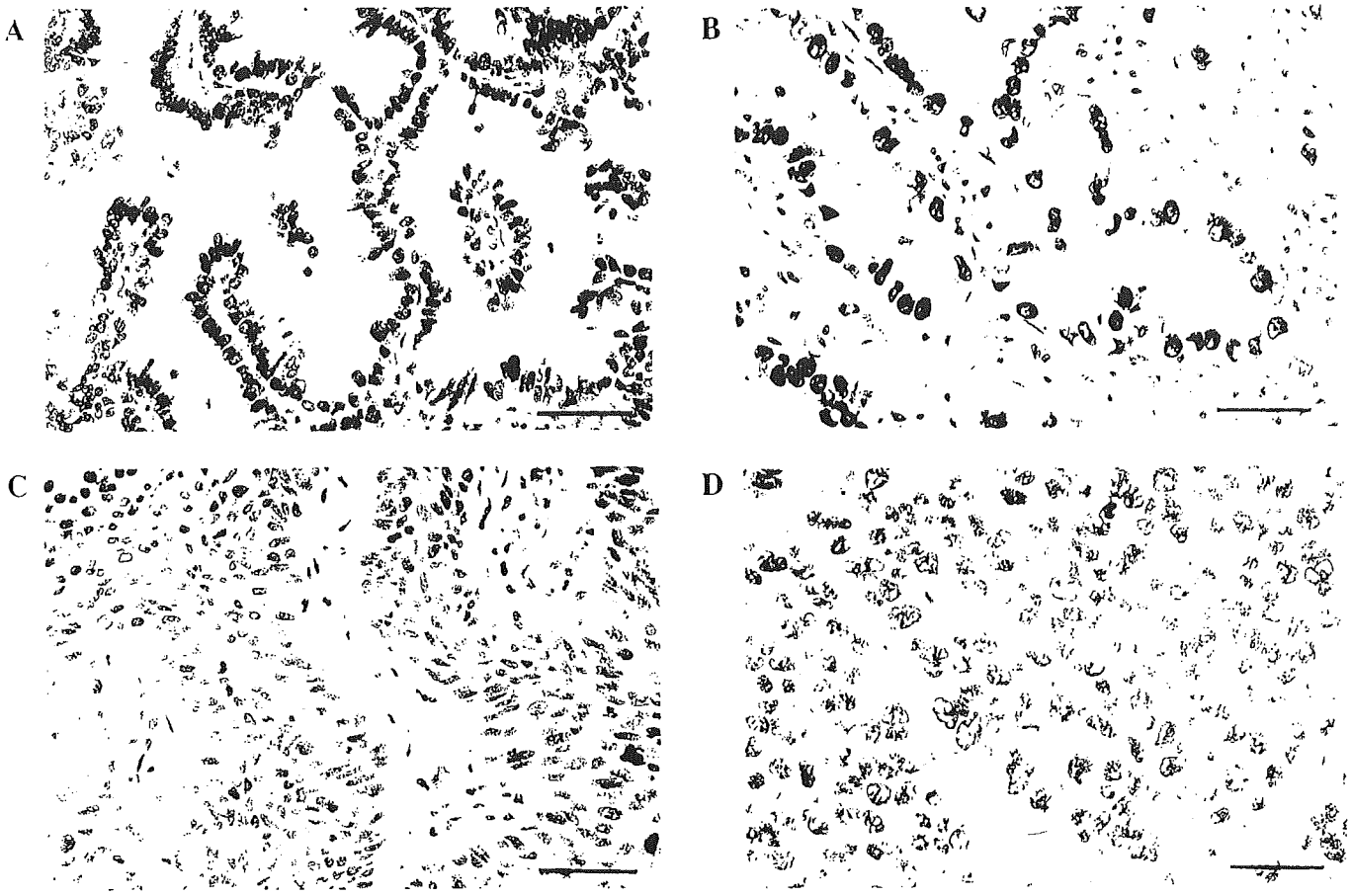

Figure 1. Representative photomicrographs of immunohistochemical staining of HDGF in adenocarcinoma (A and B) and squamous cell carcinoma (C and D) cases. HDGF is expressed weakly in the nucleus of <65% of tumor cells in A and C (defined as low HDGF-expression). To the contrary, HDGF staining was more intense in the nucleus and weak in the cytoplasm of ≥65% of tumor cells in B and D (defined as high HDGF-expression). Scale bars, 50 μm.

statistical analyses, the criterion of significance was defined as $P<0.05$.

Results

HDGF expression was detected in all tumor sections in various proportions. Many cancer cells exhibited strong HDGF-staining, mainly in the nucleus, and some cancer cells presented weak staining in the cytoplasm. Representative cases of adenocarcinoma and squamous-cell carcinoma are shown in Fig. 1. The median score of HDGF-LI in all cases was 64.5% (20-95%), and therefore we defined 65% as a cut-off for low (<65%, n=51) and high (≥65%, n=51) expression. Weak staining in the endothelial cells and smooth muscle cells in the vessels was used as the internal control as mentioned above. HDGF was also detected weakly in some of the non-cancerous type II pneumocytes and ciliated columnar epithelial cells (data not shown). These findings were consistent with those of a previous report on idiopathic pulmonary fibrosis (14).

The relationship between HDGF expression and clinicopathological variables (age, sex, smoking, tumor size, stage, T-factor, N-factor, pleural involvement, vascular involvement, lymphatic involvement, histology and differentiation) in all cases is summarized in Table I. There was no significant relationship between HDGF expression and any clinicopathological variable.

Kaplan-Meier overall and disease-free curves for HDGF expression dichotomized by the median level are shown in Figs. 2 and 3, respectively. Patients with lung cancer expressing high HDGF had a significantly worse overall and disease-free survival than those with lung cancer expressing low HDGF ($P=0.0004$ and $P=0.0005$ by the log-rank test, respectively). Among 102 patients, 25 received adjuvant therapy: radiotherapy was given to 6 patients and systemic chemotherapy including cisplatin or a combination of uracil and tegafur (UFT) was given to 19 patients. There was no significant difference in the proportion of patients who received adjuvant therapy between the 2 groups (13/51 in the low-HDGF group and 12/51 in the high-HDGF group, $P>0.99$).

In the univariate analysis of correlations between prognosis and potential prognostic factors evaluated (HDGF expression, adjuvant therapy and the 12 clinicopathological variables shown in Table I), vascular involvement, smoking, N-factor, tumor size, sex, pathological stage and HDGF were significant prognostic factors ($P<0.05$) for overall and disease-free survival. Adjuvant therapy was not a significant factor for overall ($P=0.580$) or disease-free ($P=0.536$) survival in this study. These 7 significant variables were entered into the Cox proportional-hazards model and multivariate analysis was performed (Table II). Pathological stage and HDGF-expression level were significant independent prognostic factors for overall and disease-free survival, and moreover HDGF had

Table I. Association between HDGF-expression and clinicopathological variables in all cases.

Variables	High-HDGF (%)		Low-HDGF	P-value[a]
Age				
<70 years	37	(50.0)	37	
≥70 years	14	(50.0)	14	>0.999
Sex				
Male	38	(55.1)	31	
Female	13	(39.4)	20	0.204
Smoking				
<40 pack-year	28	(54.9)	23	
≥40 pack-year	23	(45.1)	28	0.428
Tumor size				
≤30 mm	17	(40.5)	25	
>30 mm	34	(59.5)	26	0.159
pStage[b]				
Stage I + II	30	(49.2)	31	
Stage III + IV	21	(51.2)	20	>0.999
pT-factor[b]				
T1 + T2	42	(49.4)	43	
T3 + T4	9	(52.9)	8	>0.999
pN-factor[b]				
N0	25	(51.0)	24	
N1 + N2	26	(49.0)	27	>0.999
Pleural involvement[c]				
P0 + P1	40	(49.4)	41	
P2 + P3	11	(50.6)	10	>0.999
Vascular involvement				
v (-)	19	(48.7)	20	
v (+)	32	(50.8)	31	>0.999
Lymphatic involvement				
ly (-)	16	(50.0)	16	
ly (+)	35	(50.0)	35	>0.999
Histology[d]				
Ad	38	(54.3)	32	
Sq	13	(40.6)	19	0.286
Differentiation				
Well	25	(45.5)	30	
Moderate/poor	26	(55.3)	21	0.427

[a]χ^2-test. [b]According to the AJCC/UICC TNM pathological classification. pStage, pathological stage; pT, pathological tumor; pN, pathological lymph node; [c]According to the general rules for clinical and pathological record of lung cancer established by the Japan Lung Cancer Sosiety. [d]Ad, adenocarcinoma; Sq, squamous cell carcinoma.

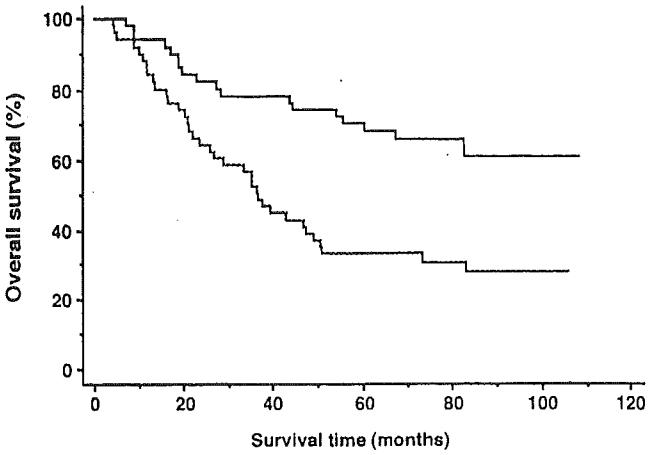

Figure 2. Kaplan-Meier analysis of the overall survival of NSCLC patients with low (a solid line) and high (a dotted line) HDGF-expression. P=0.0004 by the log-rank test.

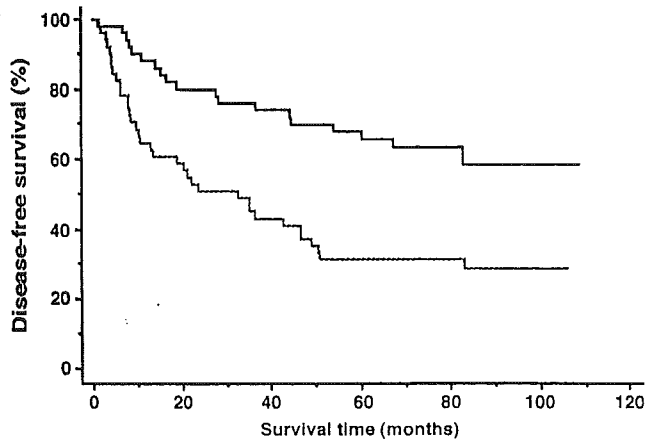

Figure 3. Kaplan-Meier analysis of the disease-free survival of NSCLC patients with low (a solid line) and high (a dotted line) HDGF-expression. P=0.0005 by the log-rank test.

higher risk-ratios than pathological stage for overall (2.976 versus 1.964) and disease-free survivals (2.970 versus 1.848).

The role of HDGF in the biological behavior of NSCLC remains to be fully elucidated. HDGF was very recently reported to be not only a mitogenic factor for lung epithelial cells but also an angiogenic factor (6,9,10,14). We, therefore, examined the relationship between HDGF-expression level and Ki-67-LI or MVD in serial sections. Ki-67-LI values for low and high HDGF-expressing cases were 19.3±14.3% and 34.1±17.7%, respectively (mean ± SD, P<0.00005 by Student's t-test). MVD values for low and high HDGF-expressing cases were 49.8±23.6 and 68.5±20.7 vessels/mm^2, respectively (mean ± SD, P<0.00005 by Student's t-test). These findings suggested that HDGF may promote the proliferation of tumor cells and intratumor angiogenesis in lung cancer.

Discussion

We demonstrated that HDGF is mainly expressed in the nucleus of NSCLC cells and that high expression of HDGF

Table II. Multivariate analysis of prognostic factors.

Variables	Unfav./Fav.[a]	Overall survival Risk ratio (95% CI[b])	P-value	Disease-free survival Risk ratio (95% CI)	P-value
Vascular involvement	v (+)/v (-)	1.016 (0.512-2.014)	0.9644	1.044 (0.532-2.048)	0.9009
Smoking	≥40/<40 pack-year	1.398 (0.700-2.792)	0.3427	1.632 (0.815-3.270)	0.1667
pN-factor[c]	N1 + N2/N0	1.462 (0.760-2.811)	0.2548	1.246 (0.649-2.392)	0.5083
Tumor size	>30/≤30 mm	1.572 (0.853-2.895)	0.1468	1.443 (0.782-2.663)	0.2404
Sex	Male/female	1.957 (0.856-4.472)	0.1114	1.956 (0.860-4.450)	0.1096
pStage[c]	III + IV/I + II	1.964 (1.084-3.556)	0.0259	1.848 (1.018-3.355)	0.0436
HDGF	High/low	2.976 (1.641-5.398)	0.0003	2.970 (1.651-5.344)	0.0003

[a]Unfavorable vs. favorable characteristics. [b]CI, confidence interval. [c]According to the AJCC/UICC TNM pathological classification. pStage, pathological stage; pN, pathological lymph node.

is an independent significant factor for worse overall and disease-free survival of patients with completely resected NSCLC. We also showed that HDGF-expression level was associated with both a high Ki-67-LI and a high intratumor MVD.

Recently, Ren *et al* (23) reported that overexpression of HDGF was a marker of poor prognosis only in patients with curatively resected stage I NSCLC. They found no association between HDGF expression and Ki-67-LI of cancer cells, which is inconsistent with our results. This difference may be because our study included stage I-IV cases whereas theirs only included stage I cases: HDGF-expression correlated with Ki-67-LI in stage IB-IV but not in stage IA (data not shown). Thus, we demonstrated that HDGF-expression level is a prognostic factor independent of and more powerful than the pathological stage of NSCLC by the multivariate analysis.

Exogenous HDGF promotes *in vitro* DNA synthesis and cell proliferation in rat and human lung epithelial cells. Endogenous HDGF overexpressed via transient gene transfer was translocated into the nucleus and promoted the proliferation of human lung epithelial A549 cells. Mori *et al* (14) confirmed, using short interfering RNA technique, that endogenously produced HDGF has a mitogenic effect on A549 cells. Collectively, HDGF probably stimulates the proliferation of lung epithelial cells, at least partially, in an autocrine manner. These findings support our result that high expression of HDGF correlates with a high Ki-67-LI of cancer cells. To date it is unknown if the exogenous mitogenic effect of HDGF is mediated by a cell surface receptor or uptake of the protein (4). Further exploration of this mechanism will contribute to the precise understanding of the biological functions of HDGF.

HDGF induced tumorigenesis of NIH3T3 cells in nude mice via direct angiogenic activity and induction of VEGF (7). Moreover, HDGF is a highly expressed vascular endothelial cell protein *in vivo* and is a potent endothelial mitogen and regulator of endothelial cell migration that acts through mechanisms distinct from those of VEGF (6). Since we could not observe a remarkable enhancement of HDGF expression in endothelial cells or vascular smooth muscle cells in NSCLC sections (data not shown), overproduction of HDGF by cancer cells might induce a high intratumor MVD possibly in a paracrine manner.

HDGF shows a homology to high mobility group-1 (HMG-1), a DNA binding protein (24), but lacks the characteristics of an HMG-1 protein, especially of the HMG box responsible for DNA bindings (2). HMG-1 enhances the activity of several transcription factors, including the glucocorticoid receptor, as well as the activity of RAG recombinase (24,25). The molecules controlled by HDGF in the nucleus and subsequent functions of HDGF have not been identified. Therefore, HDGF may display other tumorigenic behavior besides tumor-cell proliferation or angiogenesis.

Bernard *et al* (26) detected HDGF expression mainly in the nucleus and much more strongly in melanoma cell lines than in melanocytes. They showed by immunohistochemical analysis of clinical samples that 54% of benign nevoid cells reacted positively, whereas 78-90% of melanoma cells were positive in all stages of melanoma. We found that HDGF was expressed in ~30-40% of non-cancerous alveolar or bronchial epithelial cells (data not shown) and 20-95% (median 64.5%) of NSCLC cells. The proportion of HDGF-positive cells seems slightly smaller in NSCLC than in melanoma, but our results were in general compatible with those of Bernard *et al* (26). With regard to other malignancies, a recent study using differential display revealed that HDGF expression was associated with radiosensitivity in esophageal cancer (27). Expression profiling of gastric adenocarcinoma using cDNA array revealed that HDGF was one of the overexpressed genes in gastric cancer as compared with normal gastric mucosa (28). Thus, HDGF probably plays a critical role in the development and progression of various malignancies.

Based on the above findings, we consider HDGF is a useful marker of poor prognosis in patients with completely resected NSCLC, and high HDGF-expression might be a potential indicator of the need for adjuvant therapy. Although further investigations need to be done on the molecular characteristics and biological functions of HDGF, this factor may be a target molecule for the treatment of NSCLC.

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Irinotecan in the treatment of small cell lung cancer: a review of patient safety considerations

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A water soluble derivative of camptothecin, irinotecan (CPT-11) is effective against small-cell lung cancer (SCLC), as well as non-SCLC and gastrointestinal cancers. This extended review of recently concluded and ongoing studies focuses on irinotecan in the treatment of limited (LD) and extensive (ED) SCLC specifically considering the safety of patients. Irinotecan-induced diarrhoea is pervasive, and can be severe and life-threatening especially in combination with neutropenia. It can have a significant impact on patient quality of life, negatively influencing compliance with therapy and dose-intensity. For LD SCLC, irinotecan can be administered with radiotherapy concurrently or sequentially. In a Phase III study for ED SCLC comparing etoposide and cisplatin (EP) and irinotecan and cisplatin (IP) regimens, severe myelosuppression was more frequent in the EP arm than in the IP arm, and conversely severe or life-threatening diarrhoea was more frequent in the IP arm than in the EP arm. IP resulted in significantly higher response rates and overall survival in Japan, and confirmatory Phase III studies are ongoing. Irinotecan should not be administered to patients with any degree of ongoing diarrhoea above their baseline. Irinotecan can be administered with relative safety for patients with SCLC only through careful patient monitoring, especially regarding diarrhoea and myelosuppression.

Keywords: chemotherapy, irinotecan (CPT-11), radiotherapy, small-cell lung cancer (SCLC), toxicity

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1. Introduction

Lung cancer is the leading cause of cancer deaths worldwide, with > 900,000 deaths per year attributed to the disease [1]. About 15 – 20% of lung cancers are small-cell lung cancer (SCLC), although the frequency has been decreasing relative to other lung cancer over the last two decades [2]. SCLC is considered distinct from other non-small cell lung cancers (NSCLC) because of its clinical and biological characteristics [3]. The clinical characteristics of SCLC tend to be aggressive behaviour with rapid growth, early spread to distant sites, but more sensitive to chemotherapy and radiation. SCLC is usually staged as either limited disease (LD), in which the tumour is confined to the hemithorax of origin, the mediastinum, or the supraclavicular lymph nodes, or extensive disease (ED), in which tumours have spread beyond the supraclavicular areas. About 30% of patients with SCLC have LD. Management of most cases of LD SCLC involves combination chemotherapy, usually with a platinum-containing regimen, and thoracic radiation therapy (TRT). If a complete response is obtained, the patient may be offered prophylactic cranial irradiation. The median survival time (MST) of LD SCLC is 16 – 24 months with current forms of treatment, such as chemoradiotherapy with or without surgery. ED SCLC patients are treated with combination

chemotherapy, but the disease remains incurable. Usually a platinum-containing regimen is chosen. For ED SCLC, the MST is less than one year with currently available chemotherapy, and long-term survivors are still rare [4,5].

Furthermore, the prognosis is exceedingly poor for patients who receive second-line therapy after relapse. Response is influenced by the time to progression after cessation of first-line therapy. Patients who relapse less than three months after the completion of first-line therapy are termed refractory; they have response rates that are lower than for those patients who relapse more than three months after therapy, who are termed sensitive. The objective for these patients is palliation and increased quality of life, and therefore salvage therapy should be limited to patients with a good performance status (PS) and without significant comorbidities [3].

A water soluble derivative of camptothecin, irinotecan hydrochloride (CPT-11), a topoisomerase I inhibitor, has been synthesised for use in chemotherapy. The chemical structures of irinotecan and its major metabolites found in plasma are shown in **Figure 1**. Irinotecan is converted by hepatic and peripheral carboxylesterase to its active metabolite 7-ethyl-10-hydroxycamptothecin (SN38). This is subsequently glucuronidated by hepatic uridine diphosphate glucuronosyl transferase-1A1 (UGT 1A1), the enzyme responsible for bilirubin glucuronidation with multi-genetic variants, to SN38-glucuronide (SN38G) [6]. The patient with UGT1A1*28 has an impaired capacity for glucuronidation of SN-38, increased exposure to SN-38, and there is increased clinical toxicity when treated with irinotecan. To measure UGT1A1*28, in August 2005, FDA in the US cleared the Invader Molecular Assay for irinotecan dosing. However, irinotecan activity is not determined by the product of one gene [7]. Irinotecan, SN-38 and SN-38 glucuronide (SN-38G) may be shunted out of the cell via members of the ATP-binding cassette transporters [8]. The metabolism and pharmacogenetics of irinotecan is beyond the scope of this review, but there are some excellent reviews on this subject [9-11].

It should be cautioned that there are drug–drug interactions [12] with irinotecan. Exposure to irinotecan and its active metabolite SN-38 is substantially reduced in patients receiving the CYP3A4 enzyme-inducing anticonvulsants phenyntoin, phenobarbital or carbamazepine [13]. Rifampin, rifabutin and St. John's Wort are also CYP 3A4 inducers [14,15]. St. John's Wort is contraindicated during irinotecan therapy. Ketoconazole, a strong inhibitor of CYP3A4 [16], and contraindicated during irinotecan therapy, should be discontinued in patients at least one week prior to starting irinotecan therapy.

In Japan, 1245 cancer patients received irinotecan as a single agent in Phase I or Phase II trials that were conducted to obtain approval for commercial use from the Ministry of Health, Labour and Welfare. Of the 1245 patients, 55 (4.4%) died from toxicities of irinotecan, mainly myelosuppression and/or diarrhoea [17].

The onset of diarrhoea can occur early or be delayed beyond 24 h after injection of irinotecan. Early-onset diarrhoea is a cholinergic effect. Anticholinergic drugs, such as atropine, seem to easily reverse this side effect. Late-onset diarrhoea represents the dose-limiting toxicity (DLT) of irinotecan; it can be severe and life-threatening, especially in combination with neutropenia. Late-onset diarrhoea is treated with loperamide, and identification of high-dose loperamide as an effective remedy for this toxic effect greatly facilitated development of irinotecan [18,19]. These studies established the usefulness of high-dose loperamide. Patients should be instructed to take high-dose loperamide at the first onset of any irinotecan-associated late-onset diarrhoea that has occurred at least 12 h after drug administration. This therapy has been widely used for the management of diarrhoea caused by irinotecan.

For the treatment of SCLC, initial irinotecan is usually administered on days 1 and 8 every 3 weeks or on days 1, 8 and 15 every 4 weeks. The dose ranges from 50 to 70 mg/m^2 when administered weekly. As an example of the dose modification of irinotecan, Kudoh *et al.* [20] used the following dose modification: irinotecan is not given on days 8 or 15 if the leukocyte or platelet counts were < 3000/μl or < 75,000/μl, respectively. It is also withheld if the patient develops diarrhoea of grade 2 (increase of 4 – 6 stools/day, or nocturnal stools) or worse (grade 3: increase of > 6 stools/day or incontinence; grade 4: physiological consequences requiring intensive care). The next course of treatment can only be initiated if the leukocyte count is ≥ 4000/μl, the platelet count is ≥ 10,000/μl, serum creatinine is less than the upper limit of normal, and diarrhoea has been resolved. There is no dose modification for the leukocyte count, platelet count or diarrhoea during the same course. The dose of irinotecan in the next course was reduced by 10 mg/m^2 if the leukocyte count was < 2000/μl, the platelet count was < 50,000/μl, or diarrhoea was grade 3 to 4. This dose modification was applied in most studies with minor variation. For example, in some studies, the delay in the irinotecan doses was applied when the leukocyte count was < 2000/μl [21] instead of 3000/μl.

Another available topoisomerase-I inhibitor, topotecan, has achieved response rates of up to 22% in previously treated patients with SCLC and survival almost double that achieved with other single agents. Compared with cyclophosphamide/doxorubicin/vincristine (CAV), single-agent topotecan achieved a higher response rate, longer survival and statistically significant improvements in dyspnoea, hoarseness, fatigue, anorexia and interference with daily activities [22,23]. The incidence of grade 3 – 4 diarrhoea was extremely low (1%). The clinical comparison of these two topoisomerase-I inhibitors has not been tried. This review focuses mainly on the recent results of irinotecan in the treatment of SCLC in connection with patient safety considerations.

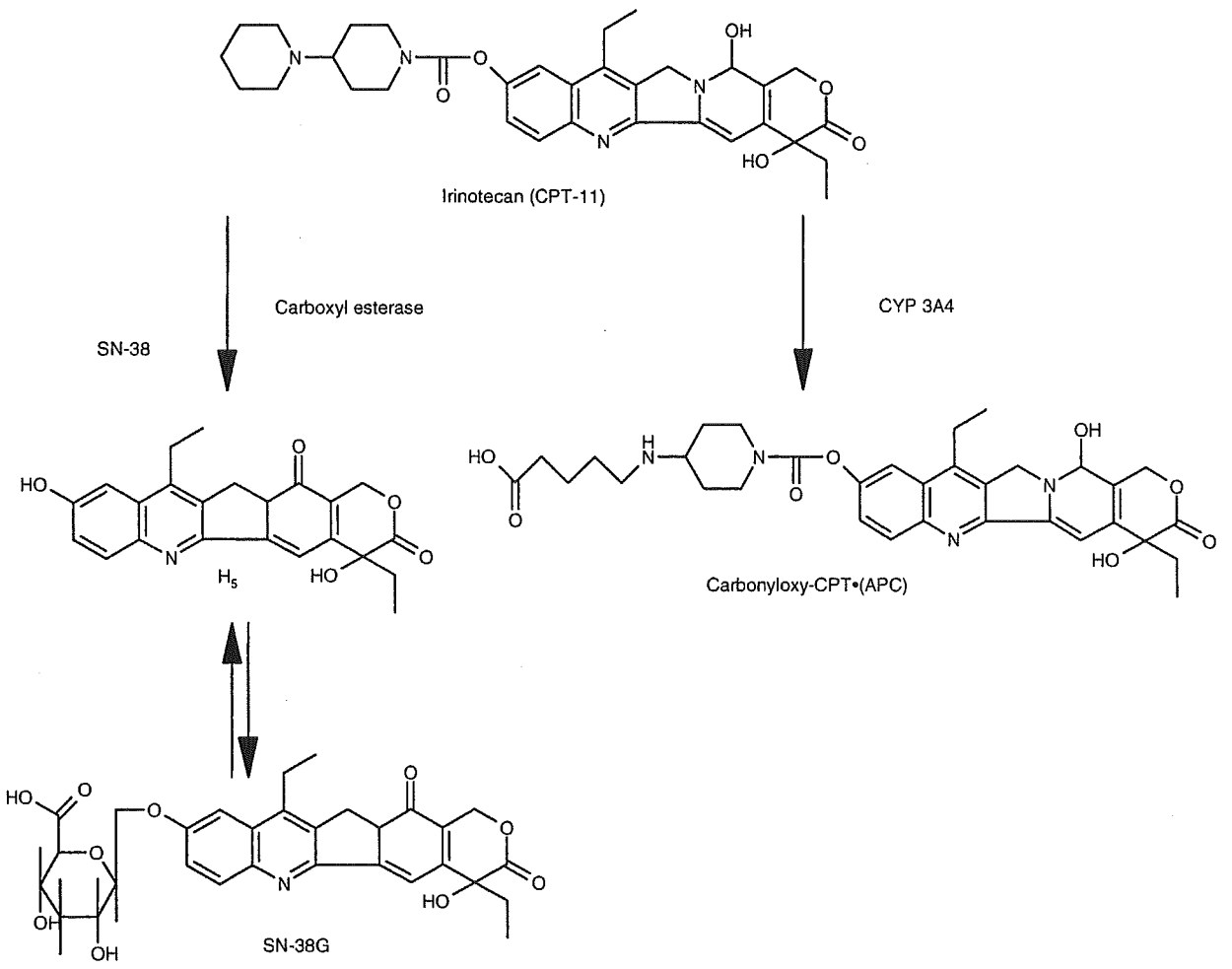

Figure 1. Metabolism of irinotecan. Chemical structures of CPT-11 and its major metabolites.

2. Irinotecan containing regimens as front-line treatment

2.1 Irinotecan plus cisplatin for ED SCLC

Clinically, irinotecan was proved to be effective against SCLC [24]. Negoro *et al.* have demonstrated that 13 (37%) out of 35 patients responded, including 33% of previously treated patients and 50% of chemotherapy-naive patients. In a Phase II trial of irinotecan for previously treated SCLC, the response rate was 47% out of 16 patients [25].

IP was tested in a Phase II trial for patients with previously untreated SCLC [20]. A total of 40 patients (53%) had LD and 35 patients (47%) had ED. Initially, irinotecan 80 mg/m^2 over 90-minutes infusion was given on days 1, 8 and 15, and cisplatin 60 mg/m^2 was given every 4 weeks. After 3 of the initial 10 patients experienced severe haematological toxicity, diarrhoea and hepatic toxicity, and one patient died of diarrhoea and neutropenia, the irinotecan dose was reduced to 60 mg/m^2. The response rate was 84%, with a complete response rate of 29%. The MST was 14.3 months for LD patients and 13.0 months for ED patients, an encouraging result. Although the survival of LD was not increased significantly, this may be due to the small number of LD SCLC patients accrued. This study prompted a Phase III study of the Japan Clinical Oncology Group (JCOG 9511).

The JCOG conducted a multi-centre, randomised, Phase III study which compared irinotecan plus cisplatin with etoposide plus cisplatin (EP) in patients with ED SCLC (JCOG 9511) (**Figure 2**) [26]. IP consisted of four 4-week cycles of 60 mg/m^2 of irinotecan on days 1, 8 and 15, and 60 mg/m^2 of cisplatin on day 1. The regimen of etoposide and cisplatin consisted of four 3-week cycles of 100 mg/m^2 of etoposide on days 1, 2 and 3, and 80 mg/m^2 of cisplatin on day 1. The delivered dose intensity for irinotecan was 80%. The results are listed in **Table 1**. This study was terminated early because an interim analysis found a statistically significant difference in survival between the two arms. The MST was 12.8 months in the IP arm and 9.4 months in the EP arm ($p = 0.002$). At two years, the proportion of patients surviving was 19.5% in the IP group and 5.2% in the EP

Table 1. IP versus EP in phase III studies.

	IP	EP	p-value	IP	EP	p-value
	JCOG9511 study [26]			*Hanna's study* [27]		
	(n = 75)	(n = 77)		(n = 210)	(n = 104)	
Irinotecan: delivered dose intensity	80%			90%		
Survival						
Median survival time (months)	12.8	9.4	0.002	9.3	10.2	0.6226
1-year survival (%)	58.4	37.7		35.4	36.7	
2-year survival (%)	19.5	5.2		8.0	7.9	
Haematological						
Neutropenia	65.3	92.2	< 0.001	36.2	86.5	< 0.0001
Anaemia	26.7	29.9	0.72	4.8	11.5	< 0.0268
Thrombocytopenia	5.3	18.2	0.002	4.3	19.2	< 0.0001
Nonhaematological				(n = 216)	(n = 106)	
Diarrhoea	16	0	< 0.001	21.3	0	0.0001
Response				(n = 221)	(n = 110)	
Complete response	2.6	9.1		3.6	2.7	
Partial response	81.8	58.4		44.3	40.9	
Overall response	84.4	67.5	0.02	48.0	43.6	
Stable disease	2.6	20.8		4.1	7.3	
Progressive disease	3.9	11.7		20.0	20.0	
Not evaluable	6.5	0		28.1	29.1	

EP: Etoposide and cisplatin; IP: Irinotecan and cisplatin.

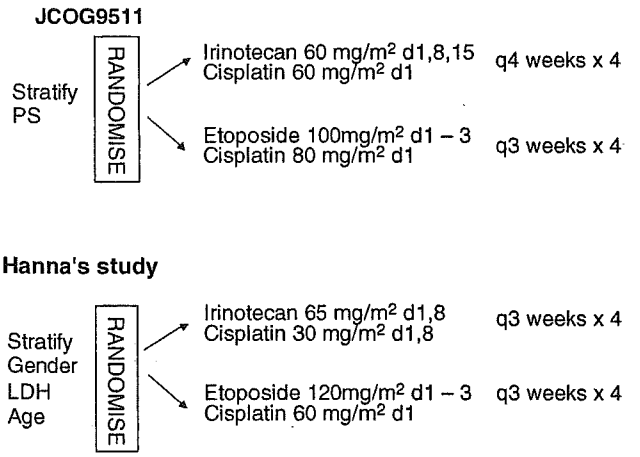

Figure 2. Two Phase III randomised trials.
LDH: Lactate dehydrogenase.

group. This was the first study to show the superiority of any one regimen over etoposide plus cisplatin for the front-line treatment of ED SCLC, and IP has become one of the standard regimens for ED SCLC in Japan. Severe myelosuppression was more frequent in the EP group than in the IP group. On the other hand, severe diarrhoea was more frequent in the IP arm than in the EP arm. Despite the dose modifications, major deviations from the protocol resulted in failure to reduce the dose of chemotherapy (in 6 patients); administration of irinotecan despite the presence of grade 1 (increase of < 4 stools/day) or 2 diarrhoea (in 9 patients); continuation of the study treatment despite grade 2 to 3 pulmonary toxicity (in 3 patients); and continuation of the treatment despite grade 3 hepatic toxicity (in 1 patient). There were 3 treatment-related deaths in the IP arm; one patient died of bleeding from a metastatic site in the lung, another patient died of sepsis associated with neutropenia and diarrhoea, and the third patient died of pneumonia associated with neutropenia. These three treatment-related deaths in the IP arm occurred during the first or second cycle of treatment and were attributed to haematological toxicities of the first cycle. This may indicate that severe haematological toxicities, as well as diarrhoea, during the first cycles of chemotherapy should be managed carefully. All cases of grade 1 to 4 diarrhoea occurred during the first and second cycles of the IP arm but early suspension of treatment may have prevented death associated with diarrhoea in all but one patient, which

involved a protocol violation because the patient was given irinotecan on day 8 of the first cycle despite the presence of grade 1 diarrhoea. This suggests that irinotecan should not be administered to patients with any degree of ongoing diarrhoea above their baseline.

Confirmatory studies are underway; currently, there is only one concluded study showing IP superiority, but it had a small sample size. Additionally, pharmacogenomic differences may exist between Japanese and Western populations.

Hanna *et al.* presented a Phase III trial comparing IP with EP in patients with previously untreated ED SCLC at the ASCO meeting in 2005 (**Figure 2, Table 1**) [27]. This was designed to confirm the JCOG9511 trial. However, the dose and schedule were modified to increase dose intensity.

The IP arm consisted of cisplatin 30 mg/m^2 and irinotecan 65 mg/m^2 on days 1 and 8 every 3 weeks. The EP arm was cisplatin 60 mg/m^2 on day 1, and etoposide 120 mg/m^2 on days 1 – 3 every 3 weeks for 4 cycles, or disease progression, or intolerable toxicity. This was planned to improve tolerability, achieve greater dose intensity and maintain or improve efficacy. The 336 patients were stratified by gender, lactate dehydrogenase level and age, and were randomised in a 2:1 fashion, with 221 treated with IP (median age, 63 years; range, 37 – 82 years; male, 57.5%) and 109 to EP (median age, 62 years; range, 38 – 83 years; male, 57.3%). Baseline characteristics were well balanced across the 2 arms, with a high representation of PS of 0 or 1 (IP, 92.3%; EP, 88.2%). After 30 patients with PS 2 were enrolled, study amendment excluded PS 2 patients. Delivered dose intensity of irinotecan was 39 mg (94%), higher than that of the JCOG9511 trial (80%). In both arms, 65% of patients received 4 or more cycles. Selected grade 3 or 4 toxicities in IP versus EP arm were: diarrhoea (21 versus 0%), neutropenia (35 versus 84%), febrile neutropenia (4 versus 11%). Grade 3 or 4 haematological toxicities were significantly more common with EP than IP. There was a trend towards more febrile neutropenia in the EP arm (10 versus 4%), and significant differences were seen in rates of dehydration (13 versus 3%; $p = 0.15$), vomiting (13 versus 4%; $p = 0.0445$), and diarrhoea (21 versus 0%; $p < 0.0001$). The survival of EP in both trials was similar (MST: 10.2 months in this study and 9.4 months in the JCOG9511 trial). However, the MST of IP was 9.3 months in this trial and 12.8 months in the JCOG9511 trial. Differences in outcome of this study from the JCOG trial may be due to pharmacogenomic or patient characteristic differences, or a change in the dose/schedule of IP. Pharmacogenomic studies among ethnic populations are needed to address this issue. It is likely that IP will prove to be at least as effective as other treatments for patients with ED SCLC.

Other Phase III trials will clarify these issues, including a SWOG S0124-randomised Phase III trial with the dose and schedule of each arm the same as the JCOG9511 trial, and a Phase III study started in June 2002 – (NCT00143455) sponsored by Pfizer. In this second study, IP consists of irinotecan 65 mg/m^2 on days 1 and 8 and cisplatin 80 mg/m^2 on day 1. EP consists of etoposide 100 mg/m^2 on days 1 – 3 and cisplatin 80 mg/m^2 on day 1 every 3 weeks. The results of these studies are awaited.

The debate continues regarding the optimal dose of combination chemotherapy as related to improvement of the outcome of SCLC. However, the author can state that too low a dose intensity may lead to poor results. Takigawa *et al.* used fractionated administration of IP in 15 patients with ED SCLC [28]. Both irinotecan at a dose of 50 mg/m^2 and cisplatin at a dose of 60 mg/m^2 were given on days 1 and 8, and repeated every 4 weeks up to 4 cycles. Although objective response rates were 80%, no complete response (CR) were obtained. The MST was 9.4 months and one-year survival was 40.0%. They stopped enrollment because of no CR and poor survival compared to Kudoh's data [20]. The dose intensity may be low because this regimen had a lower dose of irinotecan (50 mg/m^2) and a two-week rest period.

Han *et al.* reported a Phase II study of dose-intensified weekly IP in chemo-naive patients with ED SCLC [29]. The initial six patients received cisplatin 50 mg/m^2 followed by irinotecan 90 mg/m^2 on day 1 and 8 of a 21-day cycle (level I), with one treatment death and three febrile neutropenias. The doses of cisplatin and irinotecan were then reduced to 40 mg/m^2 and 80 mg/m^2, respectively (level II). The overall response rate was 97%, with a complete response rate of 26%. The MST was 11.1 months and 1- and 2-year survival rates were 44.1% and 11.8%, respectively. Major grade 3 or 4 toxicities included neutropenia (89%), anaemia (59%) and diarrhoea (27%). There were three treatment-related deaths, occurring in elderly patients aged > 60 years and/or relative poor baseline PS 2 or 3. Although they adopted the oral alkalisation and control of defecation to prevent irinotecan-induced side effects, especially delayed diarrhoea, they are uncertain whether or not this preventive treatment reduced the observed incidence of severe delayed diarrhoea.

2.2 Irinotecan plus carboplatin for ED SCLC

Schmittel *et al.* studied the DLT and maximum tolerated dose (MTD) of a dose escalation of carboplatin to a fixed dose of irinotecan (IC) in Caucasian patients [30]. They demonstrated that the maximum tolerated dose is irinotecan 50 mg/m^2 administered on day 1, 8 and 15, and carboplatin at an area under the concentration–time curve (AUC) of 5 mg/ml x min, on day 1 of a 4-week cycle. DLT (neutropenia, thrombocytopenia and diarrhoea) was comparable to the results of the Japanese trial at a dose of 60 mg/m^2 of irinotecan and AUC = 5 of carboplatin [31].

Subsequently, Schmittel *et al.* presented a randomised Phase II trial comparing IC and etoposide plus carboplatin (EC) in ED SCLC [32]. Chemotherapy-naive ED SCLC patients were randomly assigned to receive carboplatin AUC = 5 either in combination with 50 mg/m^2 of irinotecan on days 1, 8 and 15 or with etoposide 140 mg/m^2 on days 1 – 3. In the IC arm, treatment was repeated every four weeks; in the EC arm, every

three weeks. IC improved response rate (10% CR and 61% partial response (PR) in IC, 0% CR and 50% PR in EC) and progression free survival (9 months, $p = 0.03$) over standard EC (6 months). The MST was 12 months in the IC arm and 10 months in the EC arm, but with no significant difference. Patients with EC had significantly higher incidence of grade 3 to 4 leucopenia, neutropenia and thrombocytopenia. Grade 3 – 4 diarrhoea developed more frequently in the IC arm (11 versus 6%), but with no significant difference. Haematotoxicity was favourable in the IC arm. They extended into a randomised Phase III trial to assess impact on overall survival, and concluded this study showed that even when carboplatin is used instead of cisplatin, the survival of IC and EC was not significantly different, and that myelosuppression was more frequent in EC than IC.

2.3 Irinotecan plus etoposide for ED SCLC

A Phase II study of irinotecan and etoposide (IE) for chemotherapy-naive ED SCLC was recently conducted without platinum by the West Japan Thoracic Oncology Group (WJTOG) [33]. A total of 50 patients were enrolled. This regimen consisted of irinotecan 60 mg/m^2 on days 1, 8 and 15, and etoposide 80 mg/m^2 on days 2 – 4. The overall response rate was 66% with a complete response rate of 10%. The MST was 11.5 months and the 1-year survival rate was 43.2%. Grade 3 – 4 neutropenia, thrombocytopenia and diarrhoea were 62.9, 4 and 2%, respectively. There was no treatment-related death. This regimen seems to be equal to the EP regimen. The dose intensity of irinotecan and etoposide achieved with this regimen was not adequate. This may be the reason for the low incidence of diarrhoea (2%). A schedule of irinotecan administered on days 1 and 8 at 3-week intervals may be preferred.

2.4 Triplets including irinotecan for ED SCLC

JCOG9902-DI was a randomised Phase II trial to compare two kinds of three-drug combinations of cisplatin, etoposide and irinotecan (PEI regimens) for the treatment of ED SCLC [34]. A total of 60 patients were randomised to receive either arm A (cisplatin 25 mg/m^2 on day 1, on weeks 1, 3, 5, 7 and 9 and etoposide 60 mg/m^2 on days 1 – 3, on weeks 2, 4, 6, 8), or arm B (cisplatin 60 mg/m^2 on day1, irinotecan 60 mg/m^2 on days 1, 8, 15 and etoposide 50 mg/m^2 on days 1 – 3, every week for 4 cycles). Prophylactic G-CSF support was provided in both arms. This study suggested that the PEI combinations in both schedules have significant activity against ED SCLC with acceptable toxicity. The CR rate of 17% and MST of 12.9 months in arm B were much more promising compared with the CR rate of 7% and MST of 8.9 months in arm A. They concluded that arm B should be selected for future Phase III studies. However, because irinotecan administration often needed to be skipped, especially on day 15, they suggested a 3-week schedule in which irinotecan is administered only on days 1 and 8.

Briasoulis *et al.* showed that irinotecan can be safely combined with cisplatin and etoposide in a convenient and simple schedule of administration over three days [35]. They treated 36 patients with irinotecan on day 1 in combination with fixed doses of cisplatin (20 mg/m^2) and etoposide (75 mg/m^2), both for 3 consecutive days. Irinotecan dose was escalated from 60 mg/m^2 by increments of 40 mg/m^2 in this Phase I trial. The MTD of irinotecan was 140 mg/m^2 and the recommended optimal dose 120 mg/m^2. DLTs were febrile neutropenia and grade 3 diarrhoea. This same regimen is being studied with concurrent TRT in a total dose of 54 Gy in 30 fractions (1.8 Gy once daily) [36].

Thompson *et al.* reported a Phase II trial of the Minnie Pearl Cancer Research Network at the 2005 ASCO meeting [37]. They added a molecular targeted agent, imatinib (60 mg/day, per os) to chemotherapy of irinotecan (60 mg/m^2 on days 1, 8 and 15) and carboplatin (AUC = 4) every 4 weeks. Imatinib targets c-kit expression. Grade 3/4 haematological toxicity included: neutropenia (29%/16%), anaemia (13%/1%) and thrombocytopenia (7%/0%). The response rate was 66% with 10% CR. Grade 3 diarrhoea was observed in 21%. There were no treatment-related deaths. The MST was 8.5 months. This suggests that C-kit expression did not correlate with survival and that imatinib offers no efficacy at a cost of increased toxicity when combined with irinotecan and carboplatin in the treatment of ED SCLC.

3. Irinotecan-containing regimens for relapsed or refractory SCLC

Huisman *et al.* have summarised 21 Phase II studies and 3 randomised trials of second-line chemotherapy in patients with SCLC reported from 1989 to 1999 [38]. They found a cumulative response rate of 21% for multi-drug regimens and 19% for single agents. As yet there is no standard second-line treatment established for patients with SCLC who fail or relapse after front-line treatment.

Irinotecan was combined with various anticancer drugs in doublet or triplet. As doublets, these include cisplatin [39], weekly or every three weeks carboplatin [40,41], etoposide [42], gemcitabine [43,44], ifosfamide [45] and paclitaxel [46]. The responses vary from 10 to 94%, and the MST ranges from 5.8 to 8.9 months. As described earlier on triplet including irinotecan [34,47], a three-drug combination Phase II study of irinotecan, cisplatin and etoposide (PEI regimen) was conducted only for sensitive relapsed SCLC (40 patients) [48]. This Phase II regimen consisted of cisplatin 25 mg/m^2 weekly for 9 weeks, etoposide 60 mg/m^2 for 3 days on weeks 1, 3, 5, 7 and 9, and irinotecan 90 mg/m^2 on weeks 2, 4, 6 and 8 with G-CSF support after day 1 on week 2. The results showed a response rate of 78% (CR rate of 13%) and the MST of 11.8 months. A total of 39 patients (98%) had a good PS of 0 or 1. Grade 3 – 4 neutropenia, thrombocytopenia, and diarrhoea were observed in 73, 33, and 8%, respectively. Nonhaematological toxicities were mild and transient.

Another three-drug combination of cisplatin, ifosfamide and irinotecan with G-CSF was conducted by Fujita *et al.* [49].

The response rate was 94.4% and the MST was 11.1 months, encouraging result. Because of patient selections, it is difficult to make wholly valid conclusions about the most effective regimen based only on Phase II results. However, three-drug combinations containing irinotecan with G-CSF support may have better survival and feasibility than the doublets. The disadvantage is that triplet regimens require G-CSF support, which may make out-patient treatment difficult.

4. Irinotecan containing regimen for LD SCLC

Two meta-analyses showed that the addition of TRT to chemotherapy in patients with LD SCLC improves survival at two and three years by 5.4% [50,51]. In these meta-analyses, non-platinum-based combination chemotherapies were commonly used, with only a few trials using platinum-based chemotherapy. Cisplatin and etoposide plus TRT is now widely regarded as the standard regimen for LD SCLC, and presents acceptable toxicity [52]. Turrisi *et al.* reported results of once-daily versus twice-daily (b.i.d) TRT with four cycles of cisplatin and etoposide. Results showed that the MST was significantly superior in the b.i.d arm (23 versus 19 months) [53].

Irinotecan showed potent radiosensitising effects in human lung tumour xenografts which were related to the cell cycle [54]. Kubota *et al.* reported a pilot study of concurrent etoposide and cisplatin plus accelerated hyperfractionated TRT followed by irinotecan and cisplatin for LD SCLC (JCOG9903) [21]. Treatment consisted of etoposide 100 mg/m^2 on days 1 – 3, cisplatin 80 mg/m^2 on day 1, and concurrent b.i.d TRT of 45 Gy beginning on day 2. The IP regimen started on day 29 and consisted of irinotecan 60 mg/m^2, days 1, 8, 15 and cisplatin 60 mg/m^2 on day 1, with three 28-day cycles. A total of 31 patients were accrued. Although a pilot study, the MST was 20.2 months and 1-, 2- and 3-year survival rates were 76%, 41%, and 38%, respectively. This encouraging regimen proved safe with acceptable toxicities. A randomised Phase III trial comparing EP with IP following EP plus concurrent TRT for LD SCLC is now underway (JCOG0202).

The WJTOG also conducted a similar regimen [55]. Treatment included cisplatin 80 mg/m^2 on day 1 and etoposide 100 mg/m^2 on days 1 – 3 with concurrent TRT (1.5 Gy/b.i.d, a total dose of 45 Gy) followed by 3 cycles irinotecan 60 mg/m^2 on days 1, 8 and 15 and cisplatin 60 mg/m^2. The results of 51 patients were almost identical to JCOG9903; overall response and CR rate was 87.8% and 40.8%, respectively; Grade 4 toxicity included neutropenia (83.7%), anaemia (10.2%), thrombocytopenia (0%), diarrhoea (2%) and infection (2%); the MST was 21.5 months and 2-year survival rate was 45.7%.

A Phase II study of IP induction followed by concurrent b.i.d TRT with EP chemotherapy for LD SCLC was conducted [56] and also showed encouraging results. Treatment consisted of two cycles of cisplatin 40 mg/m^2 and irinotecan 80 mg/m^2 on days 1 and 8 of a 3-week cycle. This was followed by two 3-week cycles of cisplatin 60 mg/m^2 on days 43 and 64, and etoposide 100 mg/m^2 on days 43 – 45 and 64 – 66, with concurrent b.i.d TRT total of 45 Gy beginning on day 43. Thirty-five patients were accrued. The MST was 25 months (but it should be noted that this is a single institution Phase II study).

In these studies, irinotecan was used on an induction or adjuvant setting, and both regimens were encouraging. However, randomised study in which both modalities are compared has not been conducted.

There have been a few trials of concurrent chemoradiotherapy including irinotecan for patients with SCLC as well as NSCLC. Recently, a combined modality treatment of IC and TRT followed by bevacizumab (antiangiogenic anti-VEGF antibody) in the treatment of LD SCLC was conducted in a Phase II trial by the Minnie Pearl Cancer Research Network [57]. Induction therapy consisted of irinotecan 50 mg/m^2 on days 1 and 8, carboplatin AUC = 5 on day1, TRT 1.8 Gy single daily dose to total dose of 61.2 Gy (34 fractions), beginning with the 3rd cycle. Chemotherapy was repeated every three weeks for four cycles. As a maintenance therapy, bevacizumab 10 mg/kg i.v. every 2 weeks was given until disease progression, or a maximun of 10 doses (20 weeks) were administered. The response rate was 81% with 28% CR. This regimen was well tolerated with rare grade 4 toxicity and no treatment-related deaths. One-year progression-free and overall survival were 68% and 71%, respectively. These results suggest that irinotecan can be safely administered with TRT concurrently.

Sohn *et al.* also reported a Phase II study of IP with concurrent TRT in LD SCLC [58]. Chemotherapy of irinotecan 60 mg/m^2 on days 1, 8 and 15 and cisplatin 40 mg/m^2 on days 1 and 8 were repeated every 4 weeks until a maximum of 6 cycles. TRT of 2 Gy/day was commenced on day 1 of the second chemotherapy cycle up to a total of 54 Gy. The results are not concluded at this time.

Langer *et al.* reported a Phase I study of IP and either b.i.d TRT (45 Gy) or once daily RT (70 Gy) to determine if irinotecan can be safely integrated with concurrent TRT and cisplatin in LD SCLC [59]. Acute DLT was defined as grade 4 oesophagitis, pneumonitis, or diarrhoea; grade 4 neutropenic fever; or any attributable grade 5 fatal toxicity (≤ 90 days after RT). Although preliminary, there has been no attributable DLT in the 26 patients that have been enrolled. In combination with cisplatin 60 mg/m^2 every 3 weeks x 4 and either b.i.d TRT or once daily TRT, irinotecan 40 mg/m^2 on days 1 and 8 was safe and feasible. Irinotecan at 50 mg/m^2 on days 1 and 8 every 3 weeks x4 was also feasible in combination with cisplatin and b.i.d TRT. These reports allow us to conclude that irinotecan can be administered with radiotherapy sequentially or concurrently.

5. Expert opinion and conclusion

Irinotecan is effective against SCLC. For the treatment of ED SCLC, IP regimen is at least comparable to EP regimen.

The degree of myelosuppression in the IP regimens was less than that of the EP regimens. However, diarrhoea was more often observed in the IP than the EP regimens, and can lead to severe side effects when the IP regimen is used incautiously. Pharmacogenetic study of irinotecan may prompt one to use the drug in a safer way to avoid severe toxicities. McLeod suggests that at the least, irinotecan 300 – 350 mg/m^2 every 3 weeks should not be given to patients with a known UGT1A1*28 genotype until more definitive guidelines are established [60]. However, the use of UGT1A1*28 genotyping to predict toxicity is controversial and its clinical implications are unclear. Furthermore, whether or not these recommendations are also applicable to patients with SCLC should be elucidated upon because a lower dose of irinotecan is usually administered weekly for the treatment of SCLC, rather than the every 3 or 4 weeks for colorectal cancers. In the coming decade, we must confront the metabolic and pharmacogenomic differences in various populations for the treatment of cancer. For this, international cooperative studies are warranted and indeed of immense importance.

Considering patient safety, irinotecan can indeed be administered relatively safety in patients with SCLC, provided there is careful monitoring of patients, especially regarding diarrhoea and myelosuppresion. Further studies to avoid severe toxicities are needed to advance the safe use of this otherwise promising drug.

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EGFR and ErbB2 mutation status in Japanese lung cancer patients

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Much evidence has accumulated that the epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of lung cancers. Somatic mutations of the *EGFR* gene were found in about 25–40% of Japanese lung cancer patients. More recently, *erbB2* mutations are found in about 4% of European-derived lung cancer patients. We have investigated *EGFR* and *erbB2* mutation status in 95 surgically treated nonsmall cell lung cancer (NSCLC) cases from Nagoya City University Hospital. Seventy-five adenocarcinoma cases were included. The presence or absence of *EGFR* and *ernB2* mutations of kinase domains were analyzed by reverse transcription polymerase chain reaction (RT-PCR) amplifications and direct sequences. We have also investigated *erbB2* mutation status in 27 surgically treated NSCLC cases followed by treatment with gefitinib from Kinki-chuo Chest Medical Center. *EGFR* mutations (CTG→CGG; L858R) were found from 14 of 95 lung cancer patients. We also detected the deletion 1a-type mutations from 9 patients and deletion 4-type mutations from 6 patients in exon 19. In exon 20, 4 mutations including 2 novel mutations were found. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, $p = 0.0089$) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, $p = 0.0021$). On the other hand, *erbB2* mutation was only found from 1 of 95 patients, at exon 20. This patient was female and a never smoker with adenocarcinoma. This 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). There was no *erbB2* mutation in 27 gefitinib-treated NSCLC patients. In total, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients. There was a significantly higher erbB2 positive (2+/3+) ratio in *EGFR* mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%; $p = 0.0247$). The NSCLC specimen with *erbB2* mutation showed 1+ immunoreactivity. The *EGFR* mutation status might correlate with the clinicopathologic features related to good response to gefitinib, such as gender, smoking history and pathologic subtypes of lung cancers. However, *erbB2* mutation is rare from Japanese lung cancer and is of limited value for molecular target therapy.

Key words: EGFR; lung cancer; mutations; erbB2; Japanese

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy.[1] Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan.[2] More than 15,000 patients underwent surgical operations at Japanese institutions in 1998.[2] The clinical behavior of the lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases representing an early stage of lung cancer.[3]

There is much accumulated evidence that epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer.[4,5] The erbB family comprises 4 structurally related receptors: ErbB1 (EGFR), ErbB2 (HER2-neu), ErbB3 and ErbB4. On ligand stimulation, the receptor forms either homodimers or heterodimers, which activate their cytoplasmic domain. This tyrosine-auto-phosphorylated region functions as a docking site for messenger proteins, which initiate cascades of cytoplasmic and nuclear mitogenic pathways.[6] Inhibition of this pathway is facilitated by several newly developed compounds that have shown promising results in preclinical and clinical trials.[7] The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of nonsmall cell lung cancer (NSCLC) since 2002. Trastuzumab is a recombinant DNA-derived monoclonal antibody that selectively binds to p185 HER2, the protein product of *erbB2*. Trastuzumab was approved for breast cancer[8] and clinical trials for NSCLC is underway.[9,10]

Recently, we have found that novel *EGFR* mutations' status at ATP binding pockets in Japanese NSCLC patients were correlated with the clinicopathologic features related to good response to gefitinib.[11] These *EGFR* mutations are predominantly found in Japanese lung cancer patients (about 25%) when compared to USA patients (about 8%[12–14] to 10%[15]). Kasaoka *et al.* have reported that the *EGFR* mutation ratio is 40% of Japanese lung cancer patients.[16] Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro*.[11–13] More recently, it has been reported that novel *erbB2* mutations at kinase domain were found in 4% of European-derived NSCLC patients.[17]

To determine the *EGFR* and *erbB2* mutation status in Japanese lung carcinoma for screening purposes, we investigated *EGFR* and *erbB2* mutation status by the RT-PCR amplifications and direct sequences. The findings were compared to the clinicopathologic features of lung cancer.

Material and methods

Study subjects

The study group included 95 lung cancer patients who had undergone surgery (but did not receive gefitinib) at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2002. We have also investigated erbB2 mutation status for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathologic record of lung cancer in Japan.[18] All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathologic characteristics of the 95 lung cancer patients are as follows: 52 cases at stage I, 9 at stage II and 34 at stage III–IV. The mean age was 64.9 years (range, 42–82). Among the 95 lung cancer patients, 71 (74.7%) were diagnosed as adeno-

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carcinoma, 17 (17.9%) were squamous cell carcinoma and 4 (4.2%) were adenosquamous cell carcinoma. The samples from these patients had never been sequenced for *EGFR* before.

PCR assays for EGFR and erbB2

Total RNA was extracted from lung cancer tissues and adjacent nonmalignant lung tissues using Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About 10 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo $(dT)_{12\text{-}16}$ (Amersham Pharmacia Biotech, Piscataway, NJ). The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 μl of each DNA for PCR analyses. The PCR reactions were performed using LA-Taq kit (Takara Bio, Shiga, Japan) in a 25 μl reaction volume. The primer sequences for *EGFR* gene for kinase domain (exons 18–21) were as follows: the forward primer, 5-CTCTTACACCCAGTGGAGAA-3 and the reverse primer, 5-CATCCACTTGATAGGCACTT-3 (572 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec The primer sequences for *erbB2* gene for kinase domain (exons 19–22) were as follows: the forward primer, 5-CGCTTTTGGCACAGTCTACA-3 and the reverse primer, 5-GGGATCCCATCGTAAGGTTT-3 (594bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan, Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

Immunohistochemistry

Tissue blocks were cut into 4 mm sections and mounted on silane-coated slides. The slides were then deparaffinized in xylene, dehydrated in a grade alcohol series and blocked for endogenous peroxidase with 3% H_2O_2 in absolute methanol. After microwave pretreatment in Blockace solution, immunostaining was done at 4°C overnight with a rabbit polyclonal c-erbB2 oncoprotein antibody (A04085, DakoCytomation, Glostrup, Denmark) at a 1:200 dilution. The expression of erbB2 was scored as follows: −, no discernible staining or <10% of cell stained; 1+, >10% of cytoplasmic staining or plasma membrane staining with weak intensity; 2+, >10% of plasma membrane staining with moderate intensity; and 3+, >10% of plasma membrane staining with strong intensity.

Statistical methods

Statistical analyses were done using the Mann-Whitney U-test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2test. The overall survival of lung cancer patients was examined by the Kaplan-Meier methods, and differences were examined by the log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts, Berkeley, CA) and was considered significant when the p-value was less than 0.05.

Results

EGFR gene mutation status in Japanese lung cancer patients

Using the primer sets for *EGFR* kinase domain, a PCR product of 572 bp was obtained. When we visualized the PCR products with 1% agarose gel, these samples were further studied. In exon 18, there was no G719S mutation found from this study. In exon 19, 9 patients had the del 1a type mutation, 6 patients had the deletion 4 type mutation and 1 patient had the del 1b type mutation. Seven were male and 10 were female. Thirteen were nonsmokers and 4 were smokers. Fifteen patients had adenocarcinoma, 1 had squamous cell carcinoma and 1 had adenosquamous cell carcinoma. Three of the tumors were moderately differentiated, 2 were poorly differentiated and 11 were well differentiated. Five of 15 adenocarcinomas showed bronchioloalveolar carcinoma (BAC) pattern at the edge of tumor. Thus *EGFR* mutation status at exon 19 was significantly correlated with gender ($p = 0.0172$) and tobacco-smoking ($p = 0.0008$) but not with pathologic stages (stage I *vs.* II–IV, $p = 0.9144$), subtypes (adenocarcinoma *vs.* nonadenocarcinoma, $p = 0.2675$) and differentiation of lung cancer (well *vs.* moderately or poorly differentiated, $p = 0.3812$).

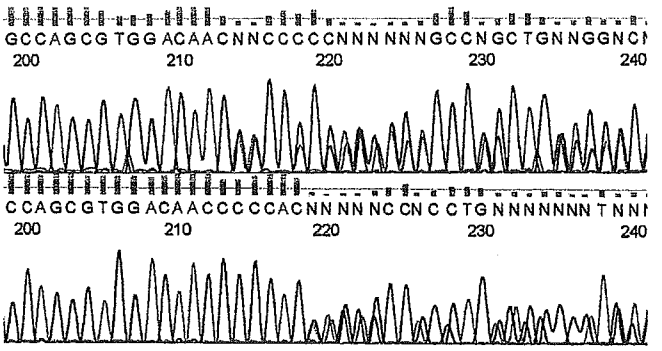

FIGURE 1 – Novel EGFR mutation at exon 20. Top: a male well-differentiated adenocarcinoma patient had the novel 2312–2313 insertion CAA. Bottom: a female, well-differentiated adenocarcinoma patient had the novel 2319–2320 insertion AACCCCCAC.

In exon 20, 3 patients had theheterozygous in-frame insertion mutations. Two were male and 1 was female. All 3 were smokers. A female, well-differentiated adenocarcinoma patient had the novel 2319-2320 insertion AACCCCCAC. A male well-differentiated adenocarcinoma patient had the novel 2312-2313 insertion CAA (Fig. 1). We have found one point mutation, C2369T (T790M). This patient also has the predominant L858R mutation (Fig. 2).

For exon 21, 14 patients had the L858R mutation and 1 patient had the L861Q mutation. Four were male and 11 were female. Twelve were nonsmokers and 3 were smokers. All 15 patients had adenocarcinoma, 1 was moderately differentiated and 14 were well differentiated. Six of 15 adenocarcinomas exhibited the BAC pattern at the edge of the tumor. Thus, exon 21 mutation status was significantly correlated with gender ($p = 0.0005$), smoking status ($p = 0.0007$), pathologic stages ($p = 0.0152$), the pathologic subtypes ($p = 0.0329$) and differentiation of lung cancer ($p = 0.0033$).

The mutations detected in lung cancer specimens from 95 lung cancer patients are summarized in Table I. Taken together, 36 mutations were found from 35 lung cancer samples in our analysis. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, $p = 0.0089$) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, $p = 0.0021$).

The overall survival of 95 lung cancer patients from Nagoya City University, with follow-up through December 30, 2003, was studied in reference to the *EGFR* mutation status. The patient with the mutation in the *EGFR* gene ($n = 35$, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* ($n = 60$, 20 were dead; log-rank test $p = 0.0143$, Breslow-Gehan-Wilcoxon test $p = 0.0220$), although the observation period was short (Fig. 3). But a multivariate analysis revealed that pathologic

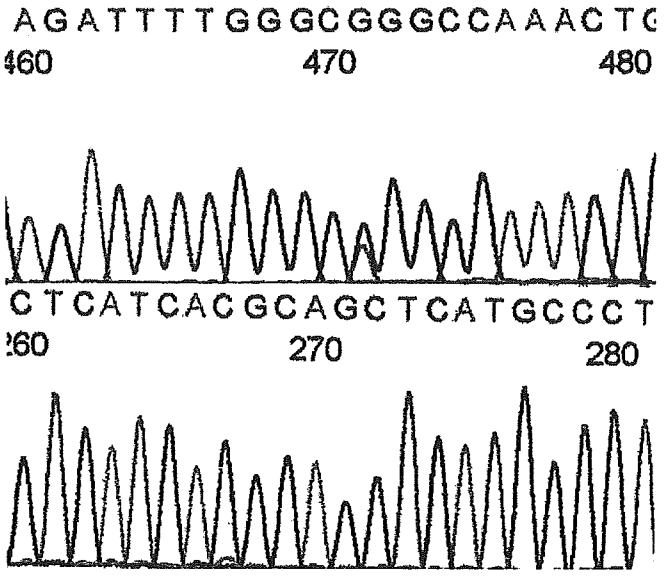

FIGURE 2 – The premoninant L858R (2573 T to G) mutation in exon 21 (top) and T790M (2369 C to T) mutation at exon 20 (bottom) within the EGFR kinase domain.

TABLE I – CLINICOPATHOLOGIC DATA OF 95 LUNG CANCER PATIENTS

Factors	*EGFR* gene status		
	Mutation patients	Wild-type patients	*p*-value
Mean age (years)			
64.9 ± 9.0	35	60	
Stage			
I	25 (72.4%)	27 (45.8%)	0.0274
II–IV	10 (28.6%)	32 (54.2%)	
Lymph node metastasis			
N0	8 (22.9%)	21 (35.0%)	0.3119
N+	27 (77.1%)	39 (65.0%)	
BI			
Never smoker	25 (71.4%)	11 (34.0%)	0.001
Smoker	10 (28.6%)	49 (66.0%)	
Differentiation			
Well	26 (78.8%)	23 (42.6%)	0.0021
Moderately or poorly	7 (21.2%)	31 (57.4%)	
Pathologic subtypes			
Adeno	32 (91.4%)	39 (74.7%)	0.0089
Nonadeno	3 (8.6%)	21 (25.3%)	
Age			
≤ 65	19 (54.3%)	29 (48.3%)	0.7269
> 65	16 (45.7%)	31 (51.7%)	
Gender			
Male	13 (37.1%)	52 (86.7%)	< 0.0001
Female	22 (62.9%)	8 (13.3%)	

N+, lymph node metastasis positive; Adeno, adenocarcinoma.

stage (p = 0.0006) was the only significant factor but not *EGFR* mutation (p = 0.1824).

ErbB2 *gene mutation status in Japanese lung cancer patients*

We identified only one *erbB2* mutation from 95 NSCLC patients. This 12-nucleotide insertion mutation (2324-2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775-776 ins YVMA) (Fig. 4). This patient was a female nonsmoker with well-differentiated adenocarcinoma, without *EGFR* mutation. Adjacent normal lung tissue exhibited a wild-type sequence for the *erbB2* gene, suggesting that this mutation was somatic. We have also done sequencing for 27 gefitinib-treated NSCLC patients. Among 27 patients, 9 patients had *EGFR* mutations (data not shown). However, no *erbB2* mutation was found within the kinase domain. Totally, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients.

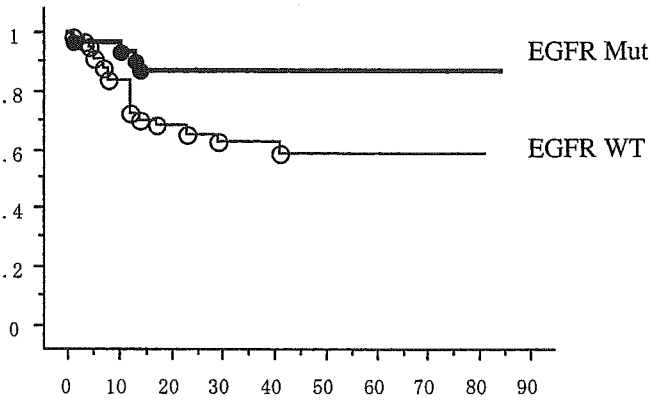

FIGURE 3 – The patient with a mutation in the *EGFR* gene (n = 35, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* (n = 60, 20 were dead) (log-rank test, p = 0.0143; Breslow-Gehan-Wilcoxon test, p = 0.0220), although the observation period was short.

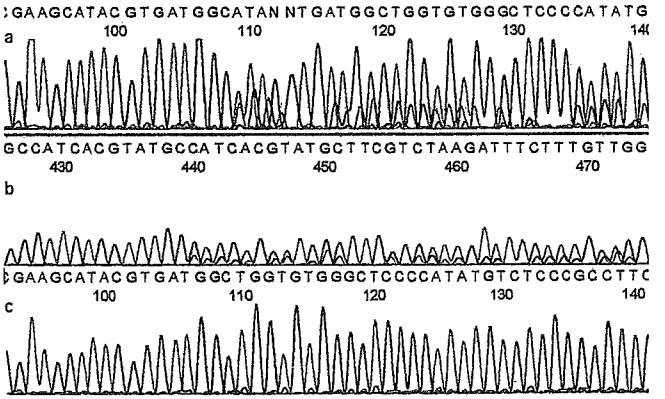

FIGURE 4 – Detection of the insertion mutation in the *erbB2* gene in genomic DNA extracted from lung cancer. *(a)* The 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). *(b)* Reverse sequence was performed and confirmed. *(c)* Adjacent normal lung tissue showed a wild-type sequence for the *erbB2* gene.

Immunohistochemistry

The immunohistochemical evaluation was done according to the scoring system described in Material and Methods. Immunohistochemistry was done only for 87 patients because the tissue blocks were not available for other patients. The erbB2-positive (2+/3+) ratio was 26.4% (23/87). There was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%) (p = 0.0247). The patient with *erbB2* mutation exhibited 1+ immunoreactivity (Fig. 5).

Discussion

We obtained findings that *EGFR* mutation status was significantly correlated with gender and smoking history of lung cancers. This was in agreement with the recent reports that *EGFR* gene mutations are common in lung cancers from never smokers[13,14] and females with adenocarcinoma.[11,14] However, our analysis also suggested that *erbB2* mutation might be less common in Japanese NSCLC patients.

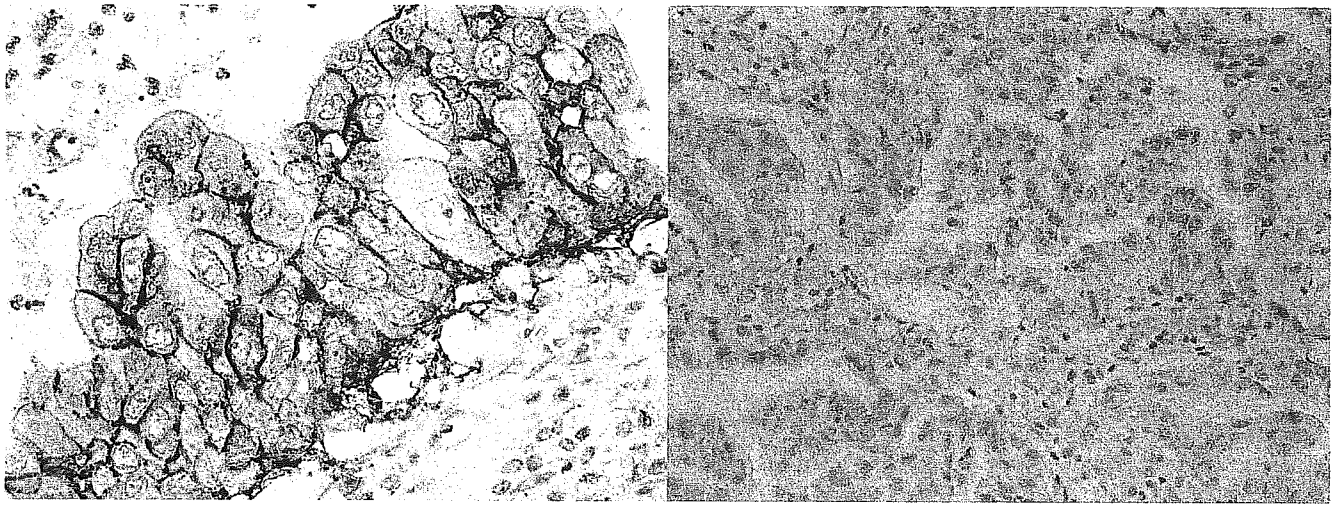

FIGURE 5 – Immunohistochemistry for ErbB2. Left: erbB2-positive (3+) section. Right: the NSCLC specimen with *erbB2* mutation exhibited 1+ immunoreactivity.

Overexpression of EGFR/ErbB2 and ErbB ligands is correlated with advanced diseases and poor patient prognosis.[19] Although *EGFR* is more abundantly expressed in lung carcinoma,[20,21] *erbB2* overexpression is less common; it is found in <35% of patients with nonsmall cell lung cancers, mainly in those with adenocarcinoma.[21] Amplification of *EGFR* and *erbB2* mRNA[22] or overexpression of their proteins[23] has been found to relate to survival in patients with NSCLC, although contradictory results have also been reported.[24,25] The drug trastuzumab, a humanized antibody against the extracellular domain of erbB2, has been approved for treatment of metastatic breast cancer and is most effective in breast cancer with *erbB2* amplification. Preliminary results suggested that the combination of chemotherapy and trastuzumab is well tolerated for NSCLC.[21] However, results from phase II trials of trastuzumab as a treatment for NSCLC have not shown any advantage for most patients[22] and have provided insufficient evidence to proceed to phase III trials.[23] Because the presence of a mutation appears to be a determination of response to therapy, as is the case with gefitinib and *EGFR* mutations, we therefore investigated the *erbB2* and *EGFR* gene mutation status. However, we have found only 1 *erbB2* mutation from 122 Japanese lung cancer patients. More recently, Shigematsu *et al.* reported that *erbB2* mutations were found in 3% (8/269) of Japanese NSCLC.[26] The single *erbB2* mutation we have found was the same as the one repeatedly found by Shigematsu *et al.*[26] Because very few NSCLC patients have gene amplification of *erbB2*, trastuzumab in the treatment of NSCLC might have a limited role.[9] Lung cancers that coexpress both EGFR and erbB2 appear to have more virulent behavior.[27] In addition, EGFR-erbB2 heterodimers are associated with a stronger and more sustained proliferative signal than EGFR homodimers.[22,28] Blockade of a signaling pathway may in theory be overcome by compensatory activation of a separate pathway in the same tumor cell. Because there was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients, blockade of both may ultimately yield superior results.

Because so many *EGFR* mutation phenotypes were discovered, it would be of interest to determine whether resistance to *EGFR* inhibition emerges through secondary mutation as is the case in imatinib-treated chronic myelogenous leukemia.[29] In our analysis, a female never smoker adenocarcinoma patient had the predominant L858R mutation as well as T790M mutation. Actually, this case was untreated with EGFR kinase inhibitors. Threonine 315 to isoleucine substitution in the Abl kinase domain was a critical structural determinant controlling inhibitor sensitivity of STI571.[29] Introduction of bulkier hydrophobic side chains at the Thr-790 position fully preserved the cellular kinase activity of the *EGFR* in the presence of selective kinase inhibitors, indicating potential mechanisms of molecular resistance formation as previously found for BCR-Abl at T315I. Previous *in vitro* study showed that mutation of T790M in the *EGFR* revealed a hotspot for resistance formation against gefitinib,[30] also *in vivo*.[31]

Over the decades, the incidence of lung adenocarcinoma has increased worldwide. Most individuals with lung adenocarcinoma (especially women) are nonsmokers,[32] who are corresponding with the sensitive population to gefitinib. In Taiwan, *EGFR* mutation ratio from adenocarcinoma was also high (55%, 38 of 69), and all of the adenocarcinomas with *EGFR* mutation were well to moderately differentiated.[33] These data were compatible for our results. Because well-differentiated adenocarcinoma patients had a better prognosis,[34] *EGFR* mutant patients showed better prognosis in our univariant analysis. The reason why many mutations were especially found in Asian, female nonsmoker adenocarcinoma remains unknown. Human papilloma virus type 16/18 infections,[35] cooking oil fume,[36] nutritional status, genetic susceptibility, immunologic infection, tuberculosis and asthma[32] have been investigated as causes of lung cancer occurring in nonsmoking women.

The findings of the breakdown of *EGFR* mutations among the 3 exons were interesting. The exon 21 mutations correlated with pathologic stage and subtype, unlike mutations in exon19. Since exon 21 mutations are more closed to the activation loop of *EGFR*, these may be more correlated with gefitinib sensitivity. Especially since 3 patients with exon 20 mutations were smokers, all of the mutations might not be equally correlated with sensitivity for gefitinib.

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Second Primary Cancers in Patients with Stage III Non-Small Cell Lung Cancer Successfully Treated with Chemo-radiotherapy

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Background: Patients successfully treated for non-small cell lung cancer (NSCLC) remain at risk for developing second primary cancer (SPC). The purpose of the current study is to assess the incidence of SPC and the impact of smoking status on the SPC in long-term survivors with stage III NSCLC after chemo-radiotherapy.
Methods: Using the database from the Japan National Hospital Lung Cancer Study Group between 1985 and 1995, information was obtained on 62 patients who were more than 3 years disease-free survivors. Details of clinical information and most smoking history were available from the questionnaire.
Results: Nine of the 62 patients developed SPC 3.9–12.2 years (median, 6.2 years) after the initiation of the treatment. The site of SPC was 2 lung, 1 esophagus, 2 stomach, 1 colon, 1 breast, 1 skin and 1 leukemia. Among these nine, three cancers occurred inside the radiation field. The relative risk of any SPC was 2.8 [95% confidence interval (CI) 1.3–5.3]. The risk changed with the passage of time and it increased significantly (5.2 times at or beyond 7 years) after the treatment. In univariate analysis, the patients who were male, had more cumulative smoking and continued smoking, had an increased risk of SPC [relative risk (RR) 2.7, CI 1.1–5.3; RR 3.0, CI 1.2–6.2; RR 5.2, CI 1.6–11.7, respectively]. In multivariate analysis, factors including smoking status and histological type had no effect on the development of a SPC.
Conclusion: The patients with stage III NSCLC successfully treated with chemo-radiotherapy were at risk for developing SPC and this risk increased with time.

Key words: second primary cancer – non-small cell lung cancer – chemo-radiotherapy

INTRODUCTION

The introduction of combined modality therapy as chest radiotherapy (RT) and chemotherapy for patients with stage III non-small cell lung cancer (NSCLC) has resulted in achieving ~15% long time survivors (123). However, patients successfully treated for NSCLC as well as small cell lung cancer (SCLC) remain at risk for developing second primary cancer (SPC) (4). The risk of SPC in patients with NSCLC has been studied mainly in cohorts of surgically resected patients for stage I NSCLC (567). These reports suggest that the risk of developing SPC and second primary lung cancer (SPLC) is 1–4% and 1–2% per patient per year, respectively, and it appears to increase with the passage of time. Another study including stages I and II patients treated with chest RT confirmed a similar trend that the risk of developing SPC and SPLC is 4.3 and 1.4% per patient per year, respectively (8). Unlike the studies of the patients with SCLC (9–11), these did not provide adequate follow-up information to determine relative risk. Also, there has been no report to date to evaluate the risk of SPC associated with the treatment of RT with chemotherapy as well as smoking status in stage III NSCLC patients.

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PATIENTS AND METHODS

Information was obtained on 1643 patients with stage III NSCLC between 1985 and 1995, using the database from the National Hospital Study Group for Lung Cancer, including