metastases in two patients (cases 1 and 3) and multiple liver metastases in one patient (case 2). One patient (case 1) received palliative radiotherapy for the primary relapse and metastatic bone lesions and died in 3 months after recurrence. The other two patients received systemic chemotherapy following recurrence. Although one patient (case 3) received a variety of combination chemotherapy including platinum-based chemotherapy, no response was obtained. Another patient (case 2) received four cycles of triplet chemotherapy with cisplatin (CDDP), docetaxel (DCT), and irinotecan (CPT-11) and exhibited a partial response (PR). One patient (case 4) could not undergo curative surgery because of poor pulmonary function and received consisting of CDDP plus etoposide (ETP) and concurrent thoracic irradiation at a dose of 44 Gy. He achieved a PR that has continued for 4 years or more.

Table 2 Serum tumor markers (*LDH* lactate dehydrogenase, *CEA* carcinoembryonic antigen, *SCC* squamous cell carcinoma-related antigen, *CYFRA* cytokeratin 19-fragments, *SLX* sialyl Lewis X-i, *CA19–9* cytokeratin 19-fragments, *NSE* neuron specific enolase, *ProGRP* pro-gastrin releasing peptide)

	Unit	Number (%)	
		All cases	Stage IV cases
LDH	≤ 240 IU/l	5 (45)	2 (40)
	> 240 IU/l	6 (55)	3 (60)
CEA	≤ 5 ng/ml	7 (58)	1 (20)
	> 5 ng/ml	5 (42)	4 (80)
SCC	≤ 1.5 ng/ml	9 (100)	4 (100)
	> 1.5 ng/ml	0 (0)	0 (0)
CYFRA	≤ 2.8 ng/ml	2 (33)	0 (0)
	> 2.8 ng/ml	4 (66)	4 (100)
SLX	≤ 38 U/ml	1 (50)	
	> 38 U/ml	1 (50)	
CA19–9	≤ 40 U/ml	4 (100)	3 (100)
	> 40 U/ml	0 (0)	0 (0)
NSE	≤ 10 pg/ml	3 (38)	0 (0)
	> 10 pg/ml	5 (63)	3 (100)
ProGRP	≤ 46 pg/ml	4 (67)	0 (0)
	> 46 pg/ml	2 (33)	2 (100)

Treatment outcome of patients with stage III disease

Three patients had locally advanced disease. Two of them received CDDP plus DCT (case 6) or CDDP plus ETP (case 7) and concurrent radiotherapy. Although both patients achieved a PR, they developed recurrence within 2 months after the treatments. An elderly patient with stage IIIA disease (case 5) received non-platinum single agent chemotherapy (vinorelbine: VNR) but no response was obtained.

Treatment outcome of patients with stage IV disease

Although three patients (cases 8, 9, and 10) received platinum-based chemotherapy, no objective response was obtained. Salvage chemotherapy including paclitaxel (PTX), DCT, CPT-11, GEM, VNR, and amrubicin (AMR) was also ineffective. Five patients including two patients with stage IIIB disease (cases 6 and 7) were treated with gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, as second-line treatment. One patient (case 10) achieved a PR. Two patients (cases 11 and 12) received supportive care alone because of poor PS.

Discussion

We have described twelve patients with pulmonary LCNEC but the treatment outcomes were disappointing in the majority of patients. In general, pulmonary LCNEC has been treated according to the strategy for NSCLC since LCNEC is considered a variant of large cell carcinoma (Travis et al. 1999). The treatment of our twelve cases was also based on this strategy i.e., early stage cases were treated surgically, locally advanced cases were treated with chemotherapy and/or

radiotherapy and metastatic or relapsed cases with platinum-based systemic chemotherapy.

Our three patients with stage I-II disease underwent curative surgery but relapsed with distant metastases within 20 months. No patient received any adjuvant or neoadjuvant therapy. Although Dresler et al. reported that adjuvant chemotherapy did not improve survival (Dresler et al. 1997), Iyoda et al. demonstrated the effectiveness of adjuvant therapy for cases with stage I large cell carcinoma with neuroendocrine features (Iyoda et al. 2001a). In addition, Mazieres et al. and Cerilli et al. proposed the potential efficacy of neoadjuvant or adjuvant therapy (Mazieres et al. 2002; Cerilli et al. 2001). In view of a previous report that the prognosis of stage I LCNEC was poorer than that of stage I NSCLC (Takei et al. 2002), and a recent report that adjuvant chemotherapy was effective in early stage NSCLC (Arriagada et al. 2004), adjuvant therapy might be effective in cases of early stage LCNEC. One patient with stage IIA disease in the present study who did not undergo curative surgery because of poor pulmonary function achieved a durable response for 4 years or more following chemoradiotherapy. It might suggest the potential usefulness of neoadjuvant therapy.

There are no published prospective randomized trials that have evaluated chemotherapy regimens for LCNEC. Only a few published retrospective studies exist to date. We treated inoperable or relapsed cases with chemotherapy or chemoradiotherapy including 'new' agents developed in the 1990s such as PTX, DCT, CPT-11, GEM, VNR, and AMR. Two patients with locally advanced disease responded to concurrent chemoradiotherapy but no patient with stage IV disease responded to systemic chemotherapy.

There are several important issues regarding the treatment of LCNEC. First, should LCNEC be treated as SCLC or as NSCLC? The majority of patients with LCNEC have been treated as NSCLC but chemotherapy used for SCLC has also been performed for LCNEC because of the clinical similarity of LCNEC with SCLC (Dresler et al. 1997; Carretta et al. 2000). However, these previous studies have produced conflicting results. Some authors reported similar responsiveness of LCNEC with SCLC (Mitry and Rougier 2001) whilst others reported that LCNEC were less sensitive than SCLC (Mazieres et al. 2002). In our study, three patients responded to CDDP-based chemotherapy or chemoradiotherapy including CDDP, ETP or CPT-11. These agents are active against both SCLC (Noda et al. 2002) and NSCLC (Ueoka et al. 2001).

Second, there is the difficulty regarding the diagnosis of LCNEC. In our institutes, approximately 80% of the patients are diagnosed following analysis of specimens obtained by bronchoscopy. It is often difficult to diagnose LCNEC with the small specimen obtained by bronchoscopy and a surgically resected sample may be needed to definitively confirm the diagnosis. This may be an important reason why there are few reports regarding the treatment strategy for advanced LCNEC. In previous study, three (17%) of 18 cases confirmed LCNEC were diagnosed with small specimens (Mazieres et al. 2002). Only one patient (5%) in resected 20 cases was diagnosed by the specimen with bronchoscopy (Doddoli et al. 2004). Zacharias et al. and Paci et al. failed to diagnose LCNEC pre-operatively (Zacharias et al. 2003; Paci et al. 2004). This is due to the difficulty of immunohistochemical diagnosis with small specimens. Among 1,158 resected lung tumors, five cases (0.4%) were diagnosed as LCNEC with adenocarcinoma or squamous cell carcinoma component (Ruffini et al. 2002). Hage et al. reported that four (57%) of seven LCNEC cases also had a non-LCNEC component (Hage et al. 2003). Three (12%) of 25 cases pre-operatively diagnosed LCNEC were excluded after surgery because of heterogeneity (Zacharias et al. 2003). In addition, 41 cases (38%) of 107 cases were led to the discrepancy between the diagnosis of lung cancer with bronchoscopic and operative specimens (Chuang et al.1984). Therefore, if a neuroendocrine tumor is suspected, these specimens should be carefully examined following immunohistochemical staining with various neuroendocrine markers. In addition, measurement of serum CEA, CYFRA, NSE, and ProGRP levels may be useful as these markers were elevated in the majority of our patients although the number of patients examined was small. Previously, Iyoda et al. reported that the serum NSE level was elevated in 34.5% of LCNEC (Iyoda et al. 2001b) and elevated levels in 5.9–33.3% of NSCLC was also reported (Yamaguchi et al. 1995; Takada et al. 1996) whilst serum ProGRP level was elevated in 63–76% of SCLC and 1.4–14.4% of NSCLC (Yamaguchi et al. 1995; Takada et al. 1996; Miyake et al. 1994). Goto et al. also reported that serum ProGRP level was frequently elevated in NSCLC cases with neuroendocrine differentiation (Goto et al. 1998). These results indicate that the measurement of serum levels of neuroendocrine markers, especially ProGRP, may be useful in the diagnosis of LCNEC. The simultaneous measurement of neuroendocrine markers and CEA or CYFRA which were frequently elevated in patients with NSCLC might facilitate the differential diagnosis of LCNEC from NSCLC. As mentioned above, complexity in the diagnosis of LCNEC also indicates that there might be more cases of LCNEC among those with advanced lung cancers, with whom a resected whole tumor specimen is not available. Further evaluation of serum markers is required to assist diagnosis of advanced LCNEC.

Araki et al. examined the immunohistochemical expression of c-kit, EGFR and c-erbB-2 in surgically resected specimens from patients with LCNEC (Araki et al. 2003). Although c-kit was expressed in 55% of patients, EGFR was not overexpressed at all. In our report, one patient with LCNEC responded to gefitinib, although we did not check the expression and the somatic mutations of EGFR. Further studies are required to investigate the efficacy of gefitinib and imatinib mesylate, a c-kit receptor tyrosine kinase inhibitor, as well as bcr/abl, although there was an

unexpected result of the imatinib treatment for SCLC patient (Johnson et al. 2003).

In conclusion, we retrospectively analyzed clinical outcomes of patients with LCNEC. We have to evaluate the usefulness of adjuvant or neoadjuvant therapy in patients with early-stage disease and the efficacy of systemic chemotherapy including molecular targeting agents for patients with advanced disease. The prospective evaluation of treatment strategies similar as in SCLC is worthy of consideration. In addition, further studies to elucidate the molecular characteristics of LCNEC are urgently needed.

Acknowledgement We thank Drs. Tadashi Yoshino, Department of Pathology, Okayama University Graduate School of Medicine and Density, Okayama, Japan and Yoshihiro Matsuno, Clinical Laboratory Division, National Cancer Center Research Institute and Hospital, Tokyo, Japan, for reviewing the case 3 that is the first case recognized as LCNEC in our institute

References

Araki K, Ishii G, Yokose T, Nagai K, Funai K, Kodama K, Nishiwaki Y, Ochiai A (2003) Frequent overexpression of the c-kit protein in large cell neuroendocrine carcinoma of the lung. Lung Cancer 40:173–180

Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J (2004) Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. N Engl J Med 350:351–360

Carretta A, Ceresoli GL, Arrigoni G, Canneto B, Reni M, Cigala C, Zannini P (2000) Diagnostic and therapeutic management of neuroendocrine lung tumors: a clinical study of 44 cases. Lung Cancer 29:217–225

Cerilli LA, Ritter JH, Mills SE, Wick MR (2001) Neuroendocrine neoplasms of the lung. Am J Clin Pathol 116 [Supp l]:S65–96

Chuang MT, Marchevsky A, Teirstein AS, Kirschner PA, Kleinerman J (1984) Diagnosis of lung cancer by fibreoptic bronchoscopy: problems in the histological classification of non-small cell carcinomas. Thorax 39:175–178

Doddoli C, Barlesi F, Chetaille B, Garbe L, Thomas P, Giudicelli R, Fuentes P (2004) Large cell neuroendocrine carcinoma of the lung: an aggressive disease potentially treatable with surgery. Ann Thorac Surg 77:1168–1172

Dresler CM, Ritter JH, Patterson GA, Ross E, Bailey MS, Wick MR (1997) Clinical-pathologic analysis of 40 patients with large cell neuroendocrine carcinoma of the lung. Ann Thorac Surg 63:180–185

Goto K, Kodama T, Hojo F, Kubota K, Kakinuma R, Matsumoto T, Ohmatsu H, Sekine I, Nagai K, Nishiwaki Y (1998) Clinicopathologic characteristics of patients with nonsmall cell lung carcinoma with elevated serum progastrin-releasing peptide levels. Cancer 82:1056–1061

Hage R, Seldenrijk K, de Bruin P, van Swieten H, van den Bosch J (2003) Pulmonary large-cell neuroendocrine carcinoma (LCNEC). Eur J Cardiothorac Surg 23:457–460

Iyoda A, Hiroshima K, Toyozaki T, Haga Y, Fujisawa T, Ohwada H (2001a) Clinical characterization of pulmonary large cell neuroendocrine carcinoma and large cell carcinoma with neuroendocrine morphology. Cancer 91:1992–2000

Iyoda A, Hiroshima K, Toyozaki T, Haga Y, Baba M, Fujisawa T, Ohwada H (2001b) Adjuvant chemotherapy for large cell carcinoma with neuroendocrine features. Cancer 92:1108–1112

Jiang SX, Kameya T, Shoji M, Dobashi Y, Shinada J, Yoshimura H (1998) Large cell neuroendocrine carcinoma of the lung: a histologic and immunohistochemical study of 22 cases. Am J Surg Pathol 22:526–537

Johnson BE, Fischer T, Fischer B, Dunlop D, Rischin D, Silberman S, Kowalski MO, Sayles D, Dimitrijevic S, Fletcher C, Hornick J, Salgia R, Le Chevalier T (2003) Phase II study of imatinib in patients with small cell lung cancer. Clin Cancer Res 9:5880–5887

Mazieres J, Daste G, Molinier L, Berjaud J, Dahan M, Delsol M, Carles P, Didier A, Bachaud JM (2002) Large cell neuroendocrine carcinoma of the lung: pathological study and clinical outcome of 18 resected cases. Lung Cancer 37:287–292

Miller AB, Hoogstraten B, Staquet M, Winkler A (1981) Reporting results of cancer treatment. Cancer 47:207–214

Mitry E, Rougier P (2001) The treatment of undifferentiated neuroendocrine tumors. Crit Rev Oncol Hematol 37:47–51

Miyake Y, Kodama T, Yamaguchi K (1994) Pro-gastrin-releasing peptide (31–98) is a specific tumor marker in patients with small cell lung carcinoma. Cancer Res 54:2136–2140

Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S, Saijo N (2002) Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med 346:85–91

Paci M, Cavazza A, Annessi V, Putrino I, Ferrari G, De Franco S, Sgarbi G (2004) Large cell neuroendocrine carcinoma of the lung: a 10-year clinicopathologic retrospective study. Ann Thorac Surg 77:1163–1167

Ruffini E, Rena O, Oliaro A, Filosso PL, Bongiovanni M, Arslanian A, Papalia E, Maggi G (2002) Lung tumors with mixed histologic pattern. Clin Pathol Char Progn Sig 22:701–707

Sobin LH, Wittekind CH (1997) UICC: TNM Classification of malignant tumours, 5th edn. Wiley-Liss, New York

Takada M, Kusunoki Y, Masuda N, Matui K, Yana T, Ushijima S, Iida K, Tamura K, Komiya T, Kawase I, Kikui N, Morino H, Fukuoka M (1996) Pro-gastrin-releasing peptide (31–98) as a tumour marker of small-cell lung cancer: comparative evaluation with neuron-specific enolase. Br J Cancer 73:1227–1232

Takei H, Asamura H, Maeshima A, Suzuki K, Kondo H, Niki T, Yamada T, Tsuchiya R, Matsuno Y (2002) Large cell neuroendocrine carcinoma of the lung: a clinicopathologic study of eighty-seven cases. J Thorac Cardiovasc Surg 124:285–292

Travis WD, Linnoila RI, Tsokos MG, Hitchcock CL, Cutler GB Jr, Nieman L, Chrousos G, Pass H, Doppman J (1991) Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. An ultrastructural, immunohistochemical, and flow cytometric study of 35 cases. Am J Surg Pathol 15:529–553

Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E (1999) Histological typing of lung and pleural tumours, 3rd edn. Springer, Heidelberg Berlin New York

Ueoka H, Tanimoto M, Kiura K, Tabata M, Takigawa N, Segawa Y, Takata I, Eguchi K, Okimoto N, Harita S, Kamei H, Shibayama T, Watanabe Y, Hiraki S, Harada M (2001) Fractionated administration of irinotecan and cisplatin for treatment of non-small-cell lung cancer: a phase II study of Okayama Lung Cancer Study Group. Br J Cancer 85:9–13

Yamaguchi K, Aoyagi K, Urakami K, Fukutani T, Maki N, Yamamoto S, Otsubo K, Miyake Y, Kodama T (1995) Enzyme-linked immunosorbent assay of pro-gastrin-releasing peptide for small cell lung cancer patients in comparison with neuron-specific enolase measurement. Jpn J Cancer Res 86:698–705

Zacharias J, Nicholson AG, Ladas GP, Goldstraw P (2003) Large cell neuroendocrine carcinoma and large cell carcinomas with neuroendocrine morphology of the lung: prognosis after complete resection and systematic nodal dissection. Ann Thorac Surg 75:348–352

ANTICANCER RESEARCH 24: 3893-3898 (2004)

Cisplatin Down-regulates Topoisomerase I Activity in Lung Cancer Cell Lines

KEISUKE AOE[1,2], KATSUYUKI KIURA[1], HIROSHI UEOKA[1], MASAHIRO TABATA[1], MASAKAZU CHIKAMORI[1], HIROYUKI KOHARA[1,2], MINE HARADA[1] and MITSUNE TANIMOTO[1]

[1]*Department of Internal Medicine, Okayama University Medical School, Okayama;*
[2]*Department of Respiratory Medicine and Clinical Research, National Sanyo Hospital Respiratory Disease Center, Yamaguchi, Japan*

Abstract. *Many clinical studies have reported that irinotecan has reproducible antitumor activity against lung cancer. Both cisplatin and SN-38 are key drugs in the treatment of lung cancer, and their combination is one of the most promising regimens available. Using lung cancer cell lines, ABC-1 and SBC-3, we examined the cytotoxic effect of the schedule, as well as the effect of cisplatin on topoisomerase I activity. Cytotoxicity was determined by MTT assay. ABC-1 or SBC-3 cells were incubated with or without various concentrations of both drugs in 96-well microplates for 72 or 96 hours in a humidified 5% CO_2 atmosphere at 37°C. Synergism was evaluated by median-effect plot analysis and a combination index isobologram method by Chou and Talalay. After ABC-1 or SBC-3 cells had been exposed to 10 μM cisplatin for one hour, topoisomerase I activities were determined by supercoiled-DNA relaxation assay. Synergism was observed in ABC-1 and SBC-3 cells when cisplatin was given first, followed by SN-38 (7-ethyl-10-hydroxycamptothecin) and cisplatin. Topoisomerase I activity decreased at 1-2 hours after exposure to cisplatin and recovered gradually after 4-5 hours of cisplatin exposure in both ABC-1 and SBC-3 cells. Accordingly, pretreatment with cisplatin will have an impact on the sensitivity to SN-38.*

Irinotecan (7-ethyl-10- [4-(1-piperidyl)-1-piperidino] carbonyloxy-camptothecin) is a water- soluble camptothecin analog, which reversibly inhibits DNA topoisomerase I (1). Topoisomerase I inhibitors as single agents show excellent activity to a wide variety of tumors, especially lung cancer and colon cancer (2). In addition, topoisomerase I inhibitors may also interfere with DNA repair and enhance cytotoxicity when combined with DNA-damaging agents (3). In preclinical studies, the combination of irinotecan and cisplatin showed synergistic effects in several tumor cell lines (4, 5).

Clinical studies on the combination of irinotecan and cisplatin have been undertaken in a variety of tumors (6-10). In the majority of clinical trials, irinotecan was administered first followed by cisplatin, although the schedule dependency of this combination is still controversial. For the development of combination chemotherapy, the schedules as well as the appropriate combinations should be preclinically investigated. Thus, we studied the schedule dependency of the combination with cisplatin and SN-38, which is an active metabolite of irinotecan, and the effect of cisplatin on topoisomerase I activity.

Correspondence to: Keisuke Aoe, MD, PhD, Department of Respiratory Medicine and Clinical Research, National Sanyo Hospital Respiratory Disease Center, Ube, Yamaguchi 755-0241, Japan. Tel: +81-836-58-2300, Fax: +81-836-58-5219, e-mail: keisukeaoe@mtf.biglobe.ne.jp

Key Words: Topoisomerase I, cisplatin, irinotecan, lung cancer.

Materials and Methods

Chemicals. Cisplatin and 7-ethyl-10-hydroxycamptothecin (SN-38) were provided by Bristol-Myers Squibb K.K., Tokyo and Yakult Honsha Co., Ltd., Tokyo, Japan, respectively. SN-38 was dissolved in dimethyl sulfoxide. 3-[4,5-dimethyl-thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cell lines. SBC-3 (JCRB0818) and ABC-1 (JCRB0815) cells were established in our laboratory from patients with small cell lung cancer and adenocarcinoma of the lung, respectively (11-13). The cell lines were maintained in a humidified atmosphere with 5% CO_2 in air at 37°C, in RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS, GIBCO BRL) (RPMI-FBS).

Cytotoxicity assay. The cytotoxic activities of each drug and combination effects of two drugs were determined by MTT assay (14) with a slight modification, as described previously (13, 15). Briefly, 100 μl aliquots of RPMI-FBS containing serial concentrations of chemotherapeutic agents and cells (ABC-1: 3000 cells/well, SBC-3; 2000 cells/well) were plated in 96-well

0250-7005/2004 $2.00+.40

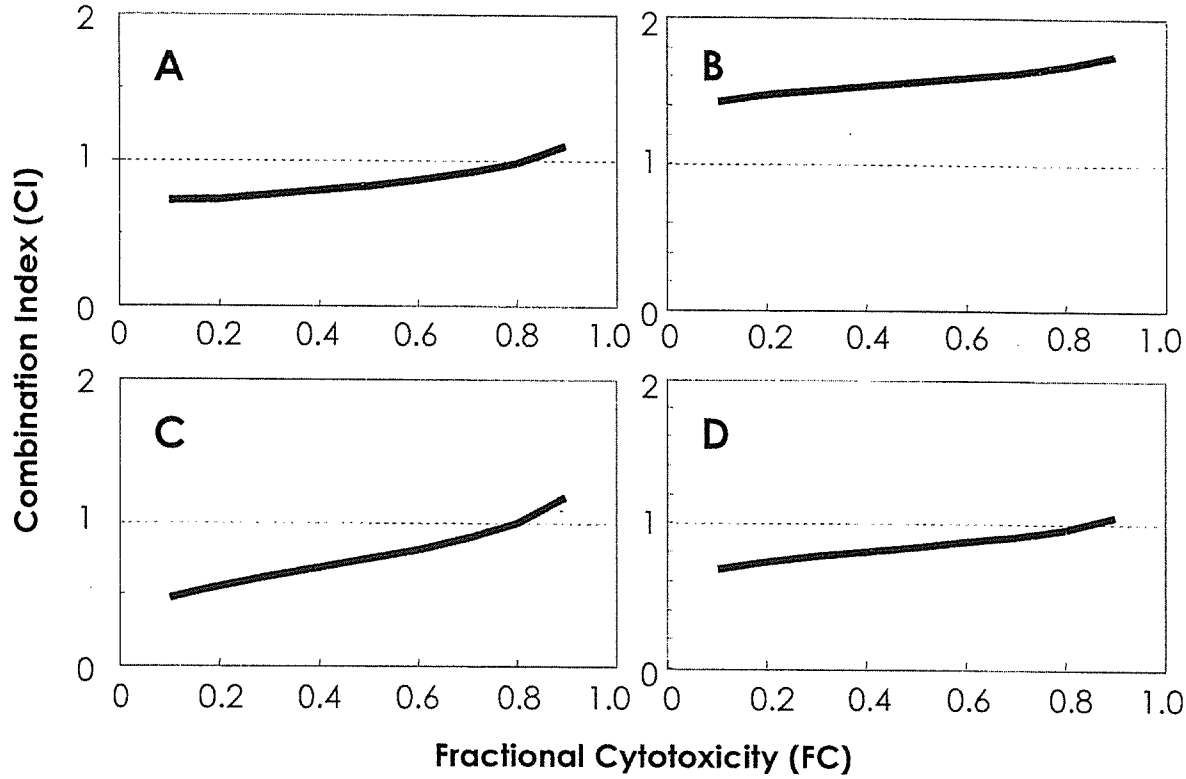

Figure 1. *A, B, C and D: Combination index isobolograms in combination with cisplatin and SN-38. Combination index (CI) < 1; synergy, CI=1; summation, CI>1; antagonism. A. ABC-1 cells: cisplatin -> SN38. B. ABC-1 cells, SN38 -> cisplatin. C. SBC-3 cells, cisplatin -> SN38. D. SBC-3 cells, SN38 -> cisplatin.*

flat-bottomed microplates and incubated at 37°C for 72 or 96 hours in a humidified atmosphere with 5% CO_2 in air. Cisplatin or SN-38 was administered for 24 hours before concurrent exposure. Then, the cells were concurrently exposed to cisplatin and SN-38 for 72 hours. MTT formazan was dissolved in fresh isopropanol. The absorbance at 560 nm was measured using a microplate reader (Model 3550; Bio-Rad Laboratories, Richmond, CA, USA). Percent growth inhibition was defined as percent absorbance inhibition within appropriate absorbance in each cell line. All experiments were repeated at least twice. The combination effects were determined by median-effect plot analysis and combination index isobolograms (16-18). Combination index (CI) < 1 indicates synergy, CI=1, summation and CI>1, antagonism.

Preparation of nuclear extract. ABC-1 or SBC-3 cells were exposed to cisplatin at a final concentration of 10 μM for one hour. After washing, the cells were resuspeneded in fresh RPMI-FBS. After 1, 2, 3, 4, 5, 6 and 14(15)-hour incubations with cisplatin, the cells were collected and a nuclear extract from the ABC-1 and SBC-3 cells was prepared, as described previously (15). The total protein concentration was determined by Bio-Rad protein assay kit.

Topoisomerase I activity assay. Nuclear extract (0.4 μg) was added to the topoisomerase I reaction mixture containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol and 0.75 μg supercoiled DNA plasmid at a final volume of 20 μl. Following incubation at 37°C for 15 minutes, the reaction was terminated by adding 5 μl of stopping buffer (final concentration; 1% Sarkosyl, 0.025% bromophenol blue and 5% glycerol). The reaction products were analyzed by electrophoresis on 0.8% agarose gel using a TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 62 mM EDTA) at 1 V/cm, stained by ethidium bromide (0.5 μg/ml) and photographed using a short wavelength UV lamp.

Results

Cytotoxicity assay and combination effects. The combination index isobolograms of the schedule-dependent interaction between cisplatin and SN-38 in ABC-1 and SBC-3 cells are shown in Figures 1A, B, C and D. In ABC-1 cells, the combination index showed lower than 1.0 in a wide range of fractional cytotoxicity when cisplatin was given first followed by SN-38 and cisplatin (Figure 1A). On the other hand, the combination index was higher than 1.5 in a wide range of fractional cytotoxicity when SN-38 was given first followed by SN-38 and cisplatin in ABC-1 cells (Figure 1B). This combination showed synergism only when cisplatin was given first in ABC-1 cells.

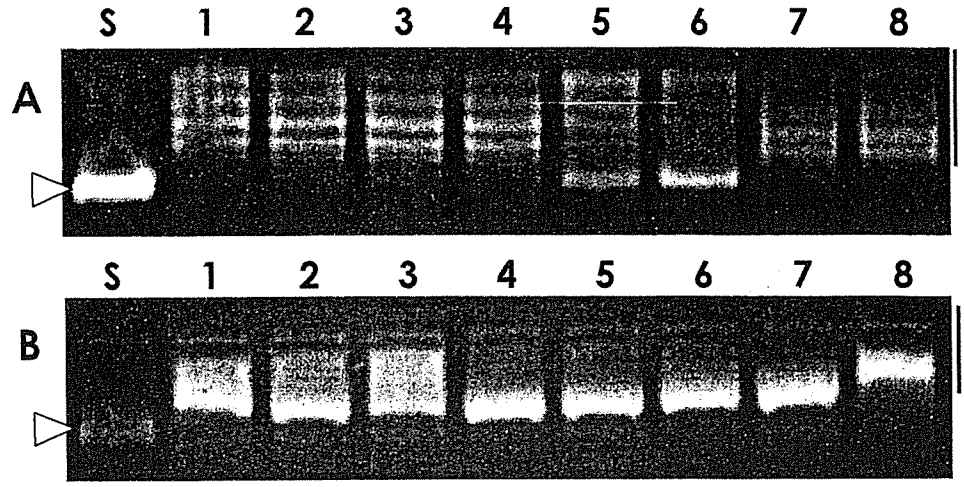

Figure 2. *A and B: Topoisomerase I activity of ABC-1 cells after cisplatin exposure. Supercoiled DNA (lane S), time after cisplatin exposure: 15h (lane 1), 6h (lane 2), 5h (lane 3), 4h (lane 4), 3h (lane 5), 2h (lane 6), and 1h (lane 7), and no exposure (lane 8). B. Topoisomerase I catalytic activity of SBC-3 cells after cisplatin exposure. Supercoiled DNA (lane S, white arrowhead), time after cisplatin exposure: 14h (lane 1), 6h (lane 2), 5h (lane 3), 4h (lane 4), 3h (lane 5), 2h (lane 6), and 1h (lane 7), and no exposure (lane 8). Supercoiled DNA and relaxed DNA are indicated with white arrowheads and bars, respectively.*

In SBC-3 cells, the combination index was lower than 1.0 in a wide range of fractional cytotoxicity without association of exposure sequence (Figures 1C and D). The combination index was lower when cisplatin was given first followed by SN-38 and cisplatin.

Topoisomerase I activity assay. Untreated ABC-1 and SBC-3 cells showed clear topoisomerase I activity, because relaxed DNA bands and no supercoiled DNA were observed (lane 8 in Figures 2A and B). As shown in Figure 2A, the topoisomerase I activity of ABC-1 cells did not change at one hour after cisplatin exposure (lane 7). Supercoiled DNA bands appeared at 2-3 hours after cisplatin exposure (lanes 5 and 6) and disappeared gradually after 4-5 hours of cisplatin exposure (lanes 1-4). Figure 2B shows the topoisomerase I activity of SBC-3 cells. Supercoiled DNA bands appeared at one hour after cisplatin exposure (lane 7), which suggests a rapid decrease in topoisomerase I activity. Relaxed DNA appeared at 5 hours after cisplatin exposure (lane 3). Thus, in both cell lines topoisomerase I activity began to decrease at 1-2 hours after cisplatin exposure and recovered gradually after 4-5 hours of cisplatin exposure.

Discussion

We found that pretreatment with cisplatin augmented the sensitivity to the combination of SN-38 and cisplatin in ABC-1 and SBC-3 cells. Pretreatment with SN-38 also enhanced sensitivity in SBC-3 cells, but did not in ABC-1 cells. Accordingly, pretreatment with cisplatin might have a great impact on the tumor cell sensitivity to irinotecan and cisplatin.

In general, tumor cells with high topoisomerase I activity are sensitive to topoisomerase I inhibitors (20, 21). Thus, we expected the increase of topoisomerase I activity after cisplatin exposure. Surprisingly, the topoisomerase I activity of both cell lines began to decrease at 1-2 hours after cisplatin exposure, but gradually recovered after 4-5 hours of cisplatin exposure. Topoisomerase I activity was down-regulated and recovered during the time course after cisplatin exposure. We presume that the topoisomerase I enzyme is transiently consumed by its binding to DNA intercalated or intracalated by cisplatin. The consumption of topoisomerase I might contribute to the synergy when cisplatin is given first. These changes of topoisomerase I activity are also observed after irradiation exposure. Boothman *et al.* (21) reported that topoisomerase I inhibitors augmented the radiation effect, although topoisomerase I activity diminished immediately after irradiation.

The relationship between cisplatin and topoisomerase I activity is very complex. Although topoisomerase I activity or mRNA was higher in four cisplatin-resistant cell lines than their respective parent cell lines, two cell lines showed collateral sensitivity to topoisomerase I inhibitors (22, 23), which the other two cell lines did not (23, 24). In addition, two cisplatin-resistant cell lines showed cross-resistance to SN-38 without an alteration of topoisomerase I activity (25, 26). The relationship between the effect of cisplatin on topoisomerase I activity and the sensitivity to topoisomerase

I inhibitors is still unclear. The sensitivity to topoisomerase I inhibitors might be controlled by multifactors such as single-mutations in the topoisomerase I enzyme (27), topoisomerase I activity (19, 20), P-glycoprotein-associated multidrug resistance (28, 29) and intercellular drug accumulation (26, 30). Accordingly, we could not explain the mechanisms of synergy in terms only of topoisomerase I activity.

A synergistic effect on tumor cells *in vitro* might induce serious adverse reactions *in vivo*. In Japan, combination chemotherapy with cisplatin and irinotecan is the standard chemotherapy for NSCLC and extensive-stage SCLC (31, 32). de Jonge *et al.* have reported that the toxicity of the combination of irinotecan and cisplatin is schedule-independent (33); we expected a stronger synergistic effect in tumor cells than that in normal cells and conducted a new regimen of cisplatin and docetaxel on day 1 and irinotecan on day 2 (34).

In conclusion, pretreatment with cisplatin indicated the sensitivity of cancer cells to SN-38, a metabolite of irinotecan. This is a promising effect which should be further investigated *in vivo*.

References

1 Hsiang YH and Liu LF: Identification of mammalian DNA topoisomerase I as an intracellular target of the antitumor drug camptothecin. Cancer Res *48*: 1722-1726, 1988.

2 de Jonge MJ, Sparreboom A and Verweij J: The development of combination therapy involving camptothecins: A review of preclinical and early clinical studies. Cancer Treat Rev *24*: 205-220, 1998.

3 Boothman DA, Trask DK and Pardee AB: Inhibition of potentially lethal DNA damage repair in human tumor cells by beta-Lapachone, an activator of topoisomerase I. Cancer Res *49*: 605-612, 1989.

4 Ma J, Maliepaard M, Nooter K, Boersma AW, Verweij J, Stoter G and Schellens JH: Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in panel of eight solid-tumor cell lines *in vitro*. Cancer Chemother Pharmacol *41*: 307-316, 1998.

5 Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S and Miura Y: Effects of CPT-11 in combination with other anti-cancer agents in culture. Int J Cancer *50*: 604-610, 1992.

6 Masuda N, Fukuoka M, Takada M, Kusunoki Y, Negoro S, Matsui K, Kudoh S, Takihuji N, Nakagawa K and Kishimoto S: CPT-11 in combination with cisplatin for advanced non-small-cell lung cancer. J Clin Oncol *10*: 1775-1780, 1992.

7 Shirao K, Shimada Y, Kondoh H, Saito D, Yamao T, Ono H, Yokoyama T, Fukuda H, Oka M, Watanabe Y, Ohtsu A, Boku N, Fujii T, Oda Y, Muro K and Yoshida S: Phase I-II study of irinotecan hydrochloride combined with cisplatin in patients with advanced gastric cancer. J Clin Oncol *15*: 921-927, 1997.

8 Ueoka H, Tabata M, Kiura K, Shibayama T, Gemba K, Segawa Y, Chikamori K, Yonei T, Hiraki S and Harada M: Fractionated administration of irinotecan and cisplatin for treatment of lung cancer: A phase I study. Br J Cancer *79*: 984-990, 1998.

9 Saltz LB, Spriggs D, Schaaf LJ, Schwartz GK, Ilson D, Kemeny N, Kanowitz J, Steger C, Eng M, Albanese P, Semple D, Hanover CK, Elfring GL, Miller LL and Kelsen D: Phase I clinical and pharmacologic study of weekly cisplatin combined with weekly irinotecan in patients with advanced solid tumors. J Clin Oncol *16*: 3858-3865, 1998.

10 Kudoh S, Fujiwara Y, Takada Y, Yamamoto H, Kinoshita A, Ariyoshi Y, Furuse K and Fukuoka M: Phase II study of irinotecan combined with cisplatin in patients with previously untreated small-cell lung cancer. J Clin Oncol *16*: 1068-1074, 1998.

11 Watanabe Y: Experimental model of human lung cancer. Part I. Establishment and characterization of new tissue culture cell lines from human squamous cell carcinoma and adenocarcinoma of the lung. Okayama Igakukai Zasshi *97*: 691-700, 1985.

12 Kishimoto N: Studies on cell biology and chemotherapy of lung cancer using tissue culture techniques Part 2. Biological characteristics of five newly established small cell lung cancer cell line. Okayama Igakukai Zasshi *104*: 905-913, 1992.

13 Kiura K, Ohnoshi T, Ueoka H, Takigawa N, Tabata M, Segawa Y, Shibayama T and Kimura I: An adriamycin-resistant subline is more sensitive than the parent human small cell lung cancer cell line to lonidamine. Anticancer Drug Des *7*: 463-470, 1992.

14 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65*: 55-63, 1983.

15 Takigawa N, Ohnoshi T, Ueoka H, Kiura K and Kimura I: Establishment and characterization of an etoposide-resistant human small cell lung cancer cell line. Acta Med Okayama *46*: 203-212, 1992.

16 Chou TC: On the determination of availability of ligand binding sites in steady-state systems. J Theor Biol *65*: 345-356, 1977.

17 Chou TC and Talalay P: Generalized equation for the analysis of inhibitors of Michaelis- Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur J Biochem *115*: 207-216, 1981.

18 Aoe K, Kiura K, Ueoka H, Tabata M, Matsumura T, Chikamori M, Matsushita A, Kohara H and Harada M: Effect of docetaxel with cisplatin or vinorelbine on lung cancer cell lines. Anticancer Res *19(1A)*: 291-299, 1999.

19 Boothman DA, Fukunaga N and Wang M: Down-regulation of topoisomerase I in mammalian cells following ionizing radiation. Cancer Res *54*: 4618-4626, 1994.

20 Jansen WJ, Zwart B, Hulscher ST, Giaccone G, Pinedo HM and Boven E: CPT-11 in human colon-cancer cell lines and xenografts: characterization of cellular sensitivity determinants. Int J Cancer *70(3)*: 335-340, 1997.

21 Jansen WJ, Kolfschoten GM, Erkelens CA, Van Ark-Otte J, Pinedo HM and Boven E: Anti-tumor activity of CPT-11 in experimental human ovarian cancer and human soft-tissue sarcoma. Int J Cancer *73(6)*: 891-896, 1997.

22 Kotoh S, Naito S, Yokomizo A, Kumazawa J, Asakuno K, Kohno K and Kuwano M: Increased expression of DNA topoisomerase I gene and collateral sensitivity to camptothecin in human cisplatin-resistant bladder cancer cells. Cancer Res *54(12)*: 3248-3252, 1994.

23 Minagawa Y, Kigawa J, Irie T, Kanamori Y, Itamochi H, Cheng X and Terakawa N: Enhanced topoisomerase I activity and increased topoisomerase II alpha content in cisplatin-resistant cancer cell lines. Jpn J Cancer Res *88(12)*: 1218-1223, 1997.

24 Moritaka T, Kiura K, Ueoka H, Tabata M, Segawa Y, Shibayama T, Takigawa N, Ohnoshi T and Harada M: Cisplatin-resistant human small cell lung cancer cell line shows collateral sensitivity to vinca alkaloids. Anticancer Res *18(2A)*: 927-933, 1998.

25 Niimi S, Nakagawa K, Sugimoto Y, Nishio K, Fujiwara Y, Yokoyama S, Terashima Y and Saijo N: Mechanism of cross-resistance to a camptothecin analogue (CPT-11) in a human ovarian cancer cell line selected by cisplatin. Cancer Res *52(2)*: 328-333, 1992.

26 Chen ZS, Sumizawa T, Furukawa T, Ono K, Tani A, Komatsu M and Akiyama S: An enhanced active efflux of CPT-11 and SN-38 in cisplatin-resistant human KB carcinoma cells. Cancer Lett *138(1-2)*: 13-22, 1999.

27 Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunoki Y, Takemoto Y and Okada K: Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc Natl Acad Sci USA *84(16)*: 5565-5569, 1987.

28 Jansen WJ, Hulscher TM, van Ark-Otte J, Giaccone G, Pinedo HM and Boven E: CPT-11 sensitivity in relation to the expression of P170-glycoprotein and multidrug resistance - associated protein. Br J Cancer *77(3)*: 359-365, 1998.

29 Chen ZS, Furukawa T, Sumizawa T, Ono K, Ueda K, Seto K and Akiyama SI: ATP-dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. Mol Pharmacol *55(5)*: 921-928, 1999.

30 Ma J, Maliepaard M, Nooter K, Loos WJ, Kolker HJ, Verweij J, Stoter G and Schellens JH: Reduced cellular accumulation of topotecan: a novel mechanism of resistance in a human ovarian cancer cell line. Br J Cancer *77(10)*: 1645-1652, 1998.

31 Negoro S, Masuda N, Takada Y, Sugiura T, Kudoh S, Katakami N, Ariyoshi Y, Ohashi Y, Niitani H and Fukuoka M: CPT-11 Lung Cancer Study Group West: Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. Br J Cancer *88(3)*: 335-341, 2003.

32 Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S and Saijo N: Japan Clinical Oncology Group. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med *346(2)*: 85-91, 2002.

33 de Jonge MJ, Verweij J, Planting AS, van der Burg ME, Stoter G, de Boer-Dennert MM, de Bruijn P, Brouwer E, Vernillet L and Sparreboom A: Drug-administration sequence does not change pharmacodynamics and kinetics of irinotecan and cisplatin. Clin Cancer Res *5(8)*: 2012-2017, 1999.

34 Kiura K, Ueoka H, Fujimoto N, Takata I, Nagata T, Kunisada K, Katayama H, Mimoto J, Nogami N, Hiraki A and Harada M: Phase I study of cisplatin, docetaxel and irinotecan for advanced non-small cell lung cancer (NSCLC). Proc Am Ass Cancer Res *41*: 692, 2000.

Received April 13, 2004
Revised September 15, 2004
Accepted October 15, 2004

ANTICANCER RESEARCH *24*: 3911-3916 (2004)

Establishment of a 7-Ethyl-10-hydroxy-camptothecin-resistant Small Cell Lung Cancer Cell Line

MASAKAZU CHIKAMORI[1], NAGIO TAKIGAWA[2], KATSUYUKI KIURA[1], MASAHIRO TABATA[1], TAKUO SHIBAYAMA[2], YOSHIHIKO SEGAWA[1], HIROSHI UEOKA[1,3], TAISUKE OHNOSHI[1*] and MITSUNE TANIMOTO[1,3]

[1]*Second Department of Internal Medicine, Okayama University Medical School (Division of Thoracic Oncology, Department of Respiratory Medicine, Okayama University Hospital), 2-5-1 Shikata-cho, Okayama 700-8558;*
[2]*Department of Internal Medicine, National Minami-Okayama Hospital, 4066 Hayashima, Tsukubo, Okayama 701-0304;*
[3]*Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

Abstract. *Irinotecan is one of the most active drugs used in the treatment of small cell lung cancer (SCLC). 7-Ethyl-10-hydroxy-camptothecin (SN-38) is an active metabolite of irinotecan. We established an SN-38-resistant subline (SBC-3/SN-38) by continuous exposure of SN-38 to a human SCLC cell line, SBC-3. Using the 3-[4, 5-dimethyl-thiazol-2-yl] 2, 5-diphenyltetrazolium bromide assay, we evaluated the cytotoxicity of 17 anticancer agents. The SBC-3/SN-38 cells were 73-fold more resistant than the parental SBC-3 cells to SN-38 and showed cross-resistance not only to topoisomerase (topo) I inhibitors (irinotecan and topotecan), but also to topo II inhibitors (adriamycin and etoposide), antimicrotubule agents (vincristine, vindesine, vinorelbine and docetaxel), alkylating agents (cyclophosphamide and ifosfamide), platinum (cisplatin and carboplatin) and antifolate (methotrexate). Interestingly, the resistant subline reserved the sensitivity to bleomycin and 5-fluorouracil. The SBC-3/SN-38 cells had decreased topo I and II activity compared to the parent cells. The SN-38-resistant cell line, SBC-3/SN-38, will be useful to elucidate the mechanism of action of the topo I inhibitors.*

The role of chemotherapy in the treatment of small cell lung cancer (SCLC) was established in the past decade. More than 80% of patients receiving current intensive chemotherapy regimens achieve an objective response. However, most responders eventually relapse and less than 20% survive longer than 3 years (1). The development of drug resistance in tumor cells is assumed to play a major role in these disappointing outcomes (2).

Irinotecan is a semi-synthetic analogue of camptothecin. In serum and tumor cells, it is converted to an active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), which has a specific mechanism of action *via* inhibition of a nuclear enzyme, topoisomerase (topo) I (3, 4). Irinotecan has exerted high activity as a single agent for SCLC (5). A recent phase III study, in extensive disease (ED) SCLC, demonstrated that a combination cisplatin and irinotecan regimen yielded a highly significant improvement in survival over the standard cisplatin and etoposide regimen (6). Accordingly, the combination is considered the standard treatment for ED SCLC. Even using a combination of irinotecan and cisplatin, the median survival and two-year survival rate were only 12.8 months and 19.5%, respectively. The emergence of irinotecan resistance has become a concern in patients with refractory ED SCLC.

Our objectives were to elucidate the mechanism of resistance to irinotecan by establishing an SN-38-resistant human SCLC cell line and to find anticancer agents to overcome the resistance.

Materials and Methods

Chemicals and reagents. The drugs used in this study were provided by the following sources: irinotecan and SN-38 from Yakult Honsha, Tokyo, Japan; topotecan from Smithcline Beecham, Tokyo, Japan; etoposide and carboplatin from Britol-Myers Sqibb, Tokyo, Japan; cisplatin and bleomycin from Nippon Kayaku Kogyo Co., Ltd, Tokyo, Japan; docetaxel from Rhone-Poulenc Rorer,

* Died on October 8, 1996

Correspondence to: Katsuyuki Kiura, M.D., Ph.D., Division of Thoracic Oncology, Department of Respiratory Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Tel: +81-86-235-7229, Fax: +81-86-232-8226, e-mail: kkiura@md.okayama-u.ac.jp

Key Words: Irinotecan, small cell lung cancer, drug resistance, topoisomerase.

0250-7005/2004 $2.00+.40

Antony, France; adriamycin, 5-fluorouracil, mitomycin C and vinorelbine from Kyowa Hakko Kogyo, Tokyo, Japan; active metabolite of cyclophosphamide: 4-hydroperoxycyclophosphamide (4-HC), active metabolite of ifosfamide: 4-hydroperoxyifosfamide (4-HI), vincristine and vindesine from Shionogi & Co., Ltd. Osaka, Japan; methotrexate from Lederle, Tokyo, Japan. 3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St Louis, MO, USA.

Cell cultures. The parent cell line, the SBC-3 cell line, was established from the bone marrow aspirate of a previously untreated SCLC patient (7). The growth medium (RPMI-FBS) was RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin-G (100 units/ml) and streptomycin (100 μg/ml). The SN-38-resistant cell line was established by continuous exposure of the SBC-3 cell line to increasing concentrations of SN-38, with subsequent cloning procedures. Initially, the SBC-3 cells were cultured continuously in RPMI-FBS containing 0.1nM SN-38. The drug concentration was gradually increased every 2 to 4 weeks. Finally, the cells growing vigorously in medium containing 10nM SN-38 were obtained 24 months later. Two weeks later, growing colonies were harvested and distributed in 24-multiwells; the cells were allowed to grow in a T25 tissue culture flask. The SN-38-resistant cell line was designated as SBC-3/SN-38.

Assay of drug sensitivity. Drug sensitivity was determined by MTT assay (8). Briefly, 50μl of RPMI-FBS containing serial concentrations of each chemotherapeutic agent was prepared in 96-well flat-bottomed microplates (Coster, Cambridge, MA, USA). Then 50μl of RPMI-FBS containing 2,000 cells for SBC-3 and 5,000 cells for SBC-3/SN-38 was added to each well. The cells were incubated for 96 h in a highly humidified incubator with 5% CO_2 and 95% air. Then 50μl of MTT (5 mg/ml phosphate-buffered saline [PBS], pH 7.2) was added to each well. After incubation at 37°C for 4 h, 125μl of fresh isopropanol with 0.04 M HCl was added to each well. The 96-well microplates were vigorously shaken by the Direct Mix Model TS-50 (Thermal Kagaku Sangyo, Tokyo, Japan). Absorbance of each well was measured at 560nm with the model 3550 microplate reader (Bio-Rad laboratories, Richmond, CA, USA). The absorbance of a well without chemotherapeutic agents was used as the control, while that of a well containing only RPMI-FBS, MTT and isopropanol was used as the background. The percent of surviving cells was calculated by the following formula: [(mean absorbance in four test wells - absorbance in background wells) / (mean absorbance in control wells - absorbance in background wells)] x 100. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of drug concentration *versus* the percent of surviving cells. Determinations were carried out in quadruplicate in each experiment and the results were confirmed by three or more separate experiments. Relative resistance was calculated by dividing the IC_{50} value of the SBC-3/SN-38 cells by the IC_{50} value of the SBC-3 cells.

Cell growth rate. The growth rate of cells was determined using the MTT assay. Cells growing in the exponential phase were seeded in 96-well microplates. The doubling-time of each cell line was estimated from the time-course of cell increments, determined by measuring the mean absorbance of 8 wells for 7 successive days (9).

Table I. *Drug sensitivity in the parent (SBC-3) and the SN-38-resistant cell lines (SBC-3/SN-38).*

	IC_{50} value (nM; mean±SD)		Relative resistance value (mean±SD)	*P*
	SBC-3	SBC-3/SN-38		
SN-38	0.83±0.11	60±9.6	73±11	0.001
Irinotecan	21±4.2	570±120	27±2.8	0.014
Topotecan	4.2±0.075	130±5.1	32±0.68	0.001
Adriamycin	20±1.6	68±8.8	3.4±0.50	0.011
Etoposide	110±36	580±260	5.5±1.6	0.071
Vincristine	1.6±0.50	5.1±1.4	3.2±0.9	0.039
Vindesine	1.1±0.18	2.6±0.80	2.5±1.1	0.111
Vinorelbine	3.8±0.99	8.7±2.7	2.3±0.14	0.038
Docetaxel	0.75±0.14	1.3±0.015	1.8±0.30	0.016
4-HC	1000±270	1500±457	1.5±0.098	0.049
4-HI	1300±46	1900±83	1.4±0.11	0.018
Cisplatin	390±67	860±43	2.3±0.68	0.026
Carboplatin	4200±940	9900±3200	2.4±0.96	0.111
Mitomycin C	39±6.0	69±22	1.8±0.38	0.129
Bleomycin	39±4.8	32±8.8	0.81±0.13	0.096
Methotrexate	20±0.71	92±7.2	4.6±0.20	0.003
5-fluorouracil	2600±660	3100±750	1.2±0.39	0.331

IC_{50}: 50% inhibitory concentration, SD: standard deviation, relative resistance value (IC_{50} value of SBC-3/SN-38 cells / IC_{50} value of SBC-3 cells) was calculated from each experiment. 4-HC: 4-hydroperoxycyclophosphamide, 4HI: 4-hydroperoxyifosfamide. *P*-value is evaluated using paired Student's *t*-test.

Intracellular glutathione and glutathione-S-transferase-π. Cells in the exponential growth phase were washed 3 times in cold PBS and sonicated with a 30-min burst using a Bioruptor (model UC100-D; Olympus, Tokyo, Japan). The glutathione (GSH) and glutathione-S-transferase-π (GST-π) concentration in the supernatant were determined after centrifuging the sonicates at 7,000g for 5 min. GSH was assayed by the method reported by Tietze (10) while GST-π was assayed using a GST-π EIA kit (Dainihon Seiyaku, Osaka, Japan). GSH and GST-π concentration were expressed as the ratio to mg protein determined by the method of Bradford (11).

Flow cytometry. A monoclonal antibody against P-glycoprotein, MRK16 was kindly provided by Dr. Tsuruo, the Applied Microbial Institute, the Tokyo University, Japan. As a negative control, mouse IgG2a was used. The cells were stained as described previously. Flow cytometric analysis was performed on a FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data were analyzed according to Consort 30 software (Becton Dickinson Immunocytometry Systems).

DNA topo activity. Crude nuclear extract was prepared according to the method of Tsutsui *et al.* (12). DNA topo I activity was determined as described by Tsutsui *et al.* (12). Plasmid DNA pBR322 was kindly provided by Dr. Tsutsui. The reaction proceeded at 30°C for 40 min in a 20μl mixture containing 10mM Tris-HCl, 0.1M NaCl, 1mM EDTA (pH 8.0), 0.5 μg of pBR322 DNA and 1μl of nuclear extract. The mixture was then treated with 0.66% SDS and 0.3mg/proteinase K prior to the analysis of DNA products by 0.8% agarose gel electrophoresis. The gels were

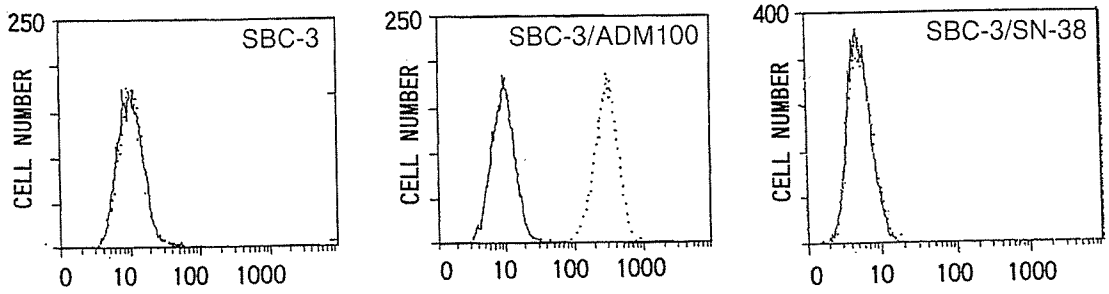

Figure 1. *Expression of P-glycoprotein in the SBC-3, SBC-3/ADM100 and SBC-3/SN-38 cells was analyzed by flow cytometry. A solid line represents a fluorescence histogram by control antibody (mouse IgG2a), while a dotted line represents a fluorescence histogram by MRK16 monoclonal antibody.*

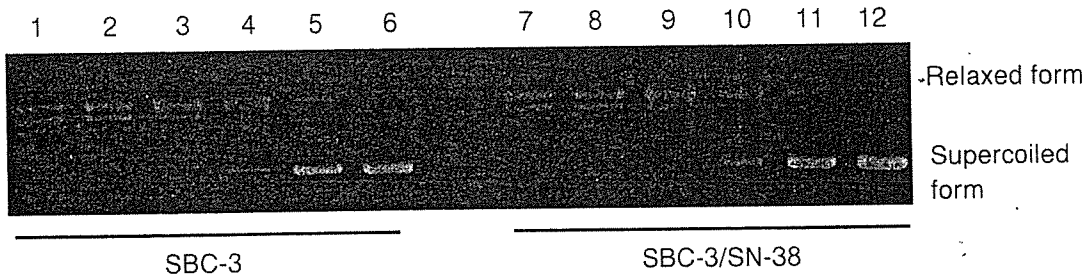

Figure 2. *DNA topo I activity determined by relaxation assay of pBR322 showing a decreased topo I activity in the SBC-3/SN-38. The amount of nuclear extracts was 3 μg for lanes 1 and 7, 1.5 μg for lanes 2 and 8, 0.75 μg for lanes 3 and 9, 0.375 μg for lanes 4 and 10, 0.18 μg for lanes 5 and 11, and 0.09 μg for lanes 6 and 12. Lanes 1-6: SBC-3; lanes 7-12: SBC-3/SN-38.*

stained with 0.5 μg/ml ethidium bromide and photographed under UV light. DNA topo II activity was assayed according to a modified technique described by Miller *et al.* (13). Kinetoplast DNA (kDNA) was also kindly provided by Dr. Tsutsui. After incubation in a total of 20-μl mixtures containing 50mM Tris-HCl (pH 8.0), 120mM KCl, 10mM $MgCl_2$, 0.5mM dithiothreitol, 0.5mM EDTA (pH 8.0), 0.5mM ATP, 30 μg/ml BSA, 0.5 μg of kDNA and 1μl of nuclear extract at 30°C for 40 min, the reaction mixture was electrophoresed. The gels were stained and photographed as mentioned above.

Statistical analysis. Values are given as means ± standard deviation. Statistical analyses were performed using the SPSS Base System™ programs (SPSS, Chicago, IL, USA). The significance of difference between two paired groups was determined by the Student's *t*-test. *P*-values less than 0.05 in two-tailed analyses were considered significant.

Results

The IC_{50} and relative resistance to 17 anticancer agents of SBC-3 and SBC-3/SN-38 cells are shown in Table I. The SBC-3/SN-38 cells were 73-fold more resistant than the SBC-3 cells to SN-38 and showed high cross-resistance: 27-fold to irinotecan and 32-fold to topotecan. The resistant subline revealed moderate cross-resistance to topo II inhibitors (adriamycin and etoposide), to antimicrotubule agents (vincristine, vindesine, vinorelbine and docetaxel), to platinum (cisplatin and carboplatin), to mitomycin C and to methotrexate. The SBC-3/SN-38 cells were less, but significantly cross-resistant to 4-HC (1.5-fold) and 4-HI (1.4-fold). The SBC-3/SN-38 cells retained the sensitivity to bleomycin and 5-fluorouracil.

The doubling-time of the SBC-3/SN-38 cells, 23.6 h, was close to that of the SBC-3 cells, 21.6 h. The intracellular GST-π was similar in the two cell types (4.24 μg/mg protein for the SBC-3 cells and 4.39 μg/mg protein for the SBC-3/SN-38 cells). Intracellular GSH was undetectable in both cell lines. Although P-glycoprotein was demonstrated in the SBC-3/ADM100 cells as positive control, it was not detected in the SBC-3/SN-38 cells (Figure 1).

The appearance of a relaxed DNA band and the disappearance of supercoiled forms are regarded as evidence for an adequate topo I activity in the nuclear extracts. In this experiment (Figure 2), supercoiled forms disappeared in the presence of nuclear extracts of over 0.375 μg of the SBC-3 (lanes 1-4) and over 0.75 μg of the SBC-3/SN-38 (lanes 7-9). Accordingly, the topo I activity of