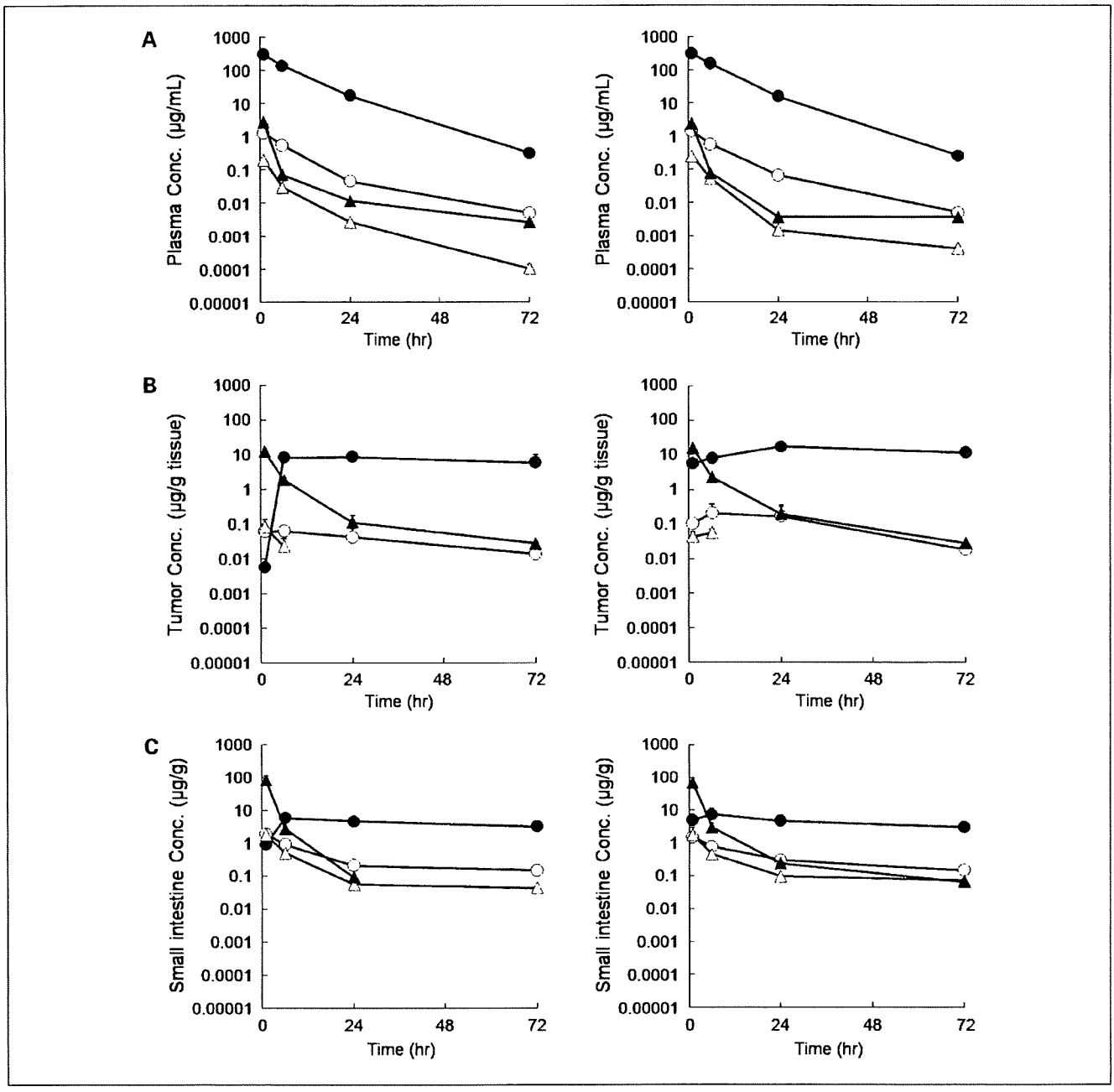

Fig. 2. Plasma, tumor, and small intestine concentrations of NK012, CPT-11, and free SN-38. Plasma (*A*), tumor (*B*), and small intestine (*C*) distribution of NK012, CPT-11, and free SN-38 after i.v. administration of CPT-11 (30 mg/kg) combined with CDDP (2.5 mg/kg) or NK012 (20 mg/kg) combined with CDDP (2.5 mg/kg). Left, SBC-3/Neo; right, SBC-3/VEGF. ●, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); Δ, SN-38 converted from CPT-11; ▲, CPT-11.

Statistical analysis. Data were analyzed with Student's *t* test when groups showed equal variances (*F* test) or with Welch's test when they showed unequal variances (*F* test). $P < 0.05$ was considered significant. All statistical tests were two sided, and data were expressed as mean ± SD.

Results

Cellular sensitivity of SCLC cells to NK012, CPT-11, SN-38, and CDDP. The IC_{50} values of NK012 for the SCLC cell lines ranged from 0.004 μmol/L (SBC-3/VEGF) to 0.041 μmol/L (H69; Table 1). The cytotoxic effects of NK012 were 198- to 532-fold higher than those of CPT-11, whereas those of NK012 were 1.10- to 2.00-fold lower than those of SN-38. These features were comparable with those reported previously (12, 13).

The molar ratios of NK012 to CDDP of 1:600 in SBC-3/VEGF, 1:120 in SBC-3/Neo, 1:150 in H69, and 1:400 in H82 were used for the drug combination studies based on the IC_{50} values of NK012 and CDDP (Table 1). The synergic to additive

effect between NK012 and CDDP was observed in these SCLC cell lines (data not shown).

Antitumor activity of NK012/CDDP and CPT-11/CDDP against SBC-3/Neo and SBC-3/VEGF tumors. SBC-3/Neo and SBC-3/VEGF tumors treated with 5 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 10 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 (P = 0.0024, SBC-3/Neo; P = 0.0437, SBC-3/VEGF). Moreover, both tumors treated with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were significantly smaller than those treated with 22 mg/kg/d CPT-11 plus 2.5 mg/kg/d CDDP on day 30 (P = 0.0058, SBC-3/Neo; P = 0.0478, SBC-3/VEGF; Fig. 1A and B). Although treatment-related BW loss was observed in mice treated with each drug combination, BW recovered to the normal level in each group by day 30 (Fig. 1C and D). A stronger antitumor activity against SBC-3/VEGF tumors was observed than against SBC-3/Neo tumors. The complete response rates achieved with 10 mg/kg/d NK012 plus 2.5 mg/kg/d CDDP were 100% and 0% for SBC-3/VEGF and SBC-3/Neo, respectively. These results further confirm our previous findings that a more potent antitumor effect of NK012 is observed in highly vascularized tumors (12).

Pharmacokinetics of NK012 and CPT-11 after NK012/CDDP and CPT-11/CDDP administration in mice bearing SBC-3/Neo or SBC-3/VEGF tumors. After CPT-11/CDDP injection, the plasma concentrations of CPT-11 and SN-38 converted from CPT-11 decreased rapidly within 6 hours in a log-linear fashion (Fig. 2A). Those of NK012 (polymer-bound SN-38) and SN-38 released from NK012 decreased more gradually (Fig. 2A). As for the CPT-11 and free SN-38 concentrations in the SBC-3/Neo and SBC-3/VEGF tumors, they decreased rapidly within 6 hours, and almost no SN-38 converted from CPT-11 was detected at 24 hours in both tumors (Fig. 2B). In the case of NK012/CDDP administration, free SN-38 released from NK012 could be detected in the tumors even at 72 hours after administration (Fig. 2B). In contrast to the case of CPT-11/CDDP administration, the concentrations of free SN-38 released from NK012 were higher in the SBC-3/VEGF tumors than in the SBC-3/Neo tumors at any time point during the observation period (significant at 1 hour; P = 0.013).

Free SN-38 concentrations in the small intestine after NK012/CDDP or CPT-11/CDDP administration were still detectable up to 72 hours in a similar fashion. CPT-11 concentrations 1 hour after CPT-11/CDDP administration were significantly higher than NK012 concentrations after NK012/CDDP administration (P = 0.0056, SBC-3/Neo; P = 0.017, SBC-3/VEGF; Fig. 2C).

These kinetic profiles in liver, spleen, lung, and kidney of free SN-38 after NK012/CDDP or CPT-11/CDDP administration were almost similar to those of NK012 or CPT-11 when administered as a single agent, as described (data not shown; ref. 12).

Intestinal toxicity of NK012, NK012/CDDP, CPT-11, and CPT-11/CDDP. Pathologic findings and characteristic mucosal changes are shown in Table 2 and Fig. 3. The small intestinal mucosa of mice in the CPT-11 or CPT-11/CDDP treatment group showed fibrotic changes, and active inflammation with cellular invasion, healed erosion, deformed glandular alignment, and glandular duct disappearance were also found. On the other hand, the small intestinal mucosa of mice in the NK012/CDDP treatment group showed only mild shortening and decreased number of villi or mild inflammatory cell invasion.

We next analyzed the concentrations of NK012, CPT-11, and free SN-38 in the feces. CPT-11 concentrations at 1 hour were significantly higher than NK012 concentrations (P = 0.0021) and decreased rapidly within 24 hours but remained detectable up to 72 hours. On the other hand, NK012 (polymer-bound SN-38) could be detected at a low concentration from 72 hours (Fig. 4A). To evaluate drug distribution over time, sections of the small intestine treated with NK012 or CPT-11 were examined by fluorescence microscopy. In the sections of CPT-11–treated small intestine, strong fluorescence originating from CPT-11 was detected in the epithelium of the small intestine, whereas weaker fluorescence originating from NK012 was distributed uniformly in the mucosal interstitium (Fig. 4B).

Discussion

Here, we compared the antitumor activity of NK012/CDDP with CPT-11/CDDP, the latter being one of the most active regimens against SCLC and NSCLC. The present data showed that when NK012/CDDP was administered, NK012 effectively accumulated in SBC-3/VEGF tumors and sufficiently exerted antitumor effects. This suggests that CDDP did not affect the permeability of tumor vessels and NK012 retention in the tumors. Hasegawa et al. (29) reported that 17 of 24 patients showed positive immunoreactivity for the VEGF protein in tumor specimens and that elevated serum VEGF levels were

Table 2. Pathologic analysis of small intestine after i.v. administration of drugs

Case no.	Treatment group	Site	Fibrosis	Inflammation	
1	Control	Jejunum	-	-	
	Control	Ileum	-	-	
2	Control	Jejunum	-	-	
	Control	Ileum	-	-	
3	Control	Jejunum	-	-	
	Control	Ileum	-	-	
4	CPT-11	Jejunum	+	+	
	CPT-11	Ileum	+	++	Erosion
5	CPT-11	Jejunum	+	+	Edema
	CPT-11	Ileum	-	-	
6	CPT-11	Jejunum	-	-	
	CPT-11	Ileum	+	+	Erosion
7	CDDP + CPT	Jejunum	+	+	
	CDDP + CPT	Ileum	-	-	
8	CDDP + CPT	Jejunum	+	+	
	CDDP + CPT	Ileum	-	+	
9	CDDP + CPT	Jejunum	+	+	
	CDDP + CPT	Ileum	-	-	
10	NK012	Jejunum	-	-	
	NK012	Ileum	-	-	
11	NK012	Jejunum	-	-	
	NK012	Ileum	-	-	
12	NK012	Jejunum	-	-	
	NK012	Ileum	-	+	
13	CDDP + NK012	Jejunum	-	+	
	CDDP + NK012	Ileum	-	+	
14	CDDP + NK012	Jejunum	-	+	
	CDDP + NK012	Ileum	-	-	
15	CDDP + NK012	Jejunum	-	+	
	CDDP + NK012	Ileum	-	-	

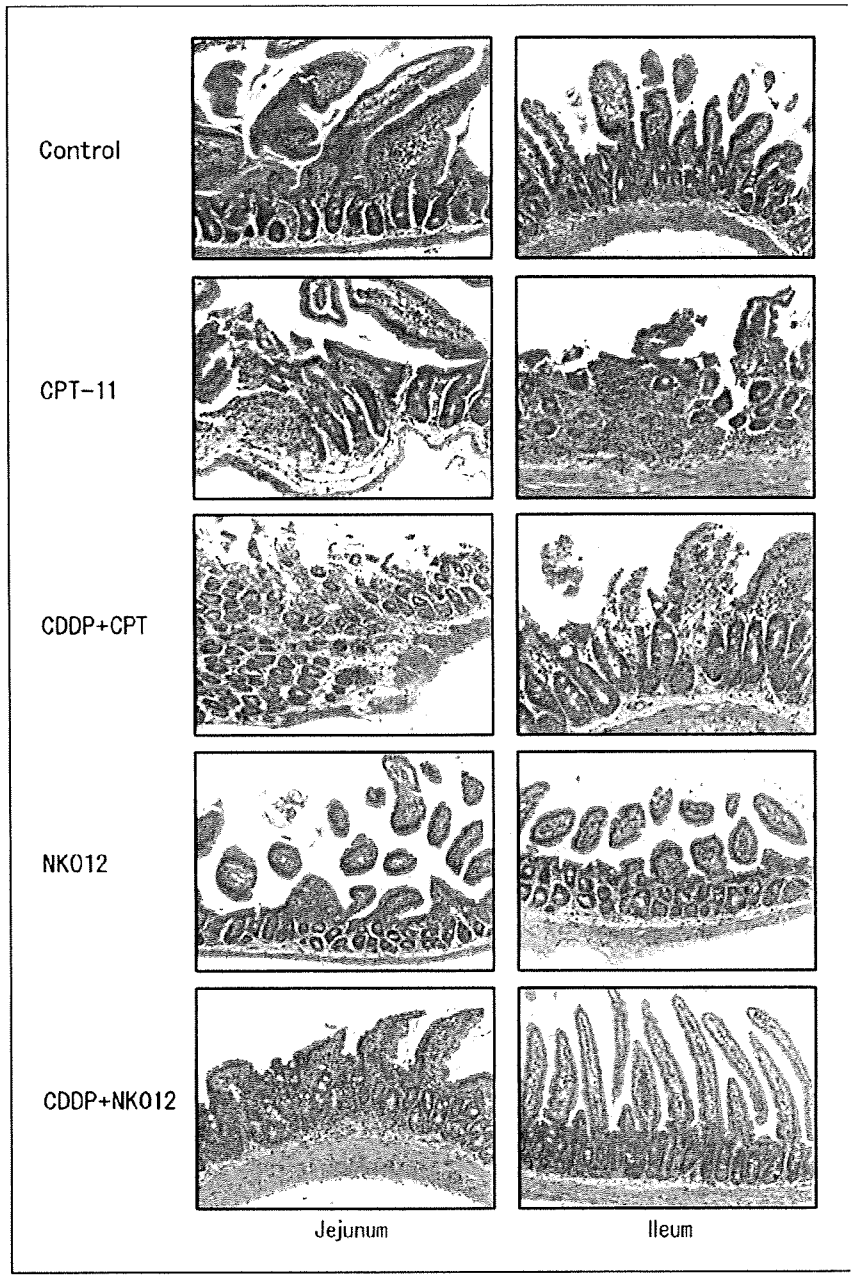

Fig. 3. Pathologic findings and characteristic mucosal changes in mouse. Jejunal and ileal mucosae from mice treated with NaCl solution (0.9%) as control, CPT-11 (22 mg/kg), CPT-11 (22 mg/kg) combined with CDDP (2.5 mg/kg), NK012 (10 mg/kg), or NK012 (10 mg/kg) combined with CDDP (2.5 mg/kg) on days 0, 7, and 14 were examined on day 28 after drug injections. The jejunal mucosa of mice in the CPT-11 treatment group showed healed erosion with fibrotic changes and lymphocytic invasion. Glandular arrangement was severely altered. Active inflammation with inflammatory cell invasion and disappearance of gland ducts were observed on the ileal mucosa in the CPT-11 treatment group. In the CPT-11/CDDP treatment group, the jejunal mucosa also showed healed erosion with scar-like fibrotic growth and mild inflammatory cell invasion into the ileal mucosa. The jejunal and ileal mucosae in the NK012 treatment group and the ileal mucosa in the NK012/CDDP treatment group were almost the same as those in the control group, that is, without inflammatory changes. The jejunal mucosa in the NK012/CDDP treatment group showed mild shortening and decreased number of villi or mild inflammatory cell invasion.

associated with poor outcome in SCLC. As for NSCLC, it was reported that the percentage of VEGF-positive cells was 52 ± 33% (95% confidence interval, 41-64%; median, 70%), and this value showed a positive association with high vascular grade (P = 0.008) and poor survival (P = 0.04; ref. 30). Taking all data together, NK012/CDDP may therefore be clinically effective against lung cancers, particularly those with high VEGF production.

Pathologic examinations were also conducted to evaluate changes in the small intestinal mucosa on day 14 after treatment. This is because diarrhea is one of the clinical dose-limiting toxicities of CPT-11, and epithelial apoptosis was reported as a mucosal change induced by CPT-11 (31). This pathologic change was observed on day 6 after i.p. administration of 100 mg/kg CPT-11 daily for 4 days. We found that the CPT-11-induced mucosal change was mainly fibrosis considered to be a form of recovery change from erosion. On the other hand, the small intestinal mucosa of the mice in the NK012/CDDP treatment group showed only mild shortening and decreased number of villi or mild inflammatory cell invasion. On comparison of these changes with those caused by CDDP (31), it was found that such alterations were mainly induced by CDDP rather than NK012.

A portion of SN-38 converted from CPT-11 undergoes subsequent conjugation as induced by UDP-glucuronyltransferase to form SN-38 β-glucuronide (SN-38-Glu; ref. 32). CPT-11, SN-38, and SN-38-Glu are excreted into the bile and then reach the small intestinal lumen (32, 33). SN-38-Glu is

deconjugated in the cecum and colon to regenerate SN-38 through bacterial β-glucuronidase (34). In this study, CPT-11 was excreted into feces much more than NK012 and a high CPT-11 concentration was detected in the small intestinal epithelium. It is speculated that the highly excreted CPT-11 is reabsorbed in the small intestinal epithelium and converted to SN-38 to cause damage to the intestinal mucosa. On the other hand, NK012 was uniformly distributed in the mucosal interstitium at a lower concentration, which may be related to the less mucosal damage and diarrhea than those induced by CPT-11, although NK012 was observed for longer period than CPT-11. About other toxic effects including bone marrow, liver,

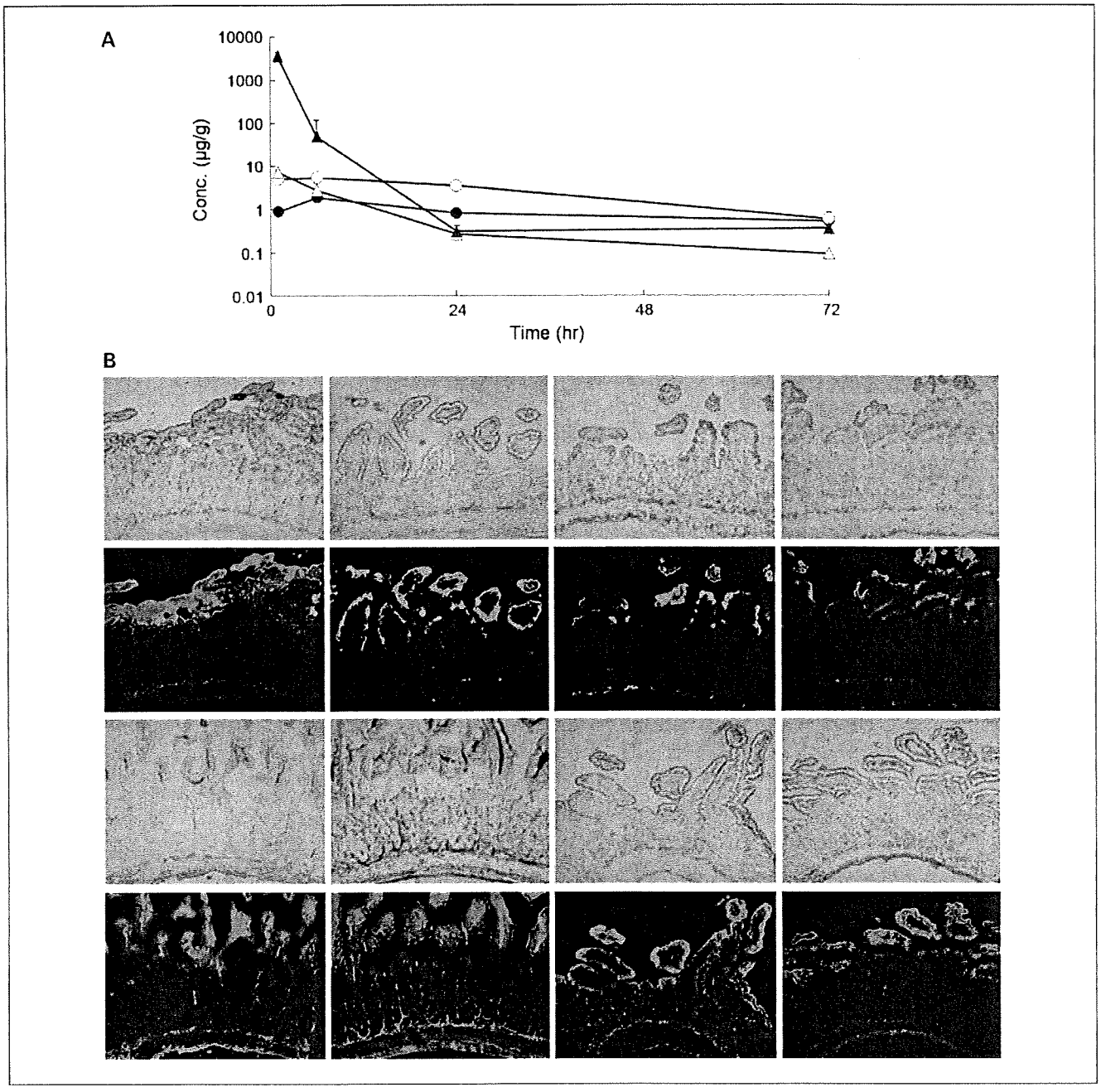

Fig. 4. Fecal concentrations of NK012, CPT-11, and free SN-38, and NK012 or CPT-11 distribution in the small intestine. *A*, distribution of NK012, CPT-11, and free SN-38 after i.v. administration of CPT-11 (30 mg/kg) or NK012 (20 mg/kg). ●, polymer-bound SN-38; ○, free SN-38 (polymer-unbound SN-38); Δ, SN-38 converted from CPT-11; ▲, CPT-11. *B*, small intestines were excised 1, 6, 24, and 72 h after i.v. administration of CPT-11 (30 mg/kg) or NK012 (20 mg/kg). Frozen sections were examined under a fluorescence microscope at a 358-nm excitation wavelength and a 461-nm emission wavelength. NK012 and CPT-11 were visualized as blue. The first or third columns are a bright-field image and the second or forth columns are a fluorescence image. Sections of small intestines were most well visualized in bright field. First, second, third, and fourth lines from the left side are images obtained 1, 6, 24, and 72 h after drug administration, respectively. CPT-11 was strongly distributed in the epithelium of the small intestine, whereas NK012 tended to be distributed weakly and uniformly in the mucosal interstitium.

and kidney toxicities, there was no significant difference between NK012/CDDP and CPT-11/CDDP in the present treatment schedule (data not shown).

In conclusion, NK012/CDDP showed a significantly higher antitumor activity with no severe diarrhea toxicity than CPT-11/CDDP, one of the most active regimens against SCLC and NSCLC. The present data suggest the clinical evaluation of NK012/CDDP in patients with SCLC and NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank N. Mie and M. Ohtsu for their technical assistance and K. Shiina and K. Abe for their secretarial assistance.

References

1. Argiris A, Murren JR. Advances in chemotherapy for small cell lung cancer: single-agent activity of newer agents. Cancer J 2001;7:228–35.
2. Bodurka DC, Levenback C, Wolf JK, et al. Phase II trial of irinotecan in patients with metastatic epithelial ovarian cancer or peritoneal cancer. J Clin Oncol 2003;21:291–7.
3. Cunningham D, Pyrhonen S, James RD, et al. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 1998;352:1413–8.
4. Negoro S, Masuda N, Takada Y, et al. Randomised phase III trial of irinotecan combined with cisplatin for advanced non-small-cell lung cancer. Br J Cancer 2003;88:335–41.
5. Mathijssen RH, van Alphen RJ, Verweij J, et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). Clin Cancer Res 2001;7:2182–94.
6. Rothenberg ML, Kuhn JG, Burris HA III, et al. Phase I and pharmacokinetic trial of weekly CPT-11. J Clin Oncol 1993;11:2194–204.
7. Slatter JG, Schaaf LJ, Sams JP, et al. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following i.v. infusion of [(14)C]CPT-11 in cancer patients. Drug Metab Dispos 2000;28:423–33.
8. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol 1988;133:95–109.
9. Maeda H, Matsumura Y. Tumoritropic and lymphotropic principles of macromolecular drugs. Crit Rev Ther Drug Carrier Syst 1989;6:193–210.
10. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 1986; 46:6387–92.
11. Matsumura Y, Maruo K, Kimura M, Yamamoto T, Konno T, Maeda H. Kinin-generating cascade in advanced cancer patients and *in vitro* study. Jpn J Cancer Res 1991;82:732–41.
12. Koizumi F, Kitagawa M, Negishi T, et al. Novel SN-38-incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor-secreting bulky tumors. Cancer Res 2006;66:10048–56.
13. Nakajima TE, Yasunaga M, Kano Y, et al. Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil. Int J Cancer 2008;122:2148–53.
14. Sumitomo M, Koizumi F, Asano T, et al. Novel SN-38-incorporated polymeric micelle, NK012, strongly suppresses renal cancer progression. Cancer Res 2008;68:1631–5.
15. Saito Y, Yasunaga M, Kuroda J, Koga Y, Matsumura Y. Enhanced distribution of NK012, a polymeric micelle-encapsulated SN-38, and sustained release of SN-38 within tumors can beat a hypovascular tumor. Cancer Sci 2008;99:1258–64.
16. Eguchi Nakajima T, Yanagihara K, Takigahira M, et al. Antitumor effect of SN-38-releasing polymeric micelles, NK012, on spontaneous peritoneal metastases from orthotopic gastric cancer in mice compared with irinotecan. Cancer Res 2008;68: 9318–22.
17. Kuroda J, Kuratsu J, Yasunaga M, Koga Y, Saito Y, Matsumura Y. Potent antitumor effect of SN-38-incorporating polymeric micelle, NK012, against malignant glioma. Int J Cancer 2009;124:2505–11.
18. Fukuda M, Nishio K, Kanzawa F, et al. Synergism between cisplatin and topoisomerase I inhibitors, NB-506 and SN-38, in human small cell lung cancer cells. Cancer Res 1996;56:789–93.
19. Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med 2002;346:85–91.
20. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol 2007;18:317–23.
21. Ohe Y, Sasaki Y, Shinkai T, et al. Phase I study and pharmacokinetics of CPT-11 with 5-day continuous infusion. J Natl Cancer Inst 1992;84:972–4.
22. Masuda N, Fukuoka M, Kusunoki Y, et al. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. J Clin Oncol 1992;10:1225–9.
23. Ohno R, Okada K, Masaoka T, et al. An early phase II study of CPT-11: a new derivative of camptothecin, for the treatment of leukemia and lymphoma. J Clin Oncol 1990;8:1907–12.
24. Kato K, Hamaguchi T, Shirao K, et al. Interim analysis of phase I study of NK012, polymer micelle SN-38, in patients with advanced cancer [abstract 485]. Proc Am Soc Clin Oncol 2008.
25. Burris III HA, Infante JR, Spigel DR, et al. A phase I dose-escalation study of NK012 [abstract 2538]. Proc Am Soc Clin Oncol 2008.
26. Natsume T, Watanabe J, Koh Y, et al. Antitumor activity of TZT-1027 (Soblidotin) against vascular endothelial growth factor-secreting human lung cancer *in vivo*. Cancer Sci 2003;94:826–33.
27. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984; 22:27–55.
28. Kudoh S, Takada M, Masuda N, et al. Enhanced antitumor efficacy of a combination of CPT-11, a new derivative of camptothecin, and cisplatin against human lung tumor xenografts. Jpn J Cancer Res 1993;84: 203–7.
29. Hasegawa Y, Takanashi S, Okudera K, et al. Vascular endothelial growth factor level as a prognostic determinant of small cell lung cancer in Japanese patients. Intern Med 2005;44:26–34.
30. Giatromanolaki A, Koukourakis MI, Kakolyris S, et al. Vascular endothelial growth factor, wild-type p53, and angiogenesis in early operable non-small cell lung cancer. Clin Cancer Res 1998; 4:3017–24.
31. Ikuno N, Soda H, Watanabe M, Oka M. Irinotecan (CPT-11) and characteristic mucosal changes in the mouse ileum and cecum. J Natl Cancer Inst 1995;87: 1876–83.
32. Atsumi R, Suzuki W, Hakusui H. Identification of the metabolites of irinotecan, a new derivative of camptothecin, in rat bile and its biliary excretion. Xenobiotica 1991;21:1159–69.
33. Chu XY, Kato Y, Sugiyama Y. Multiplicity of biliary excretion mechanisms for irinotecan, CPT-11, and its metabolites in rats. Cancer Res 1997;57:1934–8.
34. Takasuna K, Hagiwara T, Hirohashi M, et al. Involvement of β-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res 1996;56:3752–7.

Lung Cancer 64 (2009) 98–104

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Immunohistochemical expression of BCRP and ERCC1 in biopsy specimen predicts survival in advanced non-small-cell lung cancer treated with cisplatin-based chemotherapy☆

Shuji Ota[a,b,d], Genichiro Ishii[a], Koichi Goto[b], Kaoru Kubota[b], Young Hak Kim[b], Masakazu Kojika[a], Yukinori Murata[c], Manabu Yamazaki[a], Yutaka Nishiwaki[b], Kenji Eguchi[d], Atsushi Ochiai[a,*]

[a] Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan
[b] Thoracic Oncology Division, National Cancer Center Hospital East, Kashiwa, Chiba, Japan
[c] Clinical Laboratory Division, National Cancer Center Hospital East, Kashiwa, Chiba, Japan
[d] Division of Medical Oncology, Teikyo University School of Medicine, Itabashiku, Tokyo, Japan

ARTICLE INFO

Article history:
Received 18 January 2008
Received in revised form 1 July 2008
Accepted 22 July 2008

Keywords:
BCRP
MRP2
ERCC1
BRCA1
Stage IV
Non-small-cell lung cancer

ABSTRACT

Purpose: The aim of this study was to determine the prognostic value of expression of ATP binding cassette (ABC) transporter proteins and DNA repair gene proteins by immunohistochemically staining tumor biopsy specimens from patients with advanced non-small-cell lung cancer (NSCLC) being treated with platinum-based chemotherapy.
Experimental design: Expression of ABC transporter proteins, including BCRP (breast cancer resistance protein) and MRP2 (multidrug resistance proteins 2), and the DNA-repair-related proteins, ERCC1 (excision repair cross-complementation group 1) and BRCA1 (breast cancer type 1 susceptibility protein) was assessed immunohistochemically in 156 tumor samples from untreated stage IV NSCLC patients. All of the patients had received platinum-based chemotherapy. Response to chemotherapy, progression-free survival (PFS), and overall survival were compared in relation to expression of each of the proteins and to clinicopathological factors.
Results: High ERCC1 expression was associated with short survival (237 days vs. 453 days, log-rank $P = 0.03$), but not with response to chemotherapy or PFS. And high BCRP expression was associated with short survival (214 days vs. 412 days, log-rank $P = 0.02$) but not with response to chemotherapy or PFS. Multivariate analysis confirmed that negativity for the expression of BCRP tends to be an independent variable related to overall survival ($P = 0.06$).
Conclusions: This study examined ERCC1 and BCRP expression in biopsy specimens as candidates for predictors of the survival of patients with advanced NSCLC treated with platinum-based chemotherapy.

1. Introduction

More than half of all patients with non-small-cell lung cancer (NSCLC) have advanced stage IIIB or IV disease when first diagnosed, and patients with advanced NSCLC are candidates for systemic chemotherapy. Despite the increasing number of active chemotherapeutic agents available, patients with advanced NSCLC still have a median survival time of only 1 year. Intrinsic or acquired tumor-mediated drug resistance is a major clinical problem that can result in lack of tumor response to chemotherapy. Clinical investigators have recognized that several genetic abnormalities underlying NSCLC contribute to the development of the chemotherapeutic patterns that influence chemotherapeutic sensitivity to certain cytotoxic drugs. If the resistance to drugs could be explained by a simple, widely applicable method, such as immunohistochemical analysis of tumor biopsy specimens, the most effective drug candidates for the treatment could be more accurately identified.

The mechanisms of chemoresistance are likely to involve multiple gene products, and understanding of the potential modes of chemotherapeutic resistance to platinum-based chemotherapy has recently been achieved through studies that have correlated cytotoxicity with DNA repair or drug efflux [1,2]. Breast cancer

☆ This study was supported in part by a Grant-in-Aid for Cancer Research (19-10) from the Ministry of Health, Labour, and Welfare of Japan and a Grant-in-Aid for the Third Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare of Japan.

* Corresponding author at: Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa-City, Chiba 277-8577, Japan. Tel.: +81 4 7134 6855; fax: +81 7134 6865.
E-mail address: aochiai@east.ncc.go.jp (A. Ochiai).

0169-5002/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2008.07.014

resistance protein (BCRP) and multidrug resistance protein 2 (MPR2), a member of the superfamily of ATP binding cassette (ABC) transporter proteins, are involved in membrane transport during drug metabolism, and elevated expression of BCRP and MRP2 in vitro causes resistance to anticancer drugs [3–8]. Expression of BCRP and MRP2 has been found to be characterized by a reduced intracellular drug level. Moreover, there is convincing evidence that BCRP expression in biopsy specimens from patients with advanced NSCLC predicts response to chemotherapy or outcome [9].

The cytotoxic effect of anticancer platinum drugs is principally attributable to the formation of platinum-DNA adducts. Repair of these lesions in genomic DNA is mediated by both NER and Interstrand Cross-Link Repair (ICL-R) pathways, both in whose ERCC1 is a critical element [10–15]. Further, high ERCC1 expression is associated with resistance of human cancers to platinum-containing therapy [16–20].

Mutations in Breast cancer type 1 susceptibility protein (BRCA1), another DNA repair protein, can induce resistance to cisplatin-mediated apoptosis. BRCA1 is also involved in the repair of DNA damage induced by platinum drugs [21,22].

Attempts to overcome resistance have mainly involved the use of combination therapy with different classes of drugs in this study. We focused on the two different classes of proteins involved in resistance: ABC transporter proteins and DNA damage repair proteins. We quantified expression of ABC transporter (BCRP, MRP2) proteins and DNA repair genes (ERCC1, BRCA1) proteins by immunohistochemical staining of tumor biopsy, specimens collected before chemotherapy. We also evaluated the value of these proteins for predicting tumor response and survival in NSCLC patients treated with platinum-based combination therapy.

2. Materials and methods

2.1. Subjects

A total 200 of stage IV NSCLC patients received platinum-based combination chemotherapy at the National Cancer Center Hospital East between February 1996 and December 2004 because they had a PS of 0 or 1 on the Eastern Cooperative Oncology Group scale. Adequate tumor biopsy specimens collected before chemotherapy were available for 156 of these patients, and they were analyzed in this study. All tumor specimens analyzed were collected before chemotherapy. The histological classification was based on a WHO report. Clinical staging was based on an initial evaluation that consisted of a clinical assessment, chest X-ray, computed tomography of the chest and abdomen, computed tomography or magnetic resonance imaging of the brain, and bone scintigraphy. The current International Staging System was used to stage clinical disease. The clinicopathological characteristics of all of the patients are listed in Table 1. Their median age at diagnosis was 62 years (range, 39–79 years), and 44 of the 156 stage IV patients were women. All of the patients were treated with platinum-based combination chemotherapeutic regimens, which are considered standard regimens for patients with advanced NSCLC. After obtaining informed consent in accordance with institutional guidelines, all of the patients underwent tumor biopsy and chemotherapy.

2.2. Chemotherapy

All of the patients received at least 2 courses of platinum-based chemotherapy and received courses until the appearance of progressive disease. The platinum regimens were vinorelbine 25 mg/m^2 on days 1 and 8 plus cisplatin 80 mg/m^2 on day 1 of a 21-day cycle (68 patients), docetaxel 60 mg/m^2 on day 1 plus cisplatin 80 mg/m^2 on day 1 of a 21-day cycle (20 patients), irinotecan 60 mg/m^2 on days 1, 8, and 15 plus cisplatin 80 mg/m^2 on day 1 of a 28-day cycle (16 patients), gemcitabine 1000 mg/m^2 on days 1 and 8 plus cisplatin 80 mg/m^2 on day 1 of a 21-day cycle (15 patients), and paclitaxel 200 mg/m^2 administered over 3 h on day 1 plus carboplatin dosed with an area under the curve of 6 on day 1 of a 21-day cycle (28 patients). We used the standard criteria to evaluate the response to chemotherapy. Complete response was defined as the disappearance of all clinically detectable disease for at least 4 weeks. Partial response required a minimum of a 50% reduction in the sum of the products of the greatest perpendicular diameters of all measurable lesions for a minimum of 4 weeks. Progressive disease was defined as the appearance of new lesions or an increase in disease >25% measured in the same manner as for partial response. All other results were classified as "no change." The response rate was defined as the sum of the complete responses and partial responses cases expressed as a percentage of the total number of cases.

2.3. Immunohistochemistry

Immunohistochemical staining was performed on 4 μm formalin-fixed, paraffin-embedded tissue sections. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min. For antigen retrieval, the slides for BCRP (clone BXP21, dilution 1:20, Sanbio, Uden, Netherlands), MRP2 (clone M2III-6, dilution 1:20, Sanbio, Uden, Netherlands), ERCC1 (clone 8F1,dilution 1:100, Thermo Fisher, Scientific Inc., Fremont, USA), and BRCA1 (clone MS110, dilution 1:150, EMD chemicals Inc., Darmstadt, Germany) were immersed in 10 mm citric buffer solution (pH 6.0). The slides for BCRP, MRP2, and BRCA1 were heated to 95 °C by exposure to microwave irradiation for 20 min, and the slides for ERCC1 were heated to 125 °C by exposure to autoclave irradiation for 15 min. The slides were then allowed to cool for 1 h at room temperature and washed in water and PBS. Next, nonspecific binding was blocked by preincubation with 2% BSA plus 0.1% NaN_3 for 30 min. The blocking solution was drained off, and the slides were incubated overnight at 4 °C with the primary antibodies. Staining with an irrelevant mouse IgG1 or IgG2a was routinely performed as a negative control procedure. After washing three times in PBS, the slides were incubated with a labeled polymer, EnVision + Peroxidase Mouse (DAKO, Glostrup, Denmark), for 30 min. The chromogen used was 2% 3,3′-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen. Slides were counterstained with hematoxylin. Normal liver and lung tissue was used as a positive control. Staining with all antibodies was considered positive if >10% of the tumor cells stained, because a 10% cutoff level has been used in several studies using these antibodies. All of the slides were examined and scored independently by two observers (S,O and G,I) without any knowledge of the patient's clinical data. When their staining evaluations differed, the examiners discussed then, with or without reevaluating the slides, until agreement was reached.

2.4. Statistical analysis

The correlations between immunohistochemical expression and the clinical variables and response to chemotherapy were evaluated by the χ^2 test or Fisher's exact test, as appropriate. Overall survival was measured from the start of chemotherapy to the date of death from any cause or the date the patient was last known to be alive. Survival curves were estimated by the Kaplan–Meier method. The Cox proportional hazards model was used for multivariate

analysis. P values < 0.05 were considered significant. Two-sided statistical tests were used in all of the analyses. Statistical analysis software (Dr SPSSII, Windows) was used to perform the analyses

3. Results

3.1. Expression of ABC transporter and DNA damage repair proteins in NSCLC

Eighty (51%) of the 156 tumors were BCRP-positive, 26 (17%) were MRP2-positive, 100 (64%) were ERCC1-positive, and 131 (84%) were BRCA1-positive. Median percentage of staining for BCRP, ERCC1, BRCA1, and MRP2 was 20%, 40%, 50%, and 10%, respectively (the range was 0–100%).

Most of the ABC-transporter-protein-positive tumors showed mixed membranous and cytoplasmic staining. An external positive control for BCRP was canalicular membrane in liver. BCRP in the apical membrane of the bronchial layer was used an internal control, and the endothelial cells of blood vessels also stained positive. An external positive control for ERCC1 was endothelial in the tonsil and an internal positive control was stroma cells. Representative immunohistochemical BCRP and ERCC1 staining is shown in Fig. 1. The relationship between expression of ABC transporter proteins and DNA damage repair proteins and the clinical variables is shown in Table 1. ERCC1 expression and BRCA1 expression were significantly greater in the patients with a smoking history (≧20 pack years) (P = 0.015). BRCA1 expression was significantly greater in the males than in the females (P = 0.027). BRCA1 expression correlated to ERCC1 expression (P = 0.003). BCRP expression correlated to ERCC1 (P = 0.012), MRP2 (P = 0.005), but not BRCA1 (P = 0.126) (data not shown).

3.2. Expression of ABC transporter and DNA damage repair proteins and clinical outcome

It was possible to assess all 156 patients for response to chemotherapy and to analyze their survival data. The relationships between clinical variables and response to chemotherapy and survival in this study are shown in Table 2. Only "smoking history" was significantly associated with both PFS (P = 0.05) and overall survival (P = 0.02). Table 3 shows the relationships between expression of ABC transporter proteins and DNA damage repair proteins and the response to chemotherapy and survival. No significant associations were found between MRP2 expression and response to chemotherapy (P = 0.63), PFS (P = 0.94), or survival (P = 0.96), and between BRCA1 expression and response to chemotherapy (P = 0.62), PFS (P = 0.67), or survival (P = 0.06). By contrast, BCRP expression was significantly associated with both PFS (P = 0.02) and survival (P = 0.02), but not with response to chemotherapy (P = 0.15). ERCC1 expression was associated with overall survival (P = 0.03) but not with response to chemotherapy (P > 0.09) or PFS (P = . 0.06).

3.3. Multivariate analysis for PFS and overall survival

Multivariate analysis was performed by using the Cox proportional hazards model to determine whether the prognostic value of BCRP or ERCC1 disappeared when other prognostic factors were considered (Tables 4 and 5). A multivariate analysis that included gender, age, smoking history, PS, histology, BCRP, and ERCC1, showed that BCRP was not a significant independent variable correlated with PFS (P = 0.13) but overall survival was marginal (P = 0.06). The BCRP-positive value for overall survival yielded a hazard ratio of 0.72, with a 95% confidence interval of 0.51–1.01. The results show that negativity for the expression of BCRP tends to be a prognostic factor in advanced NSCLC. The PFS and overall survival curves drawn by the Kaplan–Meier method are shown according to BCRP in Fig. 2. Median survival time in the BCRP-negative group was 412 days, as opposed to 214 days in the BCRP-positive group.

4. Discussion

In this study the BCRP-positive cases had a shorter overall survival time, and BCRP expression tend to be a prognostic factor overall survival in the multivariate analysis. Expression of MRP2, on the other hand, was not an independent prognostic factor, a finding that was consistent with previous studies [9,10]. MRP2 was studied within the IALT biologic program [23]. This was the largest group of NSCLC patients used for the study of MRP2 expression and the result was that MRP2 does not predict response to adjuvant cisplatin-based chemotherapy. Yoh et al. found that the expression of BCRP in stages III and IV NSCLC patients was a significant independent variable that correlated with PFS and tend to correlated

Table 1
Relationship between clinical variables and immunohistochemical expression

	n (%)	BCRP-positive patients	MRP2-positive patients	ERCC1-positive patients	BRCA1-positive patients
Total	156	80	26	100	131
Gender					
Male	112 (72)	60	22	76	99[b]
Female	44 (28)	20	4	24	32
Age					
≧70	34 (22)	22	7	25	32
<70	122 (78)	58	19	75	99
Histology					
Ad	100 (64)	50	20	61	81
Non-ad	56 (36)	30	6	39	50
PS					
0	38 (24)	15	4	23	29
1	118 (76)	65	22	77	102
Smoking history					
≧20 pack year	99 (63)	56	19	71[a]	89[c]
<20 pack year	57 (37)	24	7	29	42

[a] P = 0.015.
[b] P = 0.027.
[c] P = 0.012.

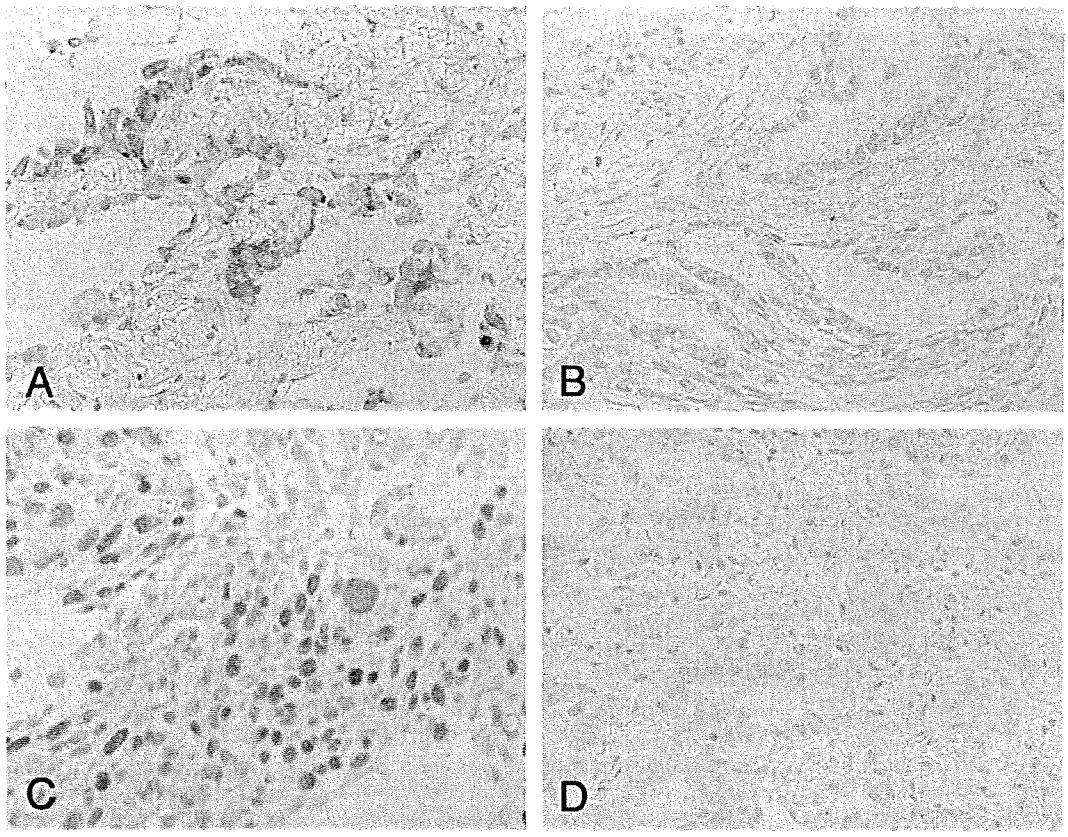

Fig. 1. Typical immunohistochemical staining patterns of NSCLC tumor biopsy specimens for BCRP (A and B) and ERCC1 (C and D). (A) Adenocarcinoma showing membrane staining for BCRP. (B) BCRP-negative adenocarcinoma. (C) Squamous cell carcinoma with positive nuclear staining for ERCC1. (D) ERCC1-negative adenocarcinoma.

with response to chemotherapy or overall survival in a multivariate analysis [9]. We think that the results of the present study reinforce the reliability of the prognostic significance of BCRP expression in stage IV NSCLC patients.

ERCC1 expression and BRCA1 expression were significantly greater in the patients with a smoking history ($P = 0.015$, $P = 0.012$ respectively). The correlation between smoking history and ERCC1, BRCA1 expression is often a lack in previous studies such as in the IALT-bio study. Fujii et al. [24] and Lee et al. [25] reported relationship between ERCC1, BRCA1 expression and smoking history, which tended to be greater in the patients with a smoking history but it was not significant statistically. Relationship between DNA repair gene protein expression and DNA damage arised from smoking could only be presumed. Interestingly, we also noticed in the present study that patients with high ERCC1 and BRCA1 double expression in tumors had shorter survival than patients who have

Table 2
Summary of relationship between clinical variables and response to chemotherapy or survival

	n	Response rate (%)	*P*	PFS (day)	*P*	MST (day)	*P*
Total	156	26		163		317	
Gender							
Male	112	24	0.32	155	0.17	307	0.23
Female	44	32		223		324	
Age							
≧70	34	18	0.27	119	0.10	261	0.14
<70	122	29		171		333	
Histology							
Ad	100	22	0.13	148	0.32	366	0.55
Non-ad	56	34		180		261	
PS							
0	38	26	>0.99	184	0.35	386	0.23
1	118	26		153		274	
Smoking history							
≧20 pack year	99	23	0.26	151	0.05	256	0.02
<20 pack year	57	32		223		426	

PFS: progression free survival, MST: median survival time.

Table 3
Relationship between immunohistochemical expression and response to chemotherapy or survival

	n	Response rate (%)	P	PFS (day)	P	MST (day)	P
BCRP							
Positive	80	21	0.15	148	0.02	214	0.02
Negative	76	32		211		412	
MRP2							
Positive	26	31	0.63	161	0.94	344	0.96
Negative	130	25		165		304	
ERCC1							
Positive	100	26	>0.99	148	0.06	237	0.03
Negative	56	27		187		453	
BRCA1							
Positive	131	27	0.62	161	0.67	261	0.06
Negative	25	20		184		461	

PFS: progression free survival, MST: median survival time.

Table 4
Multivariate analysis for overall survival of 156 patients

Variables	Category	Risk ratio	95% CI	P
Gender	Male vs. female	1.08	0.68–1.72	0.74
Age	≧70 vs. <70	1.18	0.75–1.82	0.50
PS	0 vs. 1	1.16	0.77–1.75	0.48
Histology	Ad vs. non-ad	1.33	0.93–1.90	0.12
Smoking history	≧20 vs. <20	1.47	0.96–2.27	0.08
BCRP	(−) vs. (+)	0.72	0.51–1.01	0.06
ERCC1	(−) vs. (+)	0.75	0.52–1.07	0.12

other expression pattern for those two markers ($p = 0.0027$) (data not shown).

A relation between expression of ERCC1 mRNA and resistance to platinum-based chemotherapy has been corroborated by small, retrospective studies in patients with advanced gastric, ovarian, colorectal, and esophageal cancer and in NSCLC patients [16–20]. Simon et al. reported that patients who have undergone complete resection of NSCLC with high ERCC1 mRNA expression have a better survival than patients with low ERCC1 mRNA expression [26]. Olaussen et al. found that patients who underwent complete resection of ERCC1-negative NSCLC appeared to benefit from adjuvant cisplatin-based chemotherapy, and, showed that in the group that had not received adjuvant therapy patients with ERCC1-positive tumors had a longer overall survival than patients with ERCC1-negative tumors [27]. Our findings showed that expression of ERCC1 was significantly associated with overall survival but not with response to chemotherapy or PFS. Thus, ERCC1 expressing tumor may become a poor prognostic tumor by other factors induced by administered chemotherapy (e.g., tolerance to DNA damage).

Rosell's group in Barcelona reported overexpression of BRCA1 mRNA was strongly associated with poor survival in NSCLC patients [28]. In this study, BRCA1 protein expression was borderline significance for correlation with overall survival. The reason of this discrepantly result might be explained by the difference of study population. Rosell's group study targeted for operable early stage NCSLC. To solve this problem, further investigation was needed.

In our study, BCRP expression, alone not ERCC1 expression, was of marginal significant prognostic value for MST in the multivariate analysis. Liedert et al. reported overexpression of ABC transporter protein in a cisplatin-resistant melanoma cell line [29], and this observation was accompanied by reduced formation of platinum-DNA adducts measured by an immunocytologic assay. The overexpression of ABC transporter may regulate the formation of platinum-DNA adducts in tumor cells, and BCRP may act upstream during the chemoresistance process. This mechanism may have been responsible for BCRP expression alone, not ERCC1 expression, being of marginal significant prognostic value for MST in the multivariate analysis in this study.

It is noteworthy that there was no significant association between any of the markers and response to chemotherapy. One explanation might be that response rate by itself did not correlate to survival in our study. Another consideration to answer to the discrepancy between response, PFS and overall survival, is that ERCC1 expressing tumor may become a poor prognostic tumor due to other factors than those induced by chemotherapy. Illustration of this notion came from a recent study by Hadnagy et al. [30] who recently reported a relationship between BCRP expression and cancer stem cells that seemed to play a pivotal role in tumor progression. BCRP expression by itself may be poor prognostic factor regardless of chemotherapy. A widely used flow cytometry assay for identifying cancer stem cells defines a "side-population" (SP) of cells that display Hoechst 33342 [31]. Cancer stem cells can be puri-

Table 5
Multivariate analysis for progression free survival of 156 patients

Variables	Category	Risk ratio	95% CI	P
Gender	Male vs. Female	0.90	0.56–1.49	0.70
Age	≧70 vs. < 70	1.24	0.78–1.98	0.37
PS	0 vs. 1	1.10	0.70–1.74	0.68
Histology	Ad vs. Non-ad	1.39	0.93–2.07	0.11
Smoking history	≧20 vs. <20	1.41	0.91–2.21	0.12
BCRP	(−) vs.(+)	0.72	0.48–1.10	0.13
ERCC1	(−) vs.(+)	0.82	0.54–1.26	0.37

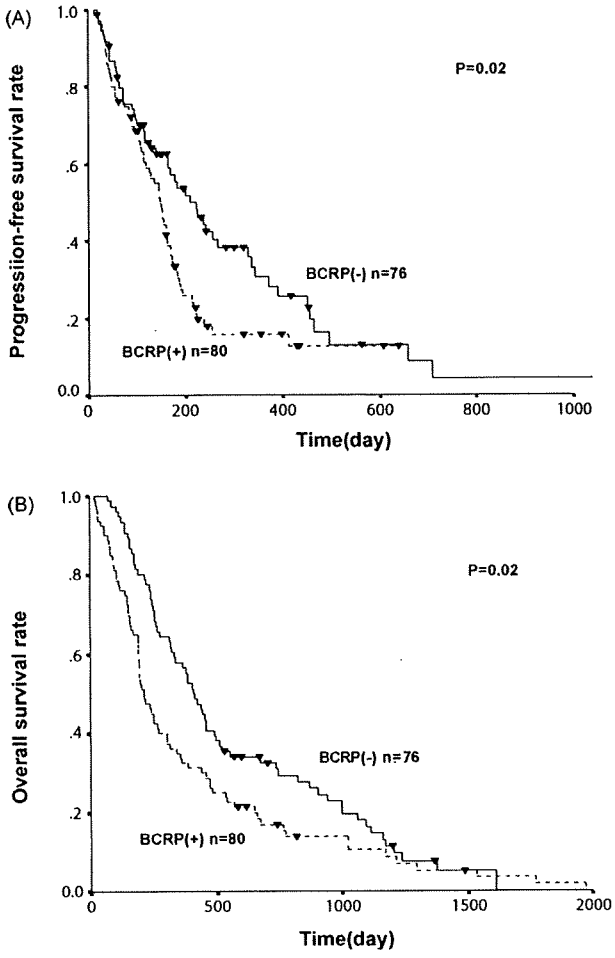

Fig. 2. (A) Progression-free survival curve of 156 patients with advanced non-small-cell lung cancer, according to BCRP expression. The median progression-free survival period of the BCRP-negative patients and BCRP-positive patients was 211 and 148 days, respectively. (B) Overall survival curves of 156 patients with advanced non-small-cell lung cancer, according to BCRP expression. Patients with BCRP-negative tumors survived longer than those with BCRP-positive tumors, and the difference was statistically significant ($P = 0.02$).

fied based on the efflux of dyes and Hoechst 33342 [32,33]. Zhou et al. reported that BCRP mRNA is expressed in a wide variety of stem cells and is a molecular determinant of the SP cells, and moreover, dyes and Hoechst 33342 efflux activity was provided by BCRP expression in mice [34]. Haraguchi et al. [35] reported significantly increased BCRP expression in SP cells in human gastrointestinal system cancer cell lines and that SP cells exhibited greater resistance to chemotherapy. The self-renewal and chemoresistance capacities of these cancer SP cells may also play important roles in maintaining cancer foci to proliferate after chemotherapy and radiotherapy. Investigating whether BCRP-positive cells have the characteristics, as cancer stem cells in NSCLC will be a future task.

We speculate, that patients with tumors that are positive for BCRP expression show drug resistance to platinum-based chemotherapy. We suggest that BCRP serve as molecular target for reducing drug resistance. Kuppens et al. reported a phase I study of Elacridar (GF120918) [36,37]. Minderman et al. [38] reported Bricodar (VX710) increases drug retention and enhances chemosensitivity in resistant cells expressing BCRP. Another approach to solving platinum-based chemotherapeutic resistance, non-platinum chemotherapy will be alternative regimen for the subgroup which over express BCRP protein.

Conflict of interest

The authors certify that there are no potential conflicts of interest.

References

[1] Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. Oncologist 2003;8:411–24.
[2] Rosell R, Lord RV, Taron M, Reguart N. DNA repair and cisplatin resistance in non-small-cell lung cancer. Lung Cancer 2002;38:217–27.
[3] Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998;95:15665–70.
[4] Brangi M, Litman T, Ciotti M, Nishiyama K, Kohlhagen G, Takimoto C, et al. Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. Cancer Res 1999;59:5938–46.
[5] Maliepaard M, van Gastelen MA, de Jong LA, Pluim D, van Waardenburg RC, Ruevekamp-Helmers MC, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res 1999;59:4559–63.
[6] Ross DD, Yang W, Abruzzo LV, Dalton WS, Schneider E, Lage H, et al. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J Natl Cancer Inst 1999;91:429–33.
[7] Wright SR, Boag AH, Valdimarsson G, Hipfner DR, Campling BG, Cole SP, et al. Immunohistochemical detection of multidrug resistance protein in human lung cancer and normal lung. Clin Cancer Res 1998;4:2279–89.
[8] Dingemans AC, van Ark-Otte J, Span S, Scagliotti GV, van der Valk P, Postmus PE, et al. Topoisomerase IIalpha and other drug resistance markers in advanced non-small cell lung cancer. Lung Cancer 2001;32:117–28.
[9] Yoh K, Ishii G, Yokose T, Minegishi Y, Tsuta K, Goto K, et al. Breast cancer resistance protein impacts clinical outcome in platinum-based chemotherapy for advanced non-small cell lung cancer. Clin Cancer Res 2004;10:1691–7.
[10] Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF, et al. The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. Mol Cell Biol 2004;24:5776–87.
[11] Grillari J, Katinger H, Voglauer R. Contributions of DNA interstrand cross-links to aging of cells and organisms. Nucleic Acids Res 2007;35:7566–76.
[12] Fisher LA, Bessho M, Bessho T. Processing of a psoralen DNA interstrand cross-link by XPF-ERCC1 complex in vitro. J Biol Chem 2008;283:1275–81.
[13] Bergstralh DT, Sekelsky J. Interstrand crosslink repair: can XPF-ERCC1 be let off the hook? Trends Genet 2008;24:70–6.
[14] Al-Minawi AZ, Saleh-Gohari N, Helleday T. The ERCC1/XPF endonuclease is required for efficient single-strand annealing and gene conversion in mammalian cells. Nucleic Acids Res 2008;36:1–9.
[15] Zhang N, Liu X, Li L, Legerski R. Double-strand breaks induce homologous recombinational repair of interstrand cross-links via cooperation of MSH2, ERCC1-XPF, REV3, and the Fanconi anemia pathway. DNA Repair (Amst) 2007;6:1670–8.
[16] Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. J Clin Invest 1994;94:703–8.
[17] Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, et al. ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. J Clin Oncol 1998;16:309–16.
[18] Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, et al. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. J Clin Oncol 2001;19:4298–304.
[19] Joshi MB, Shirota Y, Danenberg KD, Conlon DH, Salonga DS, Herndon 2nd JE, et al. High gene expression of TS1, GSTP1, and ERCC1 are risk factors for survival in patients treated with trimodality therapy for esophageal cancer. Clin Cancer Res 2005;11:2215–21.
[20] Lord RV, Brabender J, Gandara D, Alberola V, Camps C, Domine M, et al. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. Clin Cancer Res 2002;8:2286–91.
[21] Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J Biol Chem 2000;275:23899–903.
[22] Quinn JE, Kennedy RD, Mullan PB, Gilmore PM, Carty M, Johnston PG, et al. BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. Cancer Res 2003;63:6221–8.
[23] Filipits M, Haddad V, Schmid K, Huynh A, Dunant A, Andre F, et al. Multidrug resistance proteins do not predict benefit of adjuvant chemotherapy in patients

with completely resected non-small cell lung cancer: International Adjuvant Lung Cancer Trial Biologic Program. Clin Cancer Res 2007;13:3892–8.
[24] Fujii T, Toyooka S, Ichimura K, Fujiwara Y, Hotta K, Soh J, et al. ERCC1 protein expression predicts the response of cisplatin-based neoadjuvant chemotherapy in non-small-cell lung cancer. Lung Cancer 2008;59:377–84.
[25] Lee HW, Han JH, Kim JH, Lee MH, Jeong SH, Kang SY, et al. Expression of excision repair cross-complementation group 1 protein predicts poor outcome in patients with small cell lung cancer. Lung Cancer 2008;59:95–104.
[26] Simon GR, Sharma S, Cantor A, Smith P, Bepler G. ERCC1 expression is a predictor of survival in resected patients with non-small cell lung cancer. Chest 2005;127:978–83.
[27] Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med 2006;355:983–91.
[28] Rosell R, Skrzypski M, Jassem E, Taron M, Bartolucci R, Sanchez JJ, et al. A novel prognostic factor in resected non-small-cell lung cancer. PLoS ONE 2007;2:e1129.
[29] Liedert B, Materna V, Schadendorf D, Thomale J, Lage H. Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin. J Invest Dermatol 2003;121:172–6.
[30] Hadnagy A, Gaboury L, Beaulieu R, Balicki D. SP analysis may be used to identify cancer stem cell populations. Exp Cell Res 2006;312:3701–10.
[31] Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797–806.
[32] Spangrude GJ, Johnson GR. Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci USA 1990;87:7433–7.
[33] Wolf NS, Kone A, Priestley GV, Bartelmez SH. In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp Hematol 1993;21:614–22.
[34] Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028–34.
[35] Haraguchi N, Utsunomiya T, Inoue H, Tanaka F, Mimori K, Barnard GF, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 2006;24:506–13.
[36] Maliepaard M, van Gastelen MA, Tohgo A, Hausheer FH, van Waardenburg RC, de Jong LA, et al. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. Clin Cancer Res 2001;7:935–41.
[37] Kuppens IE, Witteveen EO, Jewell RC, Radema SA, Paul EM, Mangum SG, et al. A phase I, randomized, open-label, parallel-cohort, dose-finding study of Elacridar (GF120918) and oral topotecan in cancer patients. Clin Cancer Res 2007;13:3276–85.
[38] Minderman H, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. Clin Cancer Res 2004;10:1826–34.

Carcinogenesis vol.30 no.1 pp.65–70, 2009
doi:10.1093/carcin/bgn257
Advance Access publication November 12, 2008

Contribution of nicotine acetylcholine receptor polymorphisms to lung cancer risk in a smoking-independent manner in the Japanese

Kouya Shiraishi, Takashi Kohno, Hideo Kunitoh[1], Shunichi Watanabe[2], Koichi Goto[3], Yutaka Nishiwaki[3], Yoko Shimada, Hiroshi Hirose[4], Ikuo Saito[4], Aya Kuchiba[5], Seiichro Yamamoto[6] and Jun Yokota*

Biology Division, National Cancer Center Research Institute, [1]Thoracic Oncology Division and [2]Thoracic Surgery Division, National Cancer Center Hospital, Tokyo 1040045, Japan, [3]Division of Thoracic Oncology, National Cancer Center Hospital East, Chiba 2778577, Japan, [4]Health Center, Keio University School of Medicine, Tokyo 1608582, Japan, [5]Genetics Division, National Cancer Center Research Institute and [6]Cancer Information Services and Surveillance Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo 1040045, Japan

*To whom correspondence should be addressed. Tel: +81 3 3542 2511; Fax: +81 3 3542 0807;
Email: jyokota@ncc.go.jp

Recent genome wide association (GWA) studies on European and American populations revealed association with lung cancer risk of single-nucleotide polymorphisms (SNPs) in the locus containing two nicotine acetylcholine receptor (*CHRNA*) genes, whose involvement in tobacco addiction had been indicated. Association with lung cancer risk in smokers was consistently, but that in non-smokers as well as that with smoking behavior was inconsistently, observed in these studies. To obtain further information on the significance of *CHRNA* SNPs in lung cancer risk, association of seven SNPs in this locus with lung cancer risk as well as smoking status was examined in a Japanese population by a case–control study of 1250 cases (562 adenocarcinoma, 391 squamous cell carcinoma and 297 small cell carcinoma) and 936 controls. The frequency of the haplotype consisting of minor alleles for three SNPs, rs8034190, rs16969968 and rs1051730, which had been defined as a susceptible haplotype in the GWA studies, was much lower in the Japanese population (0.013) than in European and American populations (0.3–0.4). However, this haplotype was significantly associated with lung cancer risk also in Japanese (odds ratio = 2.3, 95% confidence interval = 1.5–3.7, P = 0.00028, respectively). The association was observed both in smokers and non-smokers and in all histological types of lung cancers. Individuals with this haplotype showed higher smoking doses than those without; however, the difference was not statistically significant. These results strongly indicate that *CHRNA* SNPs confer lung cancer susceptibility in a small subset of Japanese in a smoking-independent manner.

Introduction

Tobacco smoking is the major cause of lung cancer in most human populations (1). Recently, the locus containing two genes encoding nicotine acetylcholine receptor subunits, *CHRNA3* and *CHRNA5*, was shown to be associated with lung cancer risk in European and American populations by three genome wide association (GWA) studies (2–4). CHRNA proteins are expressed in lung epithelial cells and bind nicotine, an addictive compound in cigarette smoke, and nitrosamines, potential lung carcinogens in cigarette smoke and foods (5–7). Signal transduction through CHRNA proteins was suggested to cause cell proliferation and also to facilitate neoplastic transformation (8,9). Therefore, if we assume that CHRNA proteins generally transduces a signal of nicotinic substrates in cigarette smoke, a single-nucleotide polymorphism (SNP) in the *CHRNA* genes will be associated with lung cancer risk especially in smokers. Alternatively, if CHRNA proteins transduces a signal of nitrosamines in food as well, a SNP in the *CHRNA* genes could be associated in both smokers and non-smokers. On the other hand, involvement of the same locus in tobacco addiction has been also indicated (4,10,11). Particularly, the rs16969968 SNP causes a change of amino acid conserved across species in CHRNA5 protein, thus, is thought to be a responsible SNP for tobacco addiction (10). Therefore, it is also possible that *CHRNA* SNPs confer lung cancer risk in tobacco addiction-dependent manner. Association of *CHRNA* SNPs with lung cancer risk in smokers was consistently observed among three GWA studies (2–4). However, association in non-smokers was observed only in individuals of European countries and Canada (3) and was not in those of the USA and UK (2). Association of *CHRNA* SNPs with tobacco addiction was also observed inconsistently among the three studies. Association was observed in individuals of Iceland, Spain and The Netherlands (4); however, such an association was observed only in former but not in current smokers of the USA and UK (2) and was not observed in individuals of European countries and Canada (3). Therefore, the mode of association of *CHRNA* SNPs with lung cancer risk is still unclear. Thus, more information on the association in a variety of populations is necessary to elucidate the significance of *CHRNA* SNPs in lung cancer risk. In addition, in the GWA studies above, the association of *CHRNA* SNPs was not examined in Asians due to the low frequency of risk alleles, therefore, it remains also unknown whether or not and how *CHRNA* polymorphisms confer susceptibility to lung cancer in Asians. We conducted here a case–control study to examine the association of *CHRNA* polymorphisms with risks for three major histological types of lung cancer, adenocarcinoma (ADC), squamous cell carcinoma (SQC) and small cell carcinoma (SCC), as well as smoking status in a Japanese population, and the results were compared with those from European and American populations.

Abbreviations: ADC, adenocarcinoma; GWA, genome wide association; LD, linkage disequilibrium; SCC, small cell carcinoma; SNP, single-nucleotide polymorphism; SQC, squamous cell carcinoma.

Subjects and methods

Case–control study

All cases and controls were Japanese. The cases consisted of 562 ADC, 391 SQC and 216 SCC patients of the National Cancer Center Hospital located in Tokyo and 81 SCC patients of the National Cancer Center Hospital East located in Chiba, a prefecture neighboring Tokyo, from 1999 to 2007. The controls were 936 volunteers of National Cancer Center Hospital and Keio University in Tokyo. All the lung cancer cases, from whom informed consents as well as blood samples were obtained, were consecutively included in this study without any particular exclusion criteria. All the lung cancer cases were diagnosed as ADC, SQC or SCC by histological examinations according to World Health Organization classification (12,13). All the control subjects were selected with a criterion of no history of any cancer. Characteristics of a subset of cases and controls were described previously (14,15). This study was approved by the institutional review boards of the National Cancer Center.

Smoking history of cases and controls was obtained via interview using a questionnaire. Smoking dose of each subject was expressed by 'cigarettes per day', i.e. the number of cigarettes smoked per day on average on most days, whereas smoking exposure of each subject was expressed by 'pack-years', which was defined as the number of pack per day (i.e. cigarettes per day divided by 20) multiplied by years of smoking as in previous studies (2–4). Smokers were defined as those who had smoked regularly for 12 months or longer at any time in their life, whereas non-smokers were defined as those who had not. There were no individuals who had smoked regularly for <12 months. From each individual, a 10 or 20 ml whole-blood sample was obtained. Genomic DNA was extracted from whole-blood samples as described previously (14) and 10 ng of genomic DNA was subjected to genotyping using TaqMan assays and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster city, CA).

Statistical analysis

A Hardy–Weinberg equilibrium test was performed using the SNPAlyze version 3.1 software (DYNACOM Co., Ltd, Chiba, Japan). Calculation of the

D' and R^2 values as well as haplotype estimation was undertaken using the expectation-maximization algorithm of the same software. The strength of association of alleles and genotypes with ADC, SQC and SCC risks was measured as crude odds ratios (ORs). The strength of association of genotypes was also measured as ORs adjusted for gender, age and smoking using an unconditional logistic regression analysis (16). These statistical analyses were performed using the JMP version 6.0 software (SAS Institute, Cary, NC). A level of $P < 0.05$ for an OR was considered significant, whereas a level of $0.05 \leq P < 0.10$ for an OR was considered marginal.

Results

We conducted a case–control study consisting of 1250 cases and 936 controls (Table I). All the subjects were Japanese. Most of the SQC and SCC cases were male and smokers, whereas approximately half of ADC cases were smokers, as has been reported (1,17). Seven SNPs were selected from the locus containing the *CHRNA* genes (Figure 1). Two of them, rs8034191 and rs1051730, were the SNPs whose contribution to lung cancer susceptibility as well as smoking behavior was shown in previous GWA studies (2–4). Another one was rs16969968 in *CHRNA5*, whose association with nicotine dependence was previously reported (10). The remaining four were selected based on the fact that minor allele frequencies in the Japanese are reported to be >10% in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

All the cases and controls were genotyped for the seven SNPs (Table II). All these SNPs were in Hardy–Weinberg equilibrium both in cases and controls ($P > 0.05$). Similar and significant differences in the minor allele frequencies between controls and cases were observed in three SNPs, rs8034191, rs16969968 and rs1051730, all of which had minor allele frequencies of <0.02 in the controls (Table II). Two SNPs, rs16969968 and rs1051730 located in the *CHRNA5* and *CHRNA3* genes, respectively, showed significant allelic differentiations irrespective of histological types and smoking status, whereas the minor allele for the rs8034191 SNP located in the *LOC123688* gene showed significantly increased ORs for all histological types of lung cancer and for lung cancer in non-smokers but not for lung cancer in smokers. Four other SNPs, whose minor allele frequencies were >0.1 in the controls, did not show significant allelic differentiations (Table II). Thus, it was indicated that three SNPs, rs8034191, rs16969968 and rs1051730, were associated with lung cancer risk in this population. Crude ORs and ORs adjusted for age, sex and smoking for genotypes for these three SNPs were further calculated (Table III). Heterozygotes and carriers of the minor alleles showed similarly increased ORs (Table III and supplementary Table I is available at *Carcinogenesis* Online). On the other hand, ORs of homozygotes for the minor alleles were not consistently increased among populations probably due to the small number of homozygotes.

We next examined linkage disequilibrium (LD) among these seven SNPs, and haplotypes were estimated. Five of the seven SNPs examined, rs8034191, rs6495306, rs621849, rs16969968 and rs1051730, were in LD with one another ($D' > 0.8$) (Figure 1), and the size of the region with LD was 88–172 kb. The rs8034191, rs16969968 and rs1051730 SNPs, which showed significant association with lung cancer risk, showed high correlation coefficients ($R^2 > 0.6$) with

Table I. Lung cancer cases and controls used for case–control study

Variable	Control	Case			
		All	ADC	SQC	SCC
Total	936	1250	562	391	297
Age (mean ± SD; years)	50 ± 13	60 ± 8	58 ± 8	62 ± 7	62 ± 9
Sex					
Male (%)	560 (60)	910 (73)	316 (56)	355 (91)	239 (80)
Female (%)	376 (40)	340 (27)	246 (44)	37 (9)	58 (20)
Smoking habit					
Non-smoker (%)	575 (61)	264 (21)	238 (44)	13 (3)	14 (5)
Smoker (%)	361 (39)	986 (79)	324 (56)	379 (97)	283 (95)
Pack-years[a] (mean ± SD)	26 (26)	56 (31)	44 (30)	61 (29)	62 (32)

[a]Values for smokers.

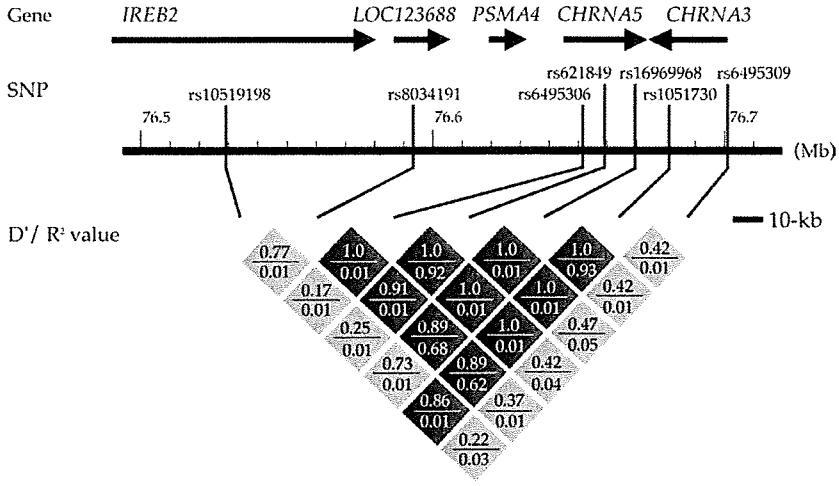

Fig. 1. SNPs in the locus containing nicotine acetylcholine receptor genes. Location of SNPs and genes are shown on the top. D' and R^2 values between two SNPs in the control subjects are shown below. Cells with D' values >0.8 are in black.

Table II. Allele and haplotype differentiation between controls and cases

SNP	Allele	Gene	Category	Frequency		OR (95% CI, *P*)[a]
				Control	Case	
rs10519198	A	*IREB2*	All	0.377	0.367	1.0 (0.9–1.1, 0.50)
			ADC		0.356	0.9 (0.8–1.1, 0.36)
			SQC		0.354	0.9 (0.8–1.1, 0.27)
			SCC		0.397	1.1 (0.9–1.3, 0.38)
			Non-smoker	0.380	0.362	0.9 (0.8–1.2, 0.93)
			Smoker	0.373	0.369	1.0 (0.8–1.2, 0.85)
rs8034191	C	*LOC123688*	All	0.018	0.035	2.0 (1.3–2.9, 0.00091)
			ADC		0.035	1.9 (1.2–3.1, 0.0045)
			SQC		0.035	1.9 (1.2–3.2, 0.010)
			SCC		0.035	2.0 (1.1–3.4, 0.013)
			Non-smoker	0.015	0.036	2.5 (1.3–4.8, 0.0056)
			Smoker	0.024	0.035	1.5 (0.9–2.5, 0.15)
rs6495306	G	*CHRNA5*	All	0.169	0.176	1.1 (0.9–1.2, 0.54)
			ADC		0.176	1.1 (0.9–1.3, 0.63)
			SQC		0.183	1.1 (0.9–1.4, 0.40)
			SCC		0.168	1.0 (0.8–1.3, 0.96)
			Non-smoker	0.175	0.181	1.0 (0.8–1.4, 0.75)
			Smoker	0.161	0.175	1.1 (0.9–1.4, 0.38)
rs621849	G	*CHRNA5*	All	0.179	0.177	1.0 (0.8–1.2, 0.82)
			ADC		0.177	1.0 (0.8–1.2, 0.87)
			SQC		0.183	1.0 (0.8–1.3, 0.84)
			SCC		0.168	0.9 (0.7–1.2, 0.54)
			Non-smoker	0.184	0.181	1.0 (0.8–1.3, 0.87)
			Smoker	0.172	0.176	1.0 (0.8–1.3, 0.81)
rs16969968	A	*CHRNA5*	All	0.015	0.034	2.2 (1.5–3.4, 0.00015)
			ADC		0.032	2.1 (1.3–3.5, 0.0026)
			SQC		0.033	2.2 (1.3–3.7, 0.0034)
			SCC		0.168	2.6 (1.5–4.5, 0.00060)
			Non-smoker	0.014	0.032	2.4 (1.2–4.7, 0.013)
			Smoker	0.015	0.033	2.2 (1.1–4.1, 0.016)
rs1051730	A	*CHRNA3*	All	0.014	0.033	2.4 (1.5–3.6, 0.000088)
			ADC		0.031	2.2 (1.3–3.7, 0.0019)
			SQC		0.033	2.4 (1.4–4.1, 0.0016)
			SCC		0.037	2.6 (1.5–4.7, 0.00058)
			Non-smoker	0.013	0.034	2.7 (1.3–5.3, 0.0041)
			Smoker	0.017	0.033	2.0 (1.1–3.8, 0.024)
rs6495309	T	*CHRNA3*	All	0.497	0.482	0.9 (0.8–1.0, 0.19)
			ADC		0.477	0.9 (0.8–1.1, 0.18)
			SQC		0.465	0.9 (0.7–1.0, 0.081)
			SCC		0.485	1.1 (0.9–1.3, 0.60)
			Non-smoker	0.493	0.455	0.9 (0.7–1.1, 0.14)
			Smoker	0.482	0.490	0.9 (0.8–1.1, 0.20)
Haplotype[b]	CAA	*LOC123688* *CHRNA5* *CHRNA3*	All	0.013	0.029	2.3 (1.5–3.7, 0.00028)
			ADC		0.028	2.3 (1.3–3.9, 0.0022)
			SQC		0.028	2.2 (1.2–4.0, 0.0059)
			SCC		0.032	2.5 (1.4–4.7, 0.0019)
			Non-smoker	0.011	0.026	2.4 (1.1–5.1, 0.022)
			Smoker	0.015	0.030	2.0 (1.0–3.8, 0.034)

[a]CI, confidence interval.
[b]rs8034191-rs16969968-rs1051730 = CAA.

one another, however, showed low coefficients (R^2 = 0.01) with two other SNPs, rs6495306 and rs621849, due to large differences in allele frequency between the former three and the latter two SNPs (Figure 1). The result of LD in this study population was consistent with the results of LD in Japanese subjects deposited in the HapMap database (http://www.hapmap.org) (supplementary Figure 1 is available at *Carcinogenesis* Online). Therefore, the lack of association of the rs6495306 and rs621849 SNPs with lung cancer risk were considered to be due to their low correlation coefficients with rs8034191, rs16969968 and rs1051730. The rs8034191, rs16969968 and rs1051730 SNPs were also in LD in European and American populations (supplementary Figure 1 is available at *Carcinogenesis* Online), and minor alleles for these three SNPs were reported to comprise a single haplotype in European and American populations with frequencies of 0.3–0.4 (2,3). Consistently, minor alleles for these three SNPs were also deduced to comprise a single haplotype in the Japanese population (supplementary Table II is available at *Carcinogenesis* Online). However, the frequency of this haplotype was much lower in the Japanese population (0.013) than in European and American populations.

ORs for the haplotype consisting of minor alleles for the three SNPs were then calculated (haplotype CAA in Table II). This haplotype showed significantly increased ORs for lung cancer risk, and the association was observed in all histological types of lung cancers and in both smokers and non-smokers. Crude ORs and ORs adjusted for age, sex and smoking for genotypes with the CAA haplotype were further calculated (haplotype CAA in Table III). Heterozygotes and carriers (1 and 1 + 2 in Table III) for the CAA haplotype showed similarly increased crude and adjusted ORs in all three histological types of lung cancer and in both smokers and non-smokers. Crude

Table III. Genotype differentiation for the rs8034191, rs1051730 SNPs and the haplotype CAA between controls and cases

SNP/haplotype	Category	Genotype	No. of controls (%)	No. of cases (%)	Crude OR (95% CI, *P*)	Adjusted OR (95% CI, *P*)
rs8034191	All	T/T	905 (96.7)	1166 (93.3)	Reference	Reference
		T/C	28 (3.0)	81 (6.4)	2.3 (1.5–3.5, 0.00021)	1.9 (1.2–3.2, 0.0058)[a]
		C/C	3 (0.3)	3 (0.2)	0.8 (0.2–3.9, 0.76)	0.6 (0.1–3.3, 0.52)[a]
		T/C + C/C	31 (3.3)	84 (6.7)	2.1 (1.4–3.2, 0.00041)	1.8 (1.1–2.9, 0.013)[a]
	ADC	T/T		526 (93.6)	Reference	Reference
		T/C		33 (5.9)	2.0 (1.2–3.4, 0.0061)	1.8 (1.1–3.1, 0.031)[a]
		C/C		3 (0.5)	1.7 (0.4–8.6, 0.50)	1.1 (0.2–6.1, 0.90)[a]
		T/C + C/C		36 (6.4)	2.0 (1.2–3.3, 0.0050)	1.7 (1.0–2.9, 0.037)[a]
	SQC	T/T		364 (93.1)	Reference	Reference
		T/C		27 (6.9)	2.4 (1.4–4.1, 0.0012)	1.9 (0.9–3.8, 0.077)[a]
		C/C		0 (0)	0 (—, 0.27)	0 (—, 0.38)[a]
		T/C + C/C		27 (6.9)	2.2 (1.3–3.7, 0.0035)	1.7 (0.9–3.5, 0.11)[a]
	SCC	T/T		276 (92.9)	Reference	Reference
		T/C		21 (7.1)	2.5 (1.4–4.4, 0.0018)	1.7 (0.8–3.5, 0.14)[a]
		C/C		0 (0)	0 (—, 0.34)	0 (—, 0.28)[a]
		T/C + C/C		21 (7.0)	2.2 (1.3–3.9, 0.0050)	1.6 (0.8–3.1, 0.21)[a]
	Non-smoker	T/T	559 (97.2)	241 (93.2)	Reference	Reference
		T/C	15 (2.6)	17 (6.4)	2.6 (1.3–5.2, 0.0073)	2.3 (1.1–4.9, 0.032)[b]
		C/C	1 (0.2)	1 (0.4)	2.3 (0.1–36, 0.55)	1.0 (0–25, 0.98)[b]
		T/C + C/C	16 (2.8)	18 (6.8)	2.6 (1.3–5.1, 0.0061)	2.2 (1.0–4.5, 0.039)[b]
	Smoker	T/T	346 (95.8)	919 (93.3)	Reference	Reference
		T/C	13 (3.6)	64 (6.5)	1.9 (1.0–3.4, 0.044)	1.7 (1.0–3.4, 0.068)[b]
		C/C	2 (0.6)	2 (0.2)	0.4 (0.1–2.7, 0.31)	0.5 (0.1–5.0, 0.54)[b]
		T/C + C/C	15 (4.2)	66 (6.7)	1.7 (0.9–2.9, 0.082)	1.6 (0.9–3.0, 0.11)[b]
rs1051730	All	G/G	910 (97.2)	1170 (93.6)	Reference	Reference
		G/A	25 (2.7)	77 (6.2)	2.4 (1.5–3.8, 0.00013)	2.2 (1.4–3.8, 0.0014)[a]
		A/A	1 (0.1)	3 (0.2)	2.3 (0.2–22, 0.45)	1.2 (0.1–25, 0.87)[a]
		G/A + A/A	26 (2.8)	80 (6.4)	2.4 (1.5–3.8, 0.000096)	2.2 (1.3–3.7, 0.0016)[a]
	ADC	G/G		530 (94.3)	Reference	Reference
		G/A		29 (5.2)	2.0 (1.2–3.4, 0.012)	1.9 (1.1–3.4, 0.029)[a]
		A/A		3 (0.5)	5.2 (0.5–50, 0.11)	2.8 (0.3–56, 0.35)[a]
		G/A + A/A		32 (5.7)	2.1 (1.3–3.6, 0.0046)	1.9 (1.1–3.4, 0.019)[a]
	SQC	G/G		365 (93.4)	Reference	Reference
		G/A		26 (6.6)	2.6 (1.5–4.6, 0.00060)	2.6 (1.2–5.5, 0.011)[a]
		A/A		0 (0)	0 (—, 0.53)	0 (—, 0.56)[a]
		G/A + A/A		26 (6.6)	2.5 (1.4–4.4, 0.00092)	2.5 (1.2–5.2, 0.014)[a]
	SCC	G/G		275 (92.6)	Reference	Reference
		G/A		22 (7.4)	2.9 (1.6–5.3, 0.00021)	2.6 (1.2–5.6, 0.012)[a]
		A/A		0 (0)	0 (—, 0.58)	0 (—, 0.46)[a]
		G/A + A/A		22 (7.4)	2.8 (1.6–5.0, 0.00033)	2.4 (1.2–5.2, 0.017)[a]
	Non-smoker	G/G	560 (97.4)	248 (93.6)	Reference	Reference
		G/A	15 (2.6)	16 (6.0)	2.4 (1.2–5.0, 0.014)	2.2 (1.0–4.7, 0.051)[b]
		A/A	0 (0)	1 (0.4)	0 (—, 0.13)	0 (—, 0.36)[b]
		G/A + A/A	15 (2.6)	17 (6.4)	2.6 (1.3–5.2, 0.0074)	2.2 (1.0–4.8, 0.040)[b]
	Smoker	G/G	350 (97.0)	922 (93.6)	Reference	Reference
		G/A	10 (2.8)	61 (6.2)	2.3 (1.2–4.6, 0.013)	2.3 (1.2–4.9, 0.012)[b]
		A/A	1 (0.2)	2 (0.2)	0.8 (0.1–8.4, 0.82)	0.8 (0.1–21, 0.90)[b]
		G/A + A/A	11 (3.0)	63 (6.4)	2.2 (1.1–4.2, 0.017)	2.2 (1.1–4.5, 0.016)[b]
Haplotype CAA[c]	All	0[d]	913 (97.5)	1179 (94.3)	Reference	Reference
		1[d]	22 (2.4)	69 (5.5)	2.4 (1.5–4.0, 0.00024)	2.2 (1.3–3.9, 0.0031)[a]
		2[d]	1 (0.1)	2 (0.2)	1.6 (0.1–17, 0.72)	0.7 (0.1–17, 0.81)[a]
		1 + 2[d]	23 (2.5)	71 (5.7)	2.4 (1.5–4.0, 0.00024)	2.1 (1.3–3.7, 0.0042)[a]
	ADC	0[d]		532 (94.7)	Reference	Reference
		1[d]		28 (5.0)	2.2 (1.2–3.9, 0.0059)	2.0 (1.1–3.6, 0.027)[a]
		2[d]		2 (0.4)	3.4 (0.3–38, 028)	1.9 (0.2–42, 0.58)[a]
		1 + 2[d]		30 (5.3)	2.2 (1.3–3.9, 0.0035)	2.0 (1.1–3.5, 0.022)[a]
	SQC	0[d]		369 (94.4)	Reference	Reference
		1[d]		22 (5.6)	2.5 (1.4–4.5, 0.0024)	2.4 (1.1–5.4, 0.029)[a]
		2[d]		0 (0)	0 (—, 0)	0 (—, 0.55)[a]
		1 + 2[d]		22 (5.6)	2.4 (1.3–4.3, 0.0036)	2.3 (1.1–5.1, 0.040)[a]
	SCC	0[d]		278 (93.6)	Reference	Reference
		1[d]		19 (6.4)	2.8 (1.5–5.3, 0.00071)	2.2 (1.0–4.9, 0.047)[a]
		2[d]		0 (0)	0 (—, 0.58)	0 (—, 0.46)[a]
		1 + 2[d]		19 (6.4)	2.7 (1.5–5.1, 0.0011)	2.1 (0.9–4.5, 0.063)[a]
	Non-smoker	0[d]	562 (97.7)	251 (94.7)	Reference	Reference
		1[d]	13 (2.3)	14 (5.3)	2.4 (1.1–5.2, 0.021)	2.0 (0.9–4.7, 0.089)[b]
		2[d]	0 (0)	0 (0)	0 (—, 0)	0 (—, 0)[b]
		1 + 2[d]	13 (2.3)	14 (5.3)	2.4 (1.1–5.2, 0.021)	2.0 (0.9–4.7, 0.089)[b]
	Smoker	0[d]	351 (97.2)	928 (94.2)	Reference	Reference
		1[d]	9 (2.5)	55 (5.6)	2.3 (1.1–4.7, 0.018)	2.3 (1.2–5.1, 0.017)[b]
		2[d]	1 (0.3)	2 (0.2)	0.8 (0.1–8.4, 0.82)	0.8 (0–20, 0.89)[b]
		1 + 2[d]	10 (2.8)	57 (5.8)	2.2 (1.1–4.3, 0.024)	2.2 (1.1–4.6, 0.022)[b]

[a]Adjusted for sex, age and smoking.
[b]Adjusted for sex and age.
[c]rs8034191-rs16969968-rs1051730 = CAA.
[d]Expressed by the number of CAA haplotype carried.

ORs were significantly increased and adjusted ORs were significantly or marginally increased. On the other hand, ORs of homozygotes for the CAA haplotype and those for the minor allele for each SNP were not consistently increased among populations probably due to the small number of homozygotes.

We next examined the association of the rs8034191, rs16969968 and rs1051730 SNPs and the CAA haplotype with smoking status. The value of cigarettes per day was used as a measure of smoking doses of subjects for this analysis since this value was commonly used in previous three GWA studies (2–4). Values of cigarettes per day of individuals with the minor alleles for these SNPs or with the CAA haplotype were higher than those without in both controls and cases; however, the difference did not reach a statistical significance (Table IV).

Discussion

In this study, minor alleles for three *CHRNA* SNPs, rs8034191, rs16969968 and rs1051730, which were defined as risky alleles for lung cancer in Europeans and Americans, also showed significantly increased ORs in Japanese. Results of recent association studies (2–4) on *CHRNA* SNPs, including this study, are summarized in Table V. The frequency of risky alleles in the Japanese population was <2% and was considerably lower than those in European and American populations as previously indicated (3). Therefore, homozygotes for risky alleles were rare (<1%) in Japanese. Accordingly, an increase in ORs of the homozygotes was not observed in the present study, although an increase in ORs of minor allele carriers (i.e. OR in dominant model) was observed. Therefore, it was suggested that *CHRNA* risky alleles makes a smaller subset of individuals more susceptible to lung cancer in Japanese than in European and American populations.

Association of *CHRNA* SNPs with lung cancer risk by dividing subjects into smokers and non-smokers was examined in two GWA studies (2,3). Association with risk in smokers was commonly observed in these two studies, whereas association in non-smoker was not [Table V, (2,3)]. In the presents study, the minor alleles for three SNPs as well as the haplotype carrying them showed similarly increased ORs in both smokers and non-smokers (Table II), indicating that *CHRNA* SNPs are associated with lung cancer risk irrespective of smoking. This result was consistent with the study by Hung *et al.* (3). The reason for the lack of association in non-smokers in the other study (2) might be a low statistical power due to a small number of non-smoking lung cancer cases (i.e. 125) as discussed (18). Alternatively, other factors that have not been taken into account, such as food intake and passive smoking, might differentiate the mode of contribution of the *CHRNA* SNPs in non-smokers.

Effects of *CHRNA* SNPs according to histological types of lung cancers were examined in a previous study [Table V, (3)] and were similar among ADC, SQC and SCC. In the present study, the minor alleles for rs8034191, rs16969968 and rs1051730 SNPs as well as the haplotype with these minor alleles also showed similarly and significantly increased ORs among them (Table II). Therefore, it was strongly indicated that *CHRNA* SNPs are associated with lung cancer risk irrespective of histological types of cancer. Recent studies suggested the presence of several types of lung ADCs according to accumulated genetic alterations, and ADCs with mutations in the epidermal growth factor gene are predominantly developed in female non-smokers (19). Therefore, we calculated ORs of genotypes for *CHRNA* SNPs for ADC risk after dividing subjects according to sex and smoking. It was found that ORs of heterozygote and minor allele carriers were significantly or marginally significantly increased in female non-smokers (supplementary Table III is available at *Carcinogenesis* Online). Similar but insignificant increases in ORs were also observed in male non-smokers, male smokers and female smokers. These results indicated that *CHRNA* SNPs confer risk for several types of lung ADCs including those developed in female non-smokers, and further validated that *CHRNA* SNPs are associated with lung cancer risk in non-smokers.

In the present study, carriers of the minor alleles for *CHRNA* SNPs as well as the CAA haplotype showed larger values of cigarettes per day than non-carriers by four to six and by one to two in the control and case populations, respectively, although the difference did not reach a statistical significance (Table IV). Associations of *CHRNA*

Table IV. Cigarettes per day values according to genotypes for *CHRNA* SNPs

Control/ case	SNP/ haplotype	Cigarettes per day (mean ± SD)		*P* by Wilcoxon test
		Non-carrier	Carrier	
Control	rs8034191	20.0 (12.2)	25.9 (15.7)	0.20
	rs1051730	20.2 (12.3)	24.2 (15.6)	0.52
	rs16969968	20.0 (12.2)	26.4 (16.6)	0.27
	CAA[a]	20.1 (12.3)	25.7 (15.7)	0.30
Case	rs8034191	28.6 (14.0)	30.6 (12.2)	0.096
	rs1051730	28.7 (14.0)	29.5 (11.6)	0.32
	rs16969968	28.7 (14.0)	29.5 (11.7)	0.32
	CAA[a]	28.6 (14.0)	30.1 (11.8)	0.19

[a]rs8034191-rs16969968-rs1051730 = CAA.

Table V. Association of *CHRNA* SNPs with lung cancer risk and smoking behavior

	SNP	Category	Minor allele frequency	OR (*P*)[a]		Association with lung cancer risk by smoking	Association with smoking status	Reference
				Homozygotes for the minor allele	Minor allele carriers			
USA and UK	rs8034191 (T/C)	NSCLC[b]	0.33	1.8 (2.5×10^{-12})		Smoker only	Yes	Amos *et al.* (2)
	rs1051730 (G/A)		0.33	1.8 (4.6×10^{-12})				
France, 15 other European countries and Canada	rs8034191 (T/C)	ADC	0.34		1.4 (2.0×10^{-10})	Smoker, non-smoker	No	Hung *et al.* (3)
		SQC			1.2 (6.0×10^{-6})			
		SCC			1.3 (2.0×10^{-4})			
Iceland, Spain and The Netherlands	rs1051730 (G/A)	NSCLC[b] + SCC	0.35	1.7 (1.1×10^{-7})		Not examined	Yes	Thorgeirsson *et al.* (4)
Japan	rs8034191 (T/C)	ADC	0.018	1.1 (0.9)	1.7 (3.7×10^{-2})	Smoker, non-smoker	No	This study
		SQC		0 (0.4)	1.7 (0.1)			
		SCC		0 (0.3)	1.6 (0.2)			
	rs1051730 (G/A)	ADC	0.014	2.8 (0.4)	1.9 (1.9×10^{-2})			
		SQC		0 (0.6)	2.5 (1.4×10^{-2})			
		SCC		0 (0.5)	2.4 (1.7×10^{-2})			

[a]OR against homozygotes for the major allele.
[b]NSCLC, non-small cell lung cancer.

SNPs with values of cigarettes per day were also investigated in control populations of the three GWA studies (2–4). In the Iceland, Spain and The Netherland study, the individuals with one and two copies of the minor allele for the rs1051730 SNP were estimated to smoke approximately one and two more cigarette per day than those without, respectively, and the association was statistically significant (4). In the USA and UK study, significant differences in the cigarettes per day values were observed only in former but not in current smokers, and the reason for the inconsistency was unclear (2). In another GWA study, difference in the values of cigarette per day according to genotypes for *CHRNA* SNPs was not observed (3). Thus, minor alleles for *CHRNA* SNPs might have an effect to make individuals more addictive to smoking and to make them consume a few more cigarettes a day. However, since the results of four studies are inconsistent, further studies are still needed to draw a conclusion on this issue.

The present study suggested that *CHRNA* SNPs contribute to lung cancer susceptibility in multiple ethnic populations, including Asians, and the contribution is irrespective of histological types of cancers. Since the association was observed in non-smokers in several populations including Japanese, *CHRNA* SNPs is probably to contribute to lung cancer risk in a smoking-independent manner. Recently, it was reported that *CHRNA* SNPs confer risk of familial lung cancer in Americans, whereas association of these SNPs with smoking status was not significant (20). Therefore, contribution of *CHRNA* SNPs to lung cancer risk through tobacco addiction remains inconclusive. Further studies on a cohort of subjects, for whom data on lung cancer development, smoking exposure, nicotine dependence and duration and intensity of smoking are available, should be done to elucidate this issue as previously discussed (18). CHRNA proteins transduce signals of not only nicotinic substrates but also non-nicotinic substrates, as described in the Introduction (5–7). Therefore, identification of SNPs in the *CHRNA* genes causing differences in the signal transduction by non-nicotinic substrates will be a way to further elucidate the involvement of *CHRNA* in lung cancer susceptibility. rs16969968 is a candidate since it causes a change of a conserved amino acid in *CHRNA5* protein (10) and other non-synonymous or regulatory polymorphisms that show high correlation coefficients with the rs8034191, rs16969968 and rs1051730 SNPs have not been found to date. Alternatively, polymorphisms in two other genes, *LOC123688* and *PSMA4*, which are in LD with these three SNPs (Figure 1), might be responsible. Further genetic and functional studies on the *CHRNA* genes are needed to elucidate the significance of the *CHRNA* locus in lung cancer susceptibility.

Funding

Ministry of Education, Culture, Sports, Science and Technology for Scientific Research on Priority Areas (KAKENHI 20014031); Ministry of Health, Labor and Welfare for Cancer Research (19S-1) and for the 3rd-term Comprehensive 10-year Strategy for Cancer Control. Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan to K.S.

Acknowledgements

We thank Dr Takashi Eguchi, Dr Matsuhiko Hayashi and Dr Keiichi Hirao of the Keio University School of Medicine and Dr Teruhiko Yoshida, Dr Hiromi Sakamoto, Dr Kimio Yoshimura and Dr Shunpei Ohnami of the National Cancer Center Research Institute for their help in collecting blood samples of the control subjects. We also thank Ms Sachiyo Mimaki and Ms Yuko Ishii for technical assistance.

Conflict of Interest Statement: None declared.

References

1. Parkin,M. *et al.* (2004) Lung cancer epidemiology and etiology. In Travis,W.D., Brambilla,E., Muller-Hermelink,H.K. and Harris,C.C. (eds) *World Health Organization Classification of Tumors: Pathology and Genetics, Tumours of Lung, Pleura, Thymus and Heart.* IARC Press, pp. 31–34.
2. Amos,C.I. *et al.* (2008) Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat. Genet.*, **40**, 616–622.
3. Hung,R.J. *et al.* (2008) A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature*, **452**, 633–637.
4. Thorgeirsson,T.E. *et al.* (2008) A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature*, **452**, 638–642.
5. Shields,P.G. (2000) Epidemiology of tobacco carcinogenesis. *Curr. Oncol. Rep.*, **2**, 257–262.
6. Minna,J.D. (2003) Nicotine exposure and bronchial epithelial cell nicotinic acetylcholine receptor expression in the pathogenesis of lung cancer. *J. Clin. Invest.*, **111**, 31–33.
7. Schuller,H.M. (2007) Nitrosamines as nicotinic receptor ligands. *Life Sci.*, **80**, 2274–2280.
8. West,K.A. *et al.* (2003) Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. *J. Clin. Invest.*, **111**, 81–90.
9. Dasgupta,P. *et al.* (2006) Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. *Cell Cycle*, **5**, 2324–2328.
10. Saccone,S.F. *et al.* (2007) Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum. Mol. Genet.*, **16**, 36–49.
11. Bierut,L.J. *et al.* (2008) Variants in nicotinic receptors and risk for nicotine dependence. *Am. J. Psychiatry*, **165**, 1163–1171.
12. Travis,W., Colby,T.V., Corrin,B., Shimosato,Y. and Brambilla,E. (eds) (1999) *Histological Typing of Lung and Pleural Tumors.* Springer-Verlag, Heidelberg, Germany.
13. Brambilla,E. *et al.* (2001) The new World Health Organization classification of lung tumours. *Eur. Respir. J.*, **18**, 1059–1068.
14. Sakiyama,T. *et al.* (2005) Association of amino acid substitution polymorphisms in DNA repair genes TP53, POLI, REV1 and LIG4 with lung cancer risk. *Int. J. Cancer*, **114**, 730–737.
15. Kohno,T. *et al.* (2006) Association of polymorphisms in the MTH1 gene with small cell lung carcinoma risk. *Carcinogenesis*, **27**, 2448–2454.
16. Breslow,N.E. *et al.* (1980) Statistical methods in cancer research. Volume I—the analysis of case-control studies. *IARC Sci. Publ.*, **32**, 5–338.
17. Yoshimi,I. *et al.* (2003) A comparison of trends in the incidence rate of lung cancer by histological type in the Osaka Cancer Registry, Japan and in the Surveillance, Epidemiology and End Results Program, USA. *Jpn. J. Clin. Oncol.*, **33**, 98–104.
18. Chanock,S.J. *et al.* (2008) Genomics: when the smoke clears. *Nature*, **452**, 537–538.
19. Sun,S. *et al.* (2007) Lung cancer in never smokers—a different disease. *Nat. Rev. Cancer*, **7**, 778–790.
20. Liu,P. *et al.* (2008) Familial aggregation of common sequence variants on 15q24-25.1 in lung cancer. *J. Natl Cancer Inst.*, **100**, 1326–1330.
21. Pritchard,J.K. *et al.* (2001) Linkage disequilibrium in humans: models and data. *Am. J. Hum. Genet.*, **69**, 1–14.

Received August 20, 2008; revised October 14, 2008; accepted November 9, 2008

A Novel Histopathological Evaluation Method Predicting the Outcome of Non-small Cell Lung Cancer Treated by Neoadjuvant Therapy

The Prognostic Importance of the Area of Residual Tumor

Yuki Yamane, MD,†‡ Genichiro Ishii, MD, PhD,* Koichi Goto, MD, PhD,† Motohiro Kojima, MD, PhD,* Masayuki Nakao, MD,*† Yoshihisa Shimada, MD,*† Yutaka Nishiwaki, MD,† Kanji Nagai, MD, PhD,† Hirotsugu Kohrogi, MD, PhD,‡ and Atsushi Ochiai, MD, PhD**

Background: Histopathological evaluation method for predicting the outcome of non-small cell lung cancer (NSCLC) treated by neoadjuvant therapy has not been fully assessed. The purpose of this study was to assess a novel histopathological evaluation method for predicting the outcome of NSCLC treated by neoadjuvant therapy.

Methods: We reviewed the histopathology of the tumors of 53 NSCLC treated by neoadjuvant chemotherapy, chemoradiotherapy, or radiotherapy followed by complete resection and identified the histologic features produced by neoadjuvant therapy by comparing them with the histologic features of the tumors in 138 NSCLC cases treated by surgery without neoadjuvant therapy. We also measured the area of residual tumor (ART) on the maximum cut surface of the tumors and analyzed the relationships between the histologic features, ART, and the outcome.

Results: The proportions of cases with the histologic features "cholesterin clefts," "foreign body reactive giant cells," "stromal hyalinosis," and "bizarre nucleus in more than 50% of the cancer cells" were significantly higher in the neoadjuvant therapy group than in the surgery alone group. However, the presence of none of these features had any significant effect on survival. Although pathologic T factor and N factor had no significant effect on overall survival, smaller ART ($\leq$400 mm^2) and absence of pleural invasion (p [−]) were predictors of a outcome (p = 0.014 and p = 0.003, respectively).

Conclusions: Smaller ART and p (−) predict a better outcome of NSCLC treated by neoadjuvant therapy. We concluded that ART is a novel histopathological evaluation method for predicting the outcome of NSCLC treated by neoadjuvant therapy.

Key Words: Non-small cell lung cancer, Neoadjuvant therapy, Histopathological evaluation method, Outcome, Histological features, ART.

(*J Thorac Oncol.* 2010;5: 49–55)

Surgical resection is the standard treatment modality for stages I to III non-small cell lung cancer (NSCLC), but many patients develop local and distant recurrence and die. The 5-year survival rates for patients with NSCLC treated by surgical resection have been disappointing, ranging from 67% for p-T1N0M0 to 23% for p-T1-3N2M0.[1]

Efforts to improve the survival of patients with resectable NSCLC and with potentially resectable more advanced local disease have included the use of chemotherapy or radiotherapy in postoperative (adjuvant) or preoperative (neoadjuvant) settings. Several trials in recent years, including in large series of patients, have demonstrated the effectiveness of adjuvant chemotherapy after complete resection.[2–6] Although several small randomized trials have reported neoadjuvant therapy to be effective,[7,8] because more recent studies have failed to confirm their data,[9,10] it remains a matter of controversy. Interest in neoadjuvant therapy has been increasing, because the efficacy of adjuvant therapy has been demonstrated.

Histopathological evaluation methods for predicting the outcome of NSCLC treated by neoadjuvant therapy have not been fully assessed. Junker et al.[11] used the following histologic tumor regression grading system: grade I, no or only slight tumor regression; grade IIA, marked but incomplete tumor regression, more than 10% vital tumor tissue; grade IIB, less than 10% vital tumor tissue; grade III, complete tumor regression without vital tumor tissue, and they found that the grade of tumor regression is a significant prognostic factor in NSCLC treated by neoadjuvant therapy.

*Pathology Division, Research Center for Innovative Oncology, †Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba; and ‡Department of Respiratory Medicine, Faculty of Medical and Pharmaceutical Sciences, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Atsushi Ochiai, MD, PhD, Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa-City, Chiba 277-8577, Japan. E-mail: aochiai@east.ncc.go.jp

ISSN: 1556-0864/10/0501-0049

The survival rate of patients with grade IIB or III was significantly better than the survival rate of patients with grade I or IIA. However, it is difficult to calculate the ratio of residual viable tumor tissue in the primary tumor, because evaluation of primary tumor area before neoadjuvant therapy is not over a level of the imagination. Moreover, the morphologic definition of "viable cancer cells" is incongruous.

Because tumor cells persist in the form of islands in the necrotic or fibrotic tissue, in some cases of NSCLC treated by neoadjuvant therapy, measurement of tumor diameter is sometimes difficult and does not always reflect residual tumor size. Therefore, we tried to measure the area of residual tumor (ART) on the maximum cut surface, and we analyzed the correlations between several histologic features considered to be attributable to neoadjuvant therapy, ART, and outcome.

In this study, we assess a more objective and reproducible histopathological evaluation method for predicting the outcome of NSCLC treated by neoadjuvant therapy. Accurate prognosis might serve as a guide to treatment after neoadjuvant therapy and surgical resection.

PATIENTS AND METHODS

Patients

A series of 53 cases of NSCLC treated by neoadjuvant chemotherapy, chemoradiotherapy, or radiotherapy followed by complete resection in our hospital between July 1992 and December 2006 were reviewed. The median follow-up period of the 25 surviving patients was 42 months. The eight patients who received neoadjuvant therapy followed by incomplete resection and the eight patients who received neoadjuvant therapy between January 2007 and February 2008 were also included in the analysis of therapy-induced histologic features. To define therapy-induced histologic features, 138 NSCLC cases in which surgery was performed without neoadjuvant therapy between January 2002 and December 2004 were also reviewed.

Pathologic Study

The surgically resected specimens from every case were fixed with 10% formalin or absolute methyl alcohol and embedded in paraffin. The tumors were cut into 5 to 10 mm slices, and serial 4-μm sections were stained with hematoxylin and eosin and by the Victoria van-Gieson method to visualize elastic fibers. All slides containing the maximum surface area of the tumor in each case were reviewed. Two pathologists (Y.Y. and G.I.) reviewed the pathology under a multiheaded microscope.

We examined the tumors for the presence of seven histologic features referring to a past study given as follows[11]: (1) coagulation necrosis, necrosis in which tissue becomes a dry, opaque, eosinophilic mass containing outlines of anucleated cells; (2) foam cell infiltration, infiltration of macrophages with foamy cytoplasm; (3) foam cell infiltration around necrotic foci, the state that a foam cell infiltration surrounds around necrotic foci; (4) cholesterin clefts, cholesterin crystalloid bodies were present within cancer tissue; (5) foreign body reactive giant cells, collections of fused macrophages (giant cell), which are generated in response to the presence of a large foreign body; (6) stromal hyalinosis, the formation of rounded masses or broad bands of homogeneous acidophilic substances that have a glassy appearance; and (7) bizarre nucleus in more than 50% of the cancer cells, the state that more than 50% of the cancer cells have enlarged, irregular, or multiple nuclei (Figure 1). We considered these histologic features to be positive when we found only a few of them within cancer tissue.

We also measured the ART by the described method below in all slides containing the maximum surface area of the tumor.

We also evaluated pathologic T stage (ypT), pathologic N stage (ypN), and pleural invasion. Pleural invasion was defined as positive when tumor cells extended beyond the elastic layer of the visceral pleura, which was stained blue with Victoria van-Gieson stain.

Measurement of ART

We identified the residual tumor cells under a microscope and outlined it on the slides with a marker pen (Figure 2*A*). Degenerated tumor cells containing a nucleus and cytoplasm were included as "residual tumor cells," but necrotic tumor cells were excluded. When a group of residual tumor cells was 2 mm from the next group, we regarded it as a

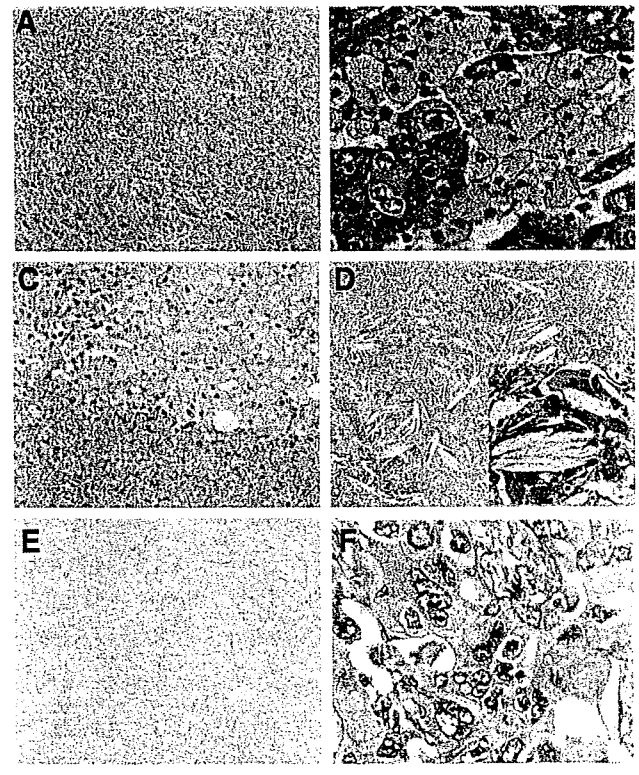

FIGURE 1. Histologic features of non-small cell lung cancer (NSCLC) treated by neoadjuvant therapy followed by surgical resection. (*A*) Coagulation necrosis, (*B*) foam cell infiltration, (*C*) foam cell infiltration around the necrotic foci, (*D*) cholesterin clefts, the inset is a higher magnification of this slide (foreign body reactive giant cells), (*E*) stromal hyalinosis, and (*F*) cancer cells containing a bizarre nucleus.