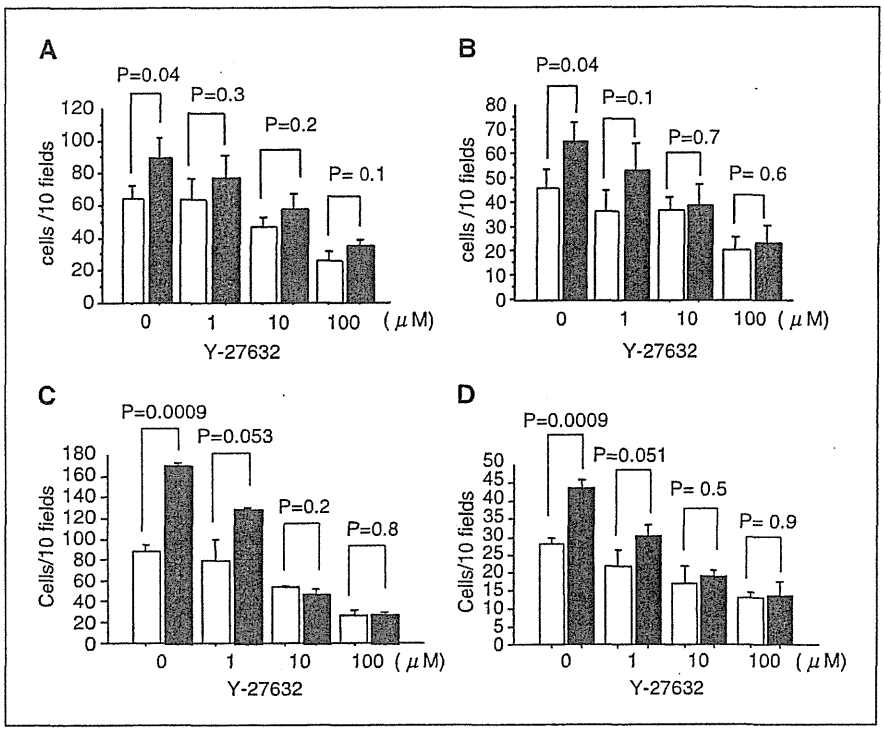

Figure 2. *In vitro* motility and invasion of LCD-vector (*open columns*) and LCD-E1AF (*closed columns*) cells with various concentrations of Y27632. Cells were incubated for 24 hours with various concentrations of Y27632, with or without HGF treatment (40 ng/mL), and the number of migrated cells was counted at a magnification of ×200. *A* and *B*, motility assay (*A*) and invasion assay (*B*) without HGF; *C* and *D*, motility assay (*C*) and invasion assay (*D*) with HGF. *Columns*, mean; *bars*, SD.

were detected using anti-rabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system. The same lysates were used in Western blotting of total Rho and actin as an internal control for the comparison of levels of GTP-bound Rho.

Western blot analysis of myosin light chain. LCD-E1AF and LCD-vector cells were cultured in RPMI 1640 with 10% FBS for 24 hours, then with or without 40 ng/mL HGF, and finally with 100 μmol/L Y27632 for 0, 1, 4, 8, 12, and 24 hours. Cells were washed twice with PBS and lysed in ice-cold lysis buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates containing 30 μg total protein were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then reacted with anti-MLC antibody from mice (Sigma Chemical) or anti-phosphorylated MLC antibody from rabbits (Cell Signaling, Beverly, MA). The primary antibodies were detected using antimouse or antirabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system.

Mice. Female BALB/c athymic nude mice, 4 to 6 weeks old, were purchased from Clea Japan, Inc. (Tokyo, Japan) and maintained in a specific pathogen-free environment throughout the experiment.

Tumor implantation. LCD-E1AF or LCD-vector cells (1×10^7) were injected i.v. into the tail vein or s.c. into the flank of 6- to 8-week-old nude mice. The mice injected s.c. were examined for localized tumor and for metastasis to mediastinal lymph node, liver, kidney, and spleen. The weight of each tumor was measured.

The implantation of LCD-E1AF and LCD-vector cells into the lung was done as previously described (33) with some modifications. Briefly, the left chest of each anesthetized mouse was incised (~5 mm incision) just below the inferior border of the scapula, and 20 μL of suspension containing 1×10^7 VMRC-LCD cells and 20 μg of Matrigel were injected into the left lung parenchyma through the intercostal space. The skin incision was closed with 3-0 silk. Mice were sacrificed on days 14, 28, and 42 after tumor cell implantation. Tumors were examined in lung, mediastinal lymph node, liver, kidney, and spleen and were weighed.

Histologic examination. The sacrificed mice were examined and then the tumors in the lungs and mediastinal lymph nodes were removed and weighed. After careful macroscopic examination, tumors were fixed with 10% formalin, embedded in paraffin, cut into 4-μm sections, and then stained with H&E.

Western blot analysis of mouse tumor homogenates. Single s.c. tumor from a mouse implanted with LCD-vector cells or a mouse with LCD-E1AF cells, single lung tumor from a mouse with LCD-vector cells, a pool of lung tumors from five mice with LCD-E1AF cells, and a pool of mediastinal lymph node tumors from four mice with LCD-E1AF cells were homogenized and sonicated in 1.0 mL of ice-cold lysis buffer. Specimens were centrifuged at 900 × *g* for 15 minutes and supernatants were filtered through sterile filters of 10 μm pore size (Toyo Roshi, Tokyo, Japan). Cell lysates containing 30 μg total protein were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then reacted with anti-MLC antibody from

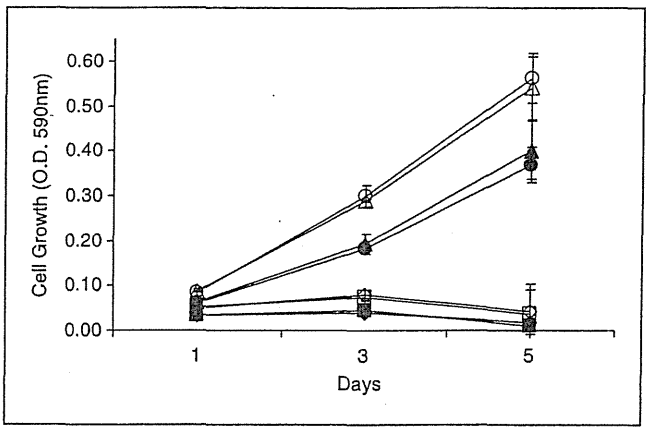

Figure 3. Effect of Y27632 on VMRC-LCD cell growth. Cells were cultured with or without 100 μmol/L Y27632 and with 10% FBS or 0.1% BSA. Cell growth was analyzed by MTT assay. ○, LCD-vector in FBS without Y27632; △, LCD-vector in FBS with Y27632; ●, LCD-E1AF in FBS without Y27632; ▲, LCD-E1AF in FBS with Y27632; □, LCD-vector in BSA without Y27632; ◇, LCD-vector in BSA with Y27632; ■, LCD-E1AF in BSA without Y27632; ♦, LCD-E1AF in BSA with Y27632. *Points*, mean; *bars*, SD.

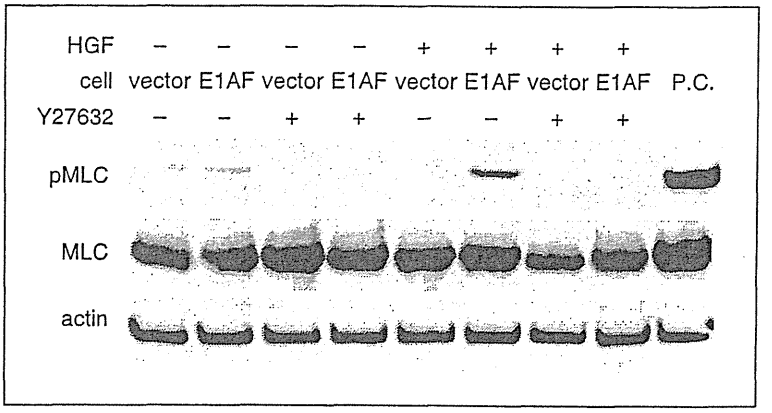

Figure 4. Western blot analysis of intracellular phosphorylated MLC, total MLC, and actin in LCD-vector and LCD-E1AF cells. Total protein was isolated from subconfluent cells cultured with or without HGF (40 ng/mL) for 24 hours and with or without Y27632 (100 μmol/L) for 24 hours. Levels of phosphorylated MLC and total MLC were determined by Western blot analysis. The protein from the lung tumors of E1AF cells in Fig. 5 was also used in this figure for positive control.

mice and anti–phosphorylated MLC antibody from rabbits. The primary antibodies were detected using antimouse or antirabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system.

Statistical analysis. Statistical differences in the means were examined by Student's unpaired two-tailed *t* test.

Results

E1AF activates Rho in VMRC-LCD cells. We have previously reported frequent overexpression of E1AF in NSCLC cell lines, except for VMRC-LCD and NCI-H226 cells, using Northern blot analysis and generation of E1AF-expressing VMRC-LCD and NCI-H226 cells (6). Western blot analysis using an anti-E1AF monoclonal antibody confirmed the presence of E1AF protein expression in LCD-E1AF and H226-E1AF cells and the absence of endogenous E1AF protein in LCD-vector and H226 vector cells (Fig. 1*A*). Endogenous E1AF protein expression was detected in A549 and NCI-H520 cells whereas it was weaker than that in LCD-E1AF and H226-E1AF cells.

To determine the effect of E1AF expression on Rho activity in VMRC-LCD, we measured the intracellular levels of GTP-bound (active) Rho using a pull-down assay system. We compared the activity of Rho in LCD-E1AF cells and LCD-vector cells with and without HGF treatment. As shown in Fig. 1*B*, the level of the GTP-bound (active) Rho was higher in LCD-E1AF cells than in LCD-vector cells. HGF enhanced the level of active Rho strongly in LCD-E1AF cells but had little effect in LCD-vector cells. Total Rho protein levels were similar under each condition in each cell line (Fig. 1*B*), showing that the activation was not caused by an increase in Rho expression.

We did the same experiments using H226-E1AF and H226-vector cells (Fig. 1*C*) as in LCD-E1AF and LCD-vector cells. H226-E1AF cells also showed higher active Rho levels than H226-vector cells in a HGF-enhanced manner whereas total Rho protein was similar between the cells, suggesting that the activation of Rho protein by E1AF and HGF without an increase in its expression is not due to variation in NSCLC cell lines.

A Rho-associated kinase inhibitor decreases cell motility and invasion. We next determined the effects of Y27632, a small-molecule inhibitor of ROCK. As in our previous report (6), LCD-E1AF cells showed significantly more motile and invasive activities than LCD-vector cells (motility, $P = 0.04$; invasion, $P = 0.04$; Fig. 2*A* and *B*) especially under HGF treatment (motility, $P = 0.009$; invasion, $P = 0.009$; Fig. 2*C* and *D*). Y27632 dose-dependently inhibited motility and invasion in both cell lines and at high Y27632 concentration, these activities became similarly low (Fig. 2), indicating that Y27632 decreased the motile and invasive activities more in LCD-E1AF cells than in LCD-vector cells.

To assess whether Y27632 was toxic, MTT assays were done with or without 10% serum. As shown in Fig. 3, Y27632 at 100 μmol/L, the highest concentration used in Fig. 2, did not affect the growth of either VMRC-LCD cell line with or without serum.

E1AF increases phosphorylation of myosin light chain whereas a Rho-associated kinase inhibitor decreases its phosphorylation. To analyze the effect of E1AF on ROCK activity in VMRC-LCD cells, we measured the phosphorylation of MLC,

Table 1. Tumorigenic and metastatic activities of LCD-vector and LCD-E1AF cells after s.c. and intrapulmonary implantation in nude mice

	S.c. implantation (day 56)		Intrapulmonary implantation (day 42)	
	Primary*	Metastasis*	Primary*	Metastasis*
LCD-vector	1/7 (0.23 ± 0.62 g)†	0/6	1/7 (9 ± 23 mg)‡	0/6
LCD-E1AF	6/6 (4.73 ± 3.29 g)†	0/6	4/7 (66 ± 65 mg)‡	3/7 (71 ± 92 mg)§

*Data are shown as no. of mice with tumor/no. of total mice (tumor weight per mouse shown as mean ± SD).
†$P < 0.01$, LCD-E1AF versus LCD-vector.
‡$P < 0.05$, LCD-E1AF versus LCD-vector.
§Metastatic site of all three mouse was mediastinal lymph node.

a downstream effector of ROCK, using Western blot analysis. As shown in Fig. 4, phosphorylated MLC was more abundant in LCD-E1AF cells than in LCD-vector cells. HGF strongly enhanced the phosphorylation of MLC in LCD-E1AF cells but not in LCD-vector cells. Y27632 inhibited the phosphorylation of MLC induced by E1AF and HGF to an undetectable level.

E1AF increases tumorigenic and metastatic activities in association with phosphorylated myosin light chain. The tumorigenicity and metastasis potential of LCD-E1AF and LCD-vector cells was investigated by injection into nude mice. Eight weeks after i.v. injection of LCD-E1AF or LCD-vector cells, no tumor was observed in any organs (data not shown). After s.c. injection, all of six mice injected with LCD-E1AF cells and one of seven mice injected with LCD-vector cells had local tumors in flank subcutis 56 days after transplantation (Table 1). The s.c. tumors of LCD-E1AF cells were significantly heavier than those of LCD-vector (4.73 ± 3.29 versus 0.23 ± 0.62 g, $P < 0.005$). No metastatic tumors were observed in any organs from either cell line. When intrapulmonarily injected, LCD-E1AF and LCD-vector cells formed local tumors in the lung in four of seven and one of seven nude mice, respectively, 42 days after transplantation (Table 1). Each tumor-bearing mouse had one lung tumor. Lung tumors of LCD-E1AF cells were significantly heavier than those of LCD-vector (66 ± 65 versus 9 ± 23 mg, $P < 0.05$). Metastatic tumors from LCD-E1AF cells were observed in mediastinal lymph nodes in three of the four mice with lung tumors at 42 days (Fig. 5*A*). The number of mediastinal metastasis was three for one mouse at 42 days and one for the other three mice. The mean tumor weight was 71 ± 92 mg at 42 days after transplantation. Histopathologic examinations confirmed adenocarcinoma occupying the mediastinal lymph node (Fig. 5*B*). In contrast, there was no metastasis in mediastinal lymph nodes from the mouse with lung tumors of LCD-vector cells. There were no metastases from tumors of either cell line in the other organs.

To test whether Rho/ROCK signal is activated by E1AF *in vivo*, we examined the phosphorylation of MLC in tumors developed from LCD-E1AF and LCD-vector cells. By Western blot analysis (Fig. 5*C*), phosphorylated MLC was much more abundant in s.c., lung, and mediastinal lymph node tumors from LCD-E1AF cells than in s.c. and lung tumors from LCD-vector cells.

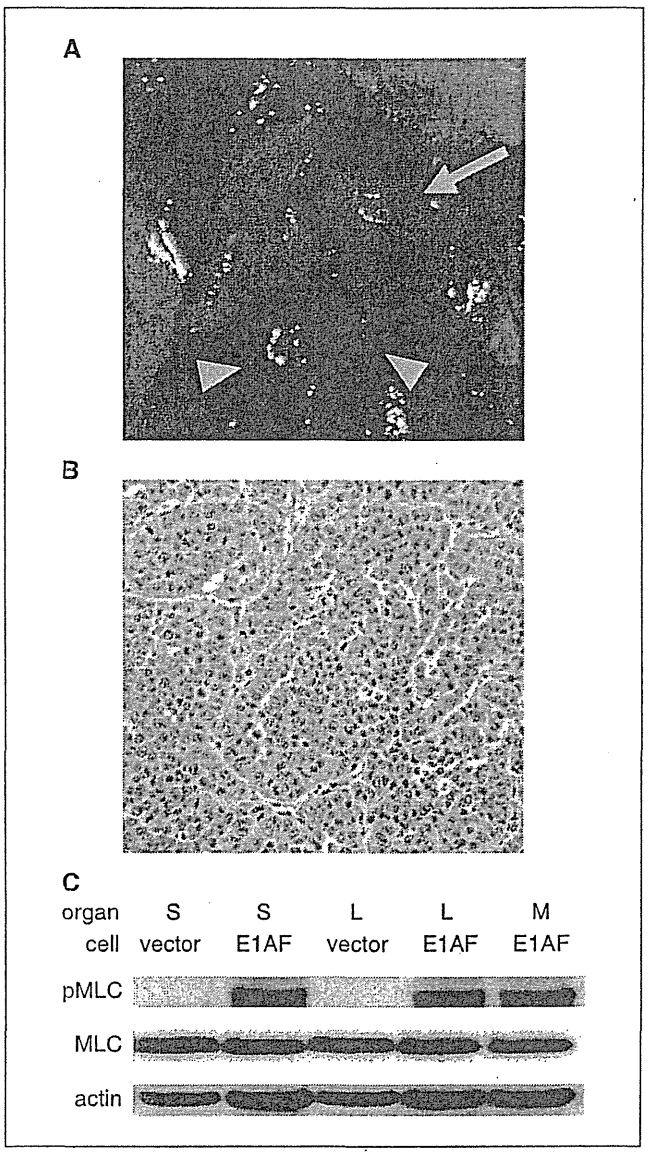

Figure 5. *In vivo* tumorigenic and metastatic activities of LCD-E1AF cells. *A*, tumor growth 42 days after intrapulmonary implantation of LCD-E1AF cells in nude mice. LCD-E1AF cells (1×10^7) were injected into the left lung parenchyma through the intercostal space. Tumors were observed in the lung (*arrow*) and in mediastinal lymph nodes (*arrowhead*). *B*, histopathologic examinations of the metastatic tumors in mediastinal lymph nodes confirmed adenocarcinoma occupying the mediastinal lymph node (magnification, ×400). *C*, Western blot analysis of phosphorylated MLC in tumors developed from LCD-E1AF and LCD-vector cells. S.c. (*S*), lung (*L*), and mediastinal lymph node (*M*) tumors from mice were homogenized and sonicated, and then total proteins (30 μg) purified from the tumors were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and reacted with anti-MLC antibody, anti–phosphorylated MLC antibody, or anti-actin antibody as control. For lung and mediastinal tumors of LCD-E1AF cells, multiple tumors from the same tumor-bearing mice were pooled for these analyses (see Materials and Methods).

Discussion

This is the first study showing that E1AF overexpression results in the activation of the Rho/ROCK signaling pathway. HGF accentuated this activation strongly. The inhibition of cell migration and invasion by a ROCK inhibitor suggests that Rho/ROCK activation is necessary for cell migration and invasion induced by E1AF. Furthermore, we have shown an increase in tumorigenesis and metastasis caused by E1AF expression, which also results in phosphorylation of MLC, a downstream target of Rho/ROCK signaling.

We have previously shown that E1AF increases migration and invasion of cancer cells and that the increased invasion is associated with E1AF-induced expression of MMPs and urokinase plasminogen activator (uPA; refs. 2, 4–6). In this study, we have shown abundant GTP-bound Rho in E1AF-transfected cells, suggesting that another mechanism, the activation of Rho, may be involved in migration and invasion enhanced by E1AF. Extensive studies have shown that increased motility and invasion induced by Rho are mediated through its downstream molecules, ROCK and MLC (15–18); MLC phosphorylation was shown in E1AF-transfected cells in the present study. Furthermore, a specific ROCK inhibitor, Y27632, inhibited cell migration and motility and decreased phosphorylation of MLC, suggesting that activation of Rho/ROCK signaling is necessary for E1AF-induced migration and invasion.

The Rho/ROCK signaling pathway has been shown to be involved in motility and invasion of various cancer cell lines, such as rat

hepatoma cells (20), human glioma cells (21), and human ovarian cancer cells (22). Studies using clinical specimens showed a relationship between the expression level of RhoC, an isoform of Rho, and tumor aggressiveness in breast cancer (23–25) and pancreatic cancer (26). Recently, NSCLCs have also been shown to express RhoC mRNA and protein at higher levels than do nontumor tissues, and the RhoC expression level is correlated to vascular permeation (27). Taken together with the accumulating data showing association of Rho/ROCK signaling with migration and invasion, the present results suggest that the Rho/ROCK signaling has a central role in migration and invasion enhanced by E1AF in NSCLC cells.

E1AF has previously been correlated with altered expression of several genes, including *uPA*, *MMP*, and another Ets family gene, *Ets-1* (2, 6). LCD-E1AF cells were found to overexpress Ets-1 and the kinetics of HGF-mediated activation of Ets-1 is better correlated with uPA level than with E1AF level (6). This may suggest that the biological changes in the cell line may be mediated by altered expression of Ets-1 or possibly other Ets family members. All of ~30 Ets family transcription factors bind GGAA/T core sequence with some specificity depending on flanking sequences, suggesting their redundant functions (34). To determine their involvement in the phenotypes of E1AF-overexpressing cells, experiments with small interfering RNA of Ets-1 and other Ets family members possibly overexpressed in the cells will be required.

To our knowledge, the relationship of not only E1AF but also of other Ets family members, including Ets-1, with the activation of Rho has not been previously reported. Although E1AF and Ets-1 are transcription factors, their expression did not increase total Rho protein, suggesting that the activation of Rho is not caused by their transcriptional regulation. Rho activity is regulated by guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP; ref. 35). Possible mechanisms for the Rho activation by E1AF include transcriptional regulation of GEFs and GAPs directly by E1AF or indirectly by potential mediators like Ets-1. A growing number of GEFs and GAPs for the Rho family have been identified, which by now include 69 Dbl-related GEF proteins, the largest family of RhoGEF, and more than 30 RhoGAPs (36, 37). To determine whether the activation of Rho by E1AF involves such mechanisms, comprehensive analyses including microarray analysis should be investigated in the near future.

The detection of endogenous E1AF protein in A549 and NCI-H520 cells is consistent with our previous study showing the E1AF mRNA expression in these cells and suggests roles of the endogenous E1AF protein in NSCLC cell lines. Loss of function studies of E1AF in the E1AF-expressing cell lines would clarify the roles of E1AF in the observed phenotypes, including Rho signaling activation, especially in a physiologic context.

The tumorigenicity potential of E1AF is indicated by the observed increase in incidence and weight of tumors in the skin and lung of nude mice developed from E1AF-transfected cells compared with vector-transfected cells. Tumorigenesis by E1AF has been investigated for breast tumors. E1AF is overexpressed in the vast majority of human breast cancers and in nearly all HER2/Neu–positive tumors (38). Using MMTV-Neu transgenic mice, Shepherd et al. (8) showed that expression of a dominant-negative E1AF transgene under the control of the MMTV promoter in mammary epithelial cells dramatically delayed the onset of mammary tumors and reduced the number and size of such tumors in individual mice. On the other hand, Xing et al. (39) reported that E1AF suppresses HER2 promoter activity in human tumor-derived cell lines, dependent on an E1AF binding site in this promoter, and inhibits tumorigenesis of the cells. However, several articles have shown that E1AF can activate E1AF-responsive promoters, including the human HER2/neu promoter in various cell types (3, 8, 40–42). Bojovic and Hassell (43) noted that the expression of these same genes is inhibited when E1AF protein is expressed at very high levels and suggested that transcriptional squelching accounts for these observations. Thus, both our results and observations in breast cancer indicate the tumorigenic activity of E1AF but this activity may depend on various conditions including cell type.

The development of mediastinal lymph node metastases from lung tumors of E1AF-transfected cells, but not from the lung tumor of vector-transfected cells, suggests that E1AF may enhance metastasis of NSCLC cells. These observations are consistent with previous reports showing that E1AF increases *in vivo* metastatic activities of fibrosarcoma cells (7) and breast cancer cells (44).

Highly phosphorylated MLC in the local and metastatic tumors developed from E1AF-transfected cells, suggesting an association between E1AF tumorigenic and metastatic activities and the increased activity of the Rho/ROCK pathway. Rho has been shown to have transforming activities (13, 14, 24) and may have some role in the observed tumorigenesis by E1AF. The association of Rho/ROCK signaling with metastasis is also consistent with recent reports showing that RhoC enhances metastasis of melanoma cells (28) and lung cancer cells (29) in association with migration and invasion.

In conclusion, these results suggest that E1AF induces activation of the Rho/ROCK pathway, which plays an important role for malignant phenotypes including motility and invasion as well as tumorigenesis and metastasis enhanced by E1AF in NSCLC cells. Inhibition of this pathway by molecules such as Y27632 may have therapeutic potential to control NSCLCs, most of which overexpress E1AF.

Acknowledgments

Received 1/7/2005; revised 8/3/2005; accepted 9/14/2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Prof. Fumiaki Sasaki (Department of Pediatric Surgery, Hokkaido University Graduate School of Medicine) for suggestions and fruitful discussion.

References

1. Wasylyk B, Hahn SL, Giovane A. The Ets family of transcription factors. Eur J Biochem 1993;211:7–18.
2. Higashino F, Yoshida K, Noumi T, Seiki M, Fujinaga K. Ets-related protein E1A-F can activate three different matrix metalloproteinase gene promoters. Oncogene 1995;10:1461–3.
3. Xin JH, Cowie A, Lachance P, Hassell JA. Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. Genes Dev 1992;6:481–96.
4. Kaya M, Yoshida K, Higashino F, Mitaka T, Ishii S, Fujinaga K. A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene 1996;12:221–7.
5. Shindoh M, Higashino F, Kaya M, et al. Correlated expression of matrix metalloproteinases and ets family transcription factor E1A-F in invasive oral squamous-cell-carcinoma-derived cell lines. Am J Pathol 1996;148:693–700.
6. Hiroumi H, Dosaka-Akita H, Yoshida K, et al. Expression of E1AF/PEA3, an Ets-related transcription factor in human non-small-cell lung cancers: its relevance in cell motility and invasion. Int J Cancer 2001;93:786–91.
7. Habelhah H, Okada F, Kobayashi M, et al. Increased

E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. Oncogene 1999;18:1771–6.
8. Shepherd TG, Kockeritz L, Szrajber MR, Muller WJ, Hassell JA. The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis. Curr Biol 2001;11:1739–48.
9. Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A. Microinjection of recombinant p21rho induces rapid changes in cell morphology. J Cell Biol 1990;111:1001–7.
10. Takaishi K, Kikuchi A, Kuroda S, Kotani K, Sasaki T, Takai Y. Involvement of rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in cell motility. Mol Cell Biol 1993;13:72–9.
11. Kishi K, Sasaki T, Kuroda S, Itoh T, Takai Y. Regulation of cytoplasmic division of *Xenopus* embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). J Cell Biol 1993;120:1187–95.
12. Mabuchi I, Hamaguchi Y, Fujimoto H, Morii N, Mishima M, Narumiya S. A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. Zygote 1993;1:325–31.
13. Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF, Der CJ. Critical role of Rho in cell transformation by oncogenic Ras. Oncogene 1995;10:2289–96.
14. Perona R, Esteve P, Jimenez B, Ballestero RP, Ramon y Cajal S, Lacal JC. Tumorigenic activity of rho genes from *Aplysia californica*. Oncogene 1993;8:1285–92.
15. Takai Y, Sasaki T, Tanaka K, Nakanishi H. Rho as a regulator of the cytoskeleton. Trends Biochem Sci 1995; 20:227–31.
16. Narumiya S. The small GTPase Rho: cellular functions and signal transduction. J Biochem (Tokyo) 1996; 120:215–28.
17. Machesky LM, Hall A. Rho: a connection between membrane receptor signalling and the cytoskeleton. Trends Cell Biol 1996;6:304–10.
18. Narumiya S, Ishizaki T, Watanabe N. Rho effectors and reorganization of actin cytoskeleton. FEBS Lett 1997;410:68–72.
19. Yoshioka K, Matsumura F, Akedo H, Itoh K. Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. J Biol Chem 1998;273:5146–54.
20. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 1999;5:221–5.
21. Manning TJ, Jr., Parker JC, Sontheimer H. Role of lysophosphatidic acid and rho in glioma cell motility. Cell Motil Cytoskeleton 2000;45:185–99.
22. Sawada K, Morishige K, Tahara M, et al. Alendronate inhibits lysophosphatidic acid-induced migration of human ovarian cancer cells by attenuating the activation of rho. Cancer Res 2002;62:6015–20.
23. van Golen KL, Davies S, Wu ZF, et al. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res 1999;5:2511–9.
24. van Golen KL, Wu ZF, Qiao XT, Bao LW, Merajver SD. RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 2000;60:5832–8.
25. Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. Am J Pathol 2002;160:579–84.
26. Suwa H, Ohshio G, Imamura T, et al. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 1998;77: 147–52.
27. Shikada Y, Yoshino I, Okamoto T, Fukuyama S, Kameyama T, Maehara Y. Higher expression of RhoC is related to invasiveness in non-small cell lung carcinoma. Clin Cancer Res 2003;9:5282–6.
28. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000;406:532–5.
29. Ikoma T, Takahashi T, Nagano S, et al. A definitive role of RhoC in metastasis of orthotopic lung cancer in mice. Clin Cancer Res 2004;10:1192–200.
30. Iwasaki M, Nishikawa A, Akutagawa N, et al. E1AF/PEA3 reduces the invasiveness of SiHa cervical cancer cells by activating serine proteinase inhibitor squamous cell carcinoma antigen. Exp Cell Res 2004;299:525–32.
31. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 1999;18:578–85.
32. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
33. Doki Y, Murakami K, Yamaura T, Sugiyama S, Misaki T, Saiki I. Mediastinal lymph node metastasis model by orthotopic intrapulmonary implantation of Lewis lung carcinoma cells in mice. Br J Cancer 1999;79:1121–6.
34. Sementchenko VI, Watson DK. Ets target genes: past, present and future. Oncogene 2000;19:6533–48.
35. Zheng Y. Dbl family guanine nucleotide exchange factors. Trends Biochem Sci 2001;26:724–32.
36. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167–80.
37. Moon SY, Zheng Y. Rho GTPase-activating proteins in cell regulation. Trends Cell Biol 2003;13:13–22.
38. Benz CC, O'Hagan RC, Richter B, et al. HER2/Neu and the Ets transcription activator PEA3 are coordinately up-regulated in human breast cancer. Oncogene 1997;15:1513–25.
39. Xing X, Wang SC, Xia W, et al. The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. Nat Med 2000;6:189–95.
40. Janknecht R, Monte D, Baert JL, de Launoit Y. The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. Oncogene 1996;13:1745–54.
41. Janknecht R. Analysis of the ERK-stimulated ETS transcription factor ER81. Mol Cell Biol 1996;16:1550–6.
42. Crawford HC, Fingleton B, Gustavson MD, et al. The PEA3 subfamily of Ets transcription factors synergizes with β-catenin-LEF-1 to activate matrilysin transcription in intestinal tumors. Mol Cell Biol 2001;21:1370–83.
43. Bojovic BB, Hassell JA. The PEA3 Ets transcription factor comprises multiple domains that regulate transactivation and DNA binding. J Biol Chem 2001;276: 4509–21.
44. de Launoit Y, Chotteau-Lelievre A, Beaudoin C, et al. The PEA3 group of ETS-related transcription factors. Role in breast cancer metastasis. Adv Exp Med Biol 2000;480:107–16.

Reprinted from
Jpn J Clin Oncol 2004;34(1)43–45

Clinical Trial Note

Feasibility Study of Neoadjuvant Chemotherapy Followed by Interval Cytoreductive Surgery for Stage III/IV Ovarian, Tubal and Peritoneal Cancers: Japan Clinical Oncology Group Study JCOG0206

Takashi Onda[1], Toshiharu Kamura[2], Naoki Ishizuka[3], Noriyuki Katsumata[4], Haruhiko Fukuda[3] and Hiroyuki Yoshikawa[5]

[1]Division of Gynecologic Oncology, National Cancer Center Hospital, Tokyo, [2]Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Fukuoka, [3]Japan Clinical Oncology Group Data Center, Cancer Information and Epidemiology Division, National Cancer Center Research Institute, Tokyo, [4]Department of Medical Oncology, National Cancer Center Hospital, Tokyo and [5]Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

Jpn J Clin Oncol 2004;34(1)43–45

Clinical Trial Note

Feasibility Study of Neoadjuvant Chemotherapy Followed by Interval Cytoreductive Surgery for Stage III/IV Ovarian, Tubal and Peritoneal Cancers: Japan Clinical Oncology Group Study JCOG0206

Takashi Onda[1], Toshiharu Kamura[2], Naoki Ishizuka[3], Noriyuki Katsumata[4], Haruhiko Fukuda[3] and Hiroyuki Yoshikawa[5]

[1]Division of Gynecologic Oncology, National Cancer Center Hospital, Tokyo, [2]Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Fukuoka, [3]Japan Clinical Oncology Group Data Center, Cancer Information and Epidemiology Division, National Cancer Center Research Institute, Tokyo, [4]Department of Medical Oncology, National Cancer Center Hospital, Tokyo and [5]Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

Received October 7, 2003; accepted December 3, 2003

A feasibility study was started in January 2003 on neoadjuvant chemotherapy (NAC) followed by interval cytoreductive surgery (ICS) and postoperative chemotherapy for stage III/IV müllerian carcinomas such as ovarian, tubal and peritoneal carcinomas. The purpose is to assess the safety and efficacy of the treatment starting with NAC and also to know whether we can accurately diagnose these advanced carcinomas by imaging studies, cytologic findings and tumor markers without staging laparotomy or laparoscopy. Fifty-six patients with advanced müllerian carcinomas will be recruited to the study. After confirmation of diagnosis by laparoscopic inspection and biopsies, patients undergo four cycles of chemotherapy as NAC, followed by ICS and an additional four cycles of post-surgical chemotherapy. The primary endpoint is proportion of clinical complete remission after accomplishment of the protocol treatment, while the major secondary endpoint is positive predictive value of diagnosis before laparoscopy regarding tumor origin, histology and stage. Based on the results of this study, we will conduct a phase III study to compare the treatment starting with NAC and primary cytoreductive surgery followed by post-surgical chemotherapy.

Key words: ovarian neoplasms – laparoscopy – neoadjuvant therapy – interval cytoreductive surgery

INTRODUCTION

Prognosis of patients with advanced epithelial ovarian, tubal and peritoneal carcinomas is known to be poor. Even using platinum compound regimens, the 5-year survival rate of stage III/IV ovarian cancer is still around 20% (1). The current standard treatment for advanced ovarian cancer is primary cytoreductive surgery followed by post-surgical chemotherapy. However, optimal cytoreduction in primary surgery can be achieved only in 40% of stage III/IV ovarian cancer patients (2). An alternative to primary surgical cytoreduction in patients with unresectable bulky tumors or poor performance status is the use of chemotherapy in the neoadjuvant setting. Recent retrospective analyses (3–6) have revealed that progression-free and overall survival were comparable between patients treated with neoadjuvant chemotherapy (NAC) followed by interval cytoreductive surgery (ICS) and those treated by primary cytoreductive surgery, though the former group was older and had a poorer performance status. Phase II and III trials have not been performed on the role of neoadjuvant-setting treatment for advanced ovarian, tubal and peritoneal cancers. Therefore, we started a phase II study to assess the safety and efficacy of NAC followed by ICS and post-surgical chemotherapy before comparing with the current standard treatment including primary cytoreductive surgery in randomized controlled trial. Neoadjuvant setting has the advantage of earlier treatment start and lower invasiveness. However, according to the current general rules for the management of ovarian cancer, it is neces-

For reprints and all correspondence: Takashi Onda, Division of Gynecologic Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: taonda@ncc.go.jp

sary to confirm the origin, histology and stage before starting treatment by staging laparotomy or laparoscopy. Thus, we also determine whether we can omit the 'extra procedure' of staging laparotomy or laparoscopy before the neoadjuvant-setting treatment in the majority of patients with advanced ovarian, tubal or peritoneal cancer.

The study protocol was designed by Gynecologic Cancer Study Group (GCSG) of the Japan Clinical Oncology Group (JCOG), approved by the Clinical Trial Review Committee of JCOG on December 6, 2002, and activated on January 14, 2003.

PROTOCOL DIGEST OF THE JCOG0206

Purpose

The purposes are to assess the safety and efficacy of the treatment starting with NAC with paclitaxel and CBDCA for phase III study, comparing NAC therapy with current standard procedure, and to know whether we can accurately diagnose these advanced carcinomas by imaging studies, cytologic findings and tumor markers without staging laparotomy or laparoscopy.

Study Setting

A multi-institutional (26 centers) non-randomized phase II trial.

Resources

Health Sciences Research Grants for Clinical Research for Evidenced Based Medicine and Grants-in Aid for Cancer Research (nos 14S-4, 14-12), from the Ministry of Health, Labor and Welfare, Japan.

Endpoints

Primary endpoint is proportion of clinical complete remission (%cCR) among all stage III or IV müllerian carcinoma confirmed by laparoscopic inspection and histopathology of biopsy specimens. Clinical complete remission is defined as disappearance of all lesions by computed tomography (CT) or magnetic resonance imaging (MRI), no pleural effusions by chest radiography and normal serum CA125 level (<20 U/ml) after completion of the protocol treatment.

Secondary endpoints are as follows: (i) positive predictive value (PPV) of pre-laparoscopic diagnosis concerning the origin and histology—proportion of the patients diagnosed as müllerian carcinoma by laparoscopic inspection and histopathology of biopsy specimen among those diagnosed by pre-laparoscopic findings; (ii) PPV of prelaparoscopic diagnosis concerning clinical stage—proportion of the patients diagnosed as stage III or IV by laparoscopic inspection among those diagnosed by pre-laparoscopic findings; (iii) PPV of overall pre-laparoscopic diagnosis—proportion of the patients diagnosed as stage III or IV müllerian carcinoma by laparoscopic inspection and histopathology of biopsy specimen among those diagnosed by pre-laparoscopic findings.

Other secondary endpoints are: (iv) response rate to NAC among patients whose clinical diagnosis is confirmed by laparoscopy; (v) proportion of patients who received ICS among patients whose clinical diagnosis is confirmed by laparoscopy; (vi) progression-free survival among patients whose clinical diagnosis is confirmed by laparoscopy; (vii) operative morbidity among all enrolled patients; (viii) adverse events among all enrolled patients: and (ix) overall survival among all enrolled patients.

Eligibility Criteria

Inclusion criteria

The study subjects are patients diagnosed as stage III or IV müllerian carcinoma by pre-laparoscopic clinical findings including imaging studies (CT, MRI or ultrasonography) and cytology of ascites, pleural effusions or fluids obtained by tumor centesis. Malignancies of other origins, such as breast and digestive tract, should be excluded by endoscopy, opaque enema or ultrasonography when these malignancies are suspected from symptoms, physical examination or imaging diagnosis. To rule out malignancy of digestive tract origin, criteria for tumor markers are set to be CA125 >200 U/ml and CEA <20 ng/ml.

Further inclusion criteria are: (i) clinically deemed to be a candidate for debulking surgery without evidence of brain, bone, bone marrow metastases, multiple lung or multiple liver metastases; (ii) presence of at least one measurable lesion; (iii) previously untreated for these malignancies and no history of treatment with chemotherapy nor radiotherapy even for other diseases; (iv) age 20–75 years; (v) Eastern Cooperative Oncology Group (ECOG) performance status of 0–3; (vi) adequate bone marrow, hepatic, renal, cardiac and respiratory functions; and (vii) written informed consent.

Exclusion criteria

These are: (i) synchronous or metachronous (within 5 years) malignancy other than carcinoma in situ; (ii) pregnant or nursing; (iii) severe mental disorders; (iv) systemic and continuous use of steroidal drugs; (v) active infections; (vi) uncontrolled hypertension; (vii) diabetes mellitus, uncontrolled or controlled with insulin; (viii) history of cardiac failure, unstable angina, myocardial infarction within 6 months prior to the registration; (ix) liver cirrhosis or bleeding tendency contraindicating debulking surgery; (x) intestinal occlusion necessary for surgical treatment; and (xi) hypersensitivity to alcohol.

Treatment Methods

Diagnostic laparoscopy

After enrolment, diagnostic laparoscopy is performed within 2 weeks. To confirm pre-laparoscopic clinical diagnosis of origin, histology and stage, inspection of peritoneal cavity and biopsy from the main tumor or metastatic tumors are per-

formed. Resection of any organs or tumors attempting to reduce tumor volume is not allowed.

NEOADJUVANT CHEMOTHERAPY (NAC)

Four cycles of combination of paclitaxel (175 mg/m^2, day 1) and carboplatin (AUC = 6, day 1) are administered every 3 weeks. NAC is initiated within 1 week after laparoscopy.

INTERVAL CYTOREDUCTIVE SURGERY (ICS)

ICS is performed in 4–7 weeks after administration of the fourth cycle of NAC unless disease progression occurs during NAC. Standard procedures of ICS consist of total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and maximal debulking of metastatic tumors. Systematic pelvic and/or aortic lymphadenectomies are allowed, but not included in standard procedures.

POST-SURGICAL CHEMOTHERAPY

An additional four cycles of chemotherapy (same regimen as NAC) is administered (eight cycles of chemotherapy in total). Post-surgical chemotherapy is initiated within 3 weeks after ICS.

STUDY DESIGN AND STATISTICAL METHODS

The study is planned as a single-stage safety and efficacy study. Sample size calculation was primarily based on binominal test for the primary endpoint, %cCR. Forty-four eligible patients are required when expected %cCR of 40% and an acceptable lowest %cCR of 20% with alpha error level of 0.05 and beta error level of 0.1. Additionally, PPV is to be confident enough to omit laparoscopy before NAC in the following phase III study. It is not possible to use sensitivity or specificity to evaluate accuracy of clinical diagnoses, because laparoscopy is performed only in patients diagnosed as stage III/IV müllerian carcinomas by clinical findings in this study setting. Thus, Bayesian monitoring PPV is planned, which requires 56 patients to have the 10% or lower Bayesian posterior probability that PPV is <90% in case of three false positive patients assuming prior distribution of beta (9,1). The target sample size was determined to be 56, which also can be expected sufficient for primary endpoint. The planned accrual period is 1 year and the follow-up period is set as 3 years after the completion of accrual.

STUDY MONITORING

In-house interim monitoring is performed by the JCOG Data Center to ensure data submission, patient eligibility, protocol compliance, safety and on-schedule study progress according to the JCOG standard procedures. The monitoring reports are submitted to the JCOG Data and Safety Monitoring Committee every 6 months.

PARTICIPATING INSTITUTIONS

Hokkaido University, Sapporo Medical University, Tohoku University, University of Tsukuba, Gunma Prefectural Cancer Center, Shinshu University, National Defense Medical College, Saitama Cancer Center, National Cancer Center Hospital, The Jikei University School of Medicine, Cancer Institute Hospital, University of Tokyo, Juntendo University, Nagaoka Red Cross Hospital, Aichi Cancer Center, National Nagoya Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, Kinki University, Niigata Cancer Center, Kure National Hospital (Chugoku District Cancer Center), National Shikoku Cancer Center, National Kyushu Cancer Center, University of Kurume, Kyushu University, Saga Medical School and Kagoshima City Hospital.

References

1. Nguyen HN, Averette HE, Hoskins W, Sevin BU, Penalver M, Steren A. National survey of ovarian carcinoma. VI. Critical assessment of current International Federation of Gynecology and Obstetrics staging system. *Cancer* 1993;72:3007–11.
2. Covens AL. A critique of surgical cytoreduction in advanced ovarian cancer. *Gynecol Oncol* 2000;78:269–74.
3. Jacob JH, Gershenson DM, Morris M, Copeland LJ, Burke TW, Wharton JT. Neoadjuvant chemotherapy and interval debulking for advanced epithelial ovarian cancer. *Gynecol Oncol* 1991;42:146–50.
4. Onnis A, Marchetti M, Padovan P, Castellan L. Neoadjuvant chemotherapy in advanced ovarian cancer. *Eur J Gynaecol Oncol* 1996;17:393–6.
5. Schwartz PE, Rutherford TJ, Chambers JT, Kohorn EI, Thiel RP. Neoadjuvant chemotherapy for advanced ovarian cancer: long-term survival. *Gynecol Oncol* 1999;72:93–9.
6. Vergote I, De Wever I, Tjalma W, Van Gramberen M, Decloedt J, van Dam P. Neoadjuvant chemotherapy or primary debulking surgery in advanced ovarian carcinoma: a retrospective analysis of 285 patients. *Gynecol Oncol* 1998;71:431–6.

Reprinted from
Jpn J Clin Oncol 2004;34(9)540–546
doi:10.1093/jjco/hyh098

Cisplatin, Paclitaxel and Escalating Doses of Doxorubicin (TAP) in Advanced Ovarian Cancer: a Phase I Trial

Takashi Onda[1], Noriyuki Katsumata[2], Ryuichiro Tsunematsu[1], Toshiharu Yasugi[3], Masafumi Mushika[4], Kaichiro Yamamoto[5], Tsuneo Fujii[6], Toshio Hirakawa[7], Toshiharu Kamura[8], Toshiaki Saito[9] and Hiroyuki Yoshikawa[10]

[1]Division of Gynecological Oncology and [2]Department of Medical Oncology, National Cancer Center Hospital, Tokyo, [3]Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, [4]Department of Obstetrics and Gynecology, National Nagoya Hospital, Nagoya, [5]Department of Obstetrics and Gynecology, Sakai Hospital, Kinki University School of Medicine, Sakai, [6]Department of Obstetrics and Gynecology, National Kure Medical Center, Kure, Hiroshima, [7]Department of Obstetrics and Gynecology, Faculty of Medicine, Kyushu University, Fukuoka, [8]Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Fukuoka, [9]Gynecology Service, National Kyushu Cancer Center, Fukuoka and [10]Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

Jpn J Clin Oncol 2004;34(9)540–546
doi:10.1093/jjco/hyh098

Cisplatin, Paclitaxel and Escalating Doses of Doxorubicin (TAP) in Advanced Ovarian Cancer: a Phase I Trial

Takashi Onda[1], Noriyuki Katsumata[2], Ryuichiro Tsunematsu[1], Toshiharu Yasugi[3], Masafumi Mushika[4], Kaichiro Yamamoto[5], Tsuneo Fujii[6], Toshio Hirakawa[7], Toshiharu Kamura[8], Toshiaki Saito[9] and Hiroyuki Yoshikawa[10]

[1]Division of Gynecological Oncology and [2]Department of Medical Oncology, National Cancer Center Hospital, Tokyo, [3]Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, [4]Department of Obstetrics and Gynecology, National Nagoya Hospital, Nagoya, [5]Department of Obstetrics and Gynecology, Sakai Hospital, Kinki University School of Medicine, Sakai, [6]Department of Obstetrics and Gynecology, National Kure Medical Center, Kure, Hiroshima, [7]Department of Obstetrics and Gynecology, Faculty of Medicine, Kyushu University, Fukuoka, [8]Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Fukuoka, [9]Gynecology Service, National Kyushu Cancer Center, Fukuoka and [10]Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

Received April 3, 2004; accepted June 10, 2004

Background: The objectives of this phase I trial were to determine the maximum tolerated dose (MTD) and the recommended dose (RD) for phase II/III trials of doxorubicin (DOX) combined with paclitaxel (PTX) and cisplatin (CDDP) in patients with advanced ovarian cancer (AOC).
Methods: Twenty-eight patients with stage III/IV AOC received fixed doses of PTX (110 mg/m^2 over 24 h on day 1) and CDDP (75 mg/m^2 on day 2) and an escalating dose of DOX (20, 30, 40 or 50 mg/m^2 on day 1) every 3 weeks. The patients received up to six cycles of chemotherapy. At level 1, one of the original dose-limiting toxicities (DLTs), grade (G) 4 neutropenia lasting for 4 days or longer, occurred in four of six patients. The criterion for DLT was amended to 'G4 neutropenia lasting for 8 days or longer accompanied with G4 leukopenia' and four additional patients were evaluated at level 1.
Results: According to the new criteria, DLT was observed only in one of nine patients except one ineligible patient at level 1and two of six patients at level 4. G4 neutropenia and G4 leukopenia occurred in 85% and 44%, respectively, in the first course of chemotherapy. Non-hematological toxicity was generally mild or moderate. MTD was not determined at the planned dose levels. A clinical response was observed in 16 of 19 (84%) evaluable patients. Further dose escalation was not performed and RD was determined as level 4 because more than 30% of cycles required some modification of chemotherapy at level 4.
Conclusion: The combination of TAP including 50 mg/m^2 of DOX is feasible and well tolerated as first line chemotherapy in AOC, warranting further study of this regimen.

Key words: ovarian cancer – chemotherapy – doxorubicin – phase I study

INTRODUCTION

Since randomized trials have demonstrated the superiority of paclitaxel (PTX) plus cisplatin (CDDP) over cisplatin plus cyclophosphamide (CPA) in overall survival and progression-free survival (1,2) and subsequent trials demonstrated similar activity of PTX plus carboplatin (CBDCA) compared with PTX plus CDDP (3), the combination regimen of PTX plus platinum, such as CDDP or CBDCA, is considered the standard regimen for advanced ovarian cancer (AOC). The two-drug combination regimen of PTX and platinum yields a high response rate and improved survival for patients with AOC. In spite of chemotherapy development, the 5-year survival of patients with stage III/IV ovarian cancer is generally less than or around 20% (4), which is far from satisfactory. Therefore, several approaches, especially new agents or new drug combinations, are being examined in clinical studies to improve further the outcome of treatment for AOC.

Doxorubicin (DOX), an anthracycline, is known to be an active agent for ovarian cancer and was used in combination with CDDP and CPA as a standard regimen for ovarian cancer before the introduction of PTX plus platinum. The benefit of

For reprints and all correspondence: Takashi Onda, Division of Gynecological Oncology, National Cancer Center Hospital, 5–1–1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: taonda@ncc.go.jp

adding DOX to CDDP and CPA was controversial. A phase III randomized trial of CDDP plus CPA with or without DOX conducted by the Gynecological Oncology Group (GOG) (5) showed no clear benefit of DOX in the pathological complete response rate and median survival time. However, three meta-analyses demonstrated that the incorporation of DOX into the CDDP-based regimen for ovarian cancer may improve the long-term survival of AOC by 7–10% (6–8). Therefore, the value of DOX in the treatment of ovarian cancer was re-examined.

The benefit of adding DOX to the current standard regimen, PTX and platinum, should be evaluated to improve further the outcome of patients with AOC. To evaluate the safety and efficacy of this combination regimen, we conducted a phase I trial in patients with AOC for first-line chemotherapy using a combination of fixed doses of CDDP and PTX with escalating doses of DOX given every 3 weeks.

PATIENTS AND METHODS

Selection of Patients

The subjects of this study were untreated patients with stage IIIC or IV epithelial ovarian cancer. The histology of tumors included serous, mucinous, endometrioid, clear cell, mixed epithelial, undifferentiated, malignant Brenner, transitional cell and unclassified types. Patients with low potential malignancies were not included.

Other eligible criteria for entry into this study were as follows: (a) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; (b) age 16–75 years; (c) adequate bone marrow function [white blood cell count (WBC) $\geq$3000/mm^3 or absolute neutrophil count (ANC) $\geq$1500/mm^3 and platelet count $\geq$100 000/mm^3], adequate hepatic function [total serum bilirubin $\leq$1.5 mg/dl and serum aspartate aminotransferase (AST) $\leq$2.5 times the upper limit of normal], adequate renal function (serum creatinine $\leq$1.5 mg/dl and creatinine clearance $\geq$50 ml/min) and adequate cardiac function (normal or minor deviation in electrocardiogram); and (d) written informed consent. Patients were ineligible if they had (a) severe mental disorders; (b) uncontrolled hypertension; (c) history of cardiac failure, unstable angina, myocardial infarction within 6 months prior to the study; (d) liver cirrhosis; (e) diabetes mellitus, controlled with insulin; (f) history of severe hypersensitivity or hypersensitivity to drugs formulated with polyoxyethylated castor oil (Cremophor EL) as an ingredient (e.g. cyclosporine or vitamin K); (g) hepatitis B e antigen (HBeAg) or antibody against hepatitis C virus (HCV); or (h) if they were pregnant.

Treatment Plan

All patients underwent staging laparotomy and, simultaneously, maximum cytoreductive surgery. Following surgery, eligible patients were enrolled into the study. Patients received up to six cycles of chemotherapy consisting of paclitaxel (PTX), doxorubicin (DOX) and cisplatin (CDDP). DOX was administered as a 30 min intravenous (IV) infusion on day 1. PTX was administered as a 24 h continuous i.v. infusion on day 1 following DOX administration. CDDP was administered as a 2 h i.v. infusion on day 2. Chemotherapy was repeated every 21 days, assuming recovery from the toxicity of the previous cycle. Four different dose levels were tested. The dose of DOX was escalated from 20 mg/m^2 (level 1) to 50 mg/m^2 (level 4) in increments of 10 mg/m^2 in sequential cohorts and doses of PTX and CDDP were fixed at 110 and 75 mg/m^2, respectively.

A pre-medication schedule consisted of a 20 mg intravenous dexamethasone infusion 12 and 6 h before chemotherapy, 50 mg oral diphenhydramine and 50 mg intravenous ranitidine administration 30 min before chemotherapy. No primary granulocyte colony-stimulating factor (G-CSF) prophylaxis was allowed. G-CSF use was allowed only when grade 4 leukopenia (<1000/m^3) or grade 4 neutropenia (<500/m^3) lasting for 3 days or longer or grade 2 fever ($\geq$38°C) during grade 3 leukopenia (<2000/m^3) or grade 3 neutropenia (<1000/m^3) was observed.

Treatment Modification

Re-treatment was delayed until the following criteria were met. (a) WBC $\geq$2500/mm^3 and platelet count $\geq$100 000/mm^3; (b) total serum bilirubin $\leq$1.5 mg/dl, serum AST $\leq$2.5 times the upper limit of normal and serum creatinine $\leq$1.5 mg/dl; (c) more than 48 h passed after the final G-CSF use; and (d) absence of active infection. Patients were taken out of the study if the treatment interval exceeded 42 days.

For patients experiencing any of the following toxicities, the doses of all three drugs were reduced to 90% of the previous dose: (a) grade 4 leukopenia (<1000/m^3); (b) grade 2 fever ($\geq$38°C) lasting for 3 days and/or bacteremia during grade 3 leukopenia (<2000/m^3) or neutropenia (<1000/m^3); (c) grade 3 thrombocytopenia (<50 000/m^3); and (d) grade 3 or 4 non-hematological toxicities other than nausea and vomiting. Toxicities were graded according to the Japan Clinical Oncology Group (JCOG) toxicity criteria (9), based on Common Toxicity Criteria of the National Cancer Institute (NCI-CTC, 1982) to extend and supplement the criteria.

Chemotherapy was discontinued if (a) response was revealed to be no change (NC) after three cycles of chemotherapy, (b) progressive disease (PD) was observed, (c) unacceptable toxicities were observed or (d) recovery from toxicities was prolonged.

Determination of Maximum Tolerated Dose and Recommended Dose

The primary objectives of the study were to determine the maximum tolerated dose (MTD) and the recommended dose (RD) of DOX when combined with 110 mg/m^2 of PTX and 75 mg/m^2 of CDDP. Initially, six patients were sequentially enrolled into the lowest dose level. Dose-limiting toxicity

(DLT) was evaluated in the first course of chemotherapy to determine MTD and in all courses of chemotherapy to determine the RD. If none or one of the six patients experienced DLT, then the following six patients would be enrolled into the next dose level. If four or more of the six patients experienced DLT and the dose level was higher than level 1, MTD was determined as the previous dose level. If two or three of the six patients experienced DLT, then an additional six patients would be enrolled into the same dose level at other than level 4. If three or fewer of 12 patients experienced DLT, then the next six patients would be enrolled into the next dose level. If four or more of 12 patients experienced DLT, then MTD was determined as that dose level. These steps were repeated until MTD was determined. RD was determined taking into account the DLT observed in the following courses of chemotherapy.

DLT was initially defined as (a) grade 4 leukopenia ($<1000/m^3$) or grade 4 neutropenia ($<500/m^3$) lasting for 4 days or longer; (b) grade 2 fever ($\geq 38°C$) lasting for 3 days and/or bacteremia during grade 3 leukopenia ($<2000/m^3$) or neutropenia ($<1000/m^3$); (c) grade 4 thrombocytopenia ($<25\,000/m^3$); and (d) grade 3 or 4 non-hematological toxicities other than nausea and vomiting. The criteria were subsequently amended as described in the next subsection.

Amendment of Criteria for Dose-Limiting Toxicity

Among six patients enrolled into dose level 1, grade 4 neutropenia lasting for 4 days or longer [criterion (a)] was observed in four patients during the first course of chemotherapy and neutrophils were not counted in one patient with grade 2 leukopenia. Therefore, the study was discontinued and the toxicities were evaluated. Grade 4 neutropenia was observed for 6–7 days in three patients and observed for 11 days in one patient, although grade 4 leukopenia was not observed. However, all six patients recovered from the toxicity and could receive the subsequent course of chemotherapy without delay. No other DLT was observed in these six patients during the first and subsequent courses. Therefore, dose level 1 was evaluated to be safe and criterion (a) was considered to be too strict. Moreover, many phase I studies for ovarian cancer adopted a criterion of 'grade 4 neutropenia lasting for 8 days or longer' (10–14). Taken together, the following amendment of criteria and study design was permitted by the Data and Safety Monitoring Committee. (1) Criterion (a) was modified to 'grade 4 neutropenia lasting for 8 days or longer accompanied by grade 4 leukopenia for at least 1 day during the period'. According to this amendment, none of the above-mentioned four patients met the criterion. (2) A patient whose neutrophils were not counted was determined to be ineligible. (3) An additional four patients would be enrolled to dose level 1 to determine the safety of the dose level. If DLT was observed in none or one of nine patients, the subsequent patients would be enrolled at dose level 2. If DLT was observed in two of nine patients, an additional three patients would be enrolled at dose level 1. If DLT was observed in three or four of nine patients, the study would be discontinued.

Response Evaluation

A secondary objective of the study was to evaluate the efficacy of the TAP regimen. The World Health Organization (WHO) criteria (15) were employed in this study. Complete response (CR) was defined as the disappearance of all gross evidence of disease for at least 4 weeks. Partial response (PR) was defined as a $\geq 50\%$ reduction in the sum of the products of the two largest perpendicular dimensions of all two-dimensionally measurable lesions and no evidence of new lesions for at least 4 weeks. No change (NC) was defined as a $<25\%$ increase or a $<50\%$ reduction in the sum of the aforementioned products and no evidence of new lesions for at least 4 weeks. Progressive disease (PD) was defined as a $\geq 25\%$ increase in the sum of the above-mentioned products or the appearance of any new lesions. Not evaluable (NE) was defined when insufficient data for response evaluation are available.

Before enrolling the patients into the study, the original protocol was approved by the Institutional Review Board (IRB) in each participating institute. The new protocol including the above-mentioned amendment was also approved by IRB in all participating institutes before restarting the study.

RESULTS

Patients' Characteristics

Between December 1998 and December 2000, 28 patients with advanced ovarian cancer were enrolled in this study. One patient was excluded from the study because sufficient laboratory data were not available for analysis. The median age of the 27 eligible patients was 56 years (range, 24–71 years) and 27 patients received 3–6 courses of chemotherapy (mean, 5.4 courses). Additional patients' characteristics are summarized in Table 1.

Dose Escalation and Dose-Limiting Toxicity

Excluding one ineligible patient, whose neutrophils were not counted during the first course of chemotherapy, nine patients were enrolled into dose level 1. Among these nine patients, only one developed DLT, grade 4 diarrhea, so the dose escalation was allowed. The following six patients were enrolled into dose level 2. These six patients developed no DLT and further dose escalation was performed. The next six patients enrolled into dose level 3 did not develop DLT and the dose was escalated to level 4. Six subsequent patients were enrolled into dose level 4. Two patients developed DLT; one patient developed febrile neutropenia matching criterion (b) and grade 4 diarrhea and another patient developed prolonged grade 4 neutropenia matching criterion (a). The MTD defined in the protocol had not been reached even at dose level 4. Therefore,

Table 1. Patients' characteristics

Characteristic	No. of patients	%
Registered patients	28	–
Eligible patients	27	–
Stage		
III	24	88.9
IV	3	11.1
Histology		
Serous	23	85.2
Endometrioid	2	7.4
Clear cell	1	3.7
Undifferentiated	1	3.7
Residual disease		
0	4	14.8
0–1 cm	6	22.2
1–2 cm	4	14.8
>2 cm	13	48.1

it was decided to determine RD taking into account the toxicities of all cycles, the necessity of G-CSF support, the actual dose delivery and efficacy.

Hematological Toxicity

The hematological toxicity results are summarized in Table 2. The major toxicities observed were neutropenia and leukopenia. Grade 4 neutropenia was observed frequently even during the first course of chemotherapy [85% (23/27)] and almost all patients developed grade 4 neutropenia during all courses of chemotherapy [96% (26/27)]. The dose level was not correlated with the frequency of neutropenia (100% in level 1 and 83% in level 4 during the first course of chemotherapy). Grade 4 leukopenia was observed in 44% (12/27) of patients during the first course and in 52% (14/27) of patients during all courses of chemotherapy. The toxicity did not seem to increase from the second to sixth courses of chemotherapy. However, the frequency of grade 4 leukopenia was correlated with the dose level during the first course [22% (2/9) in level 1 to 83% (5/6) in level 4] and all courses of chemotherapy [22% (2/9) in level 1 to 83% (5/6) in level 4]. Among these grade 4 hematological toxicities observed during the first course of chemotherapy, toxicity developed by one patient in level 4 matched the dose-limiting toxicity criterion (a). As for other hematological toxicity, grade 3 anemia was rarely observed during the first course of chemotherapy [11% (3/27)]; however, nearly half of patients developed grade 3 anemia during all courses of chemotherapy [44% (12/27)]. Grade 4 thrombocytopenia was never observed during the first course of chemotherapy and only one patient developed grade 4 thrombocytopenia during all courses of chemotherapy [4% (1/27)].

Table 2. Hematological toxicity

Toxicity	No.(%) of grade 3/grade 4 toxicity			
	Level 1 (n = 9)	Level 2 (n = 6)	Level 3 (n = 6)	Level 4 (n = 6)
(A) During first course of chemotherapy				
Leukopenia	6 (67)/2 (22)	4 (67)/2 (33)	1 (17)/3 (50)	1 (17)/5 (83)
Neutropenia	0 (0)/9 (100)	1 (17)/4 (67)	0 (0)/5 (83)	1 (17)/5 (83)
Anemia	1 (11)/NA	0 (0)/NA	1 (17)/NA	1 (17)/NA
Thrombocytopenia	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
(B) During all courses of chemotherapy				
Leukopenia	6 (67)/2 (22)	3 (50)/3 (50)	1 (17)/4 (67)	1 (17)/5 (83)
Neutropenia	0 (0)/9 (100)	1 (17)/5 (83)	0 (0)/6 (100)	0 (0)/6 (100)
Anemia	2 (22)/NA	2 (33)/NA	6 (100)/NA	2 (33)/NA
Thrombocytopenia	0 (0)/0 (0)	1 (17)/0 (0)	2 (33)/0 (0)	1 (17)/1 (17)

Table 3. Non-hematological toxicity

Toxicity	No.(%) of grade 2/grade 3 toxicity			
	Level 1 (n = 9)	Level 2 (n = 6)	Level 3 (n = 6)	Level 4 (n = 6)
(A) During first course of chemotherapy				
Nausea and vomiting	2 (22)/1 (11)	2 (33)/1 (17)	3 (50)/0 (0)	2 (33)/1 (17)
Diarrhea	1 (11)/1 (11)	2 (33)/0 (0)	0 (0)/0 (0)	1 (17)/1 (17)
Alopecia	0 (0)/NA	0 (0)/NA	1 (17)/NA	2 (33)/NA
Neuropathy-sensory	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
Hypersensitivity	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
Renal toxicity	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
Febrile neutropenia	NA/1 (11)	NA/0 (0)	NA/2 (33)	NA/2 (33)
(B) During all courses of chemotherapy				
Nausea and vomiting	5 (55)/1 (11)	2 (33)/1 (17)	4 (67)/0 (0)	4 (67)/1 (17)
Diarrhea	1 (11)/1 (11)	2 (33)/0 (0)	0 (0)/0 (0)	0 (0)/2 (33)
Alopecia	8 (88)/NA	5 (83)/NA	5 (83)/NA	5 (83)/NA
Neuropathy-sensory	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	1 (17)/0 (0)
Hypersensitivity	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
Renal toxicity	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)	0 (0)/0 (0)
Febrile neutropenia	NA/2 (22)	NA/0 (0)	NA/2 (33)	NA/2 (33)

Non-hematological Toxicity

The results of non-hematological toxicity are listed in Table 3. Generally, non-hematological toxicity was mild or moderate. The observed grade 3 toxicities during the first course or all courses of chemotherapy were nausea and vomiting in 11% (3/27) or 11% (3/27), diarrhea in 7% (2/27) or 11% (3/27) and febrile neutropenia in 19% (5/27) or 22% (6/27), respectively. The frequency of above grade 3 toxicities did not increase during the second to sixth courses of chemotherapy and was not correlated with the dose levels. Among these grade 3 toxicities observed during the first course of chemotherapy,

Table 4. Clinical response

Clinical response	Level 1 ($n = 9$)	Level 2 ($n = 6$)	Level 3 ($n = 6$)	Level 4 ($n = 6$)	Total
Complete response	4	1	4	3	12
Partial response	1	2	0	1	4
No change	0	0	0	0	0
Progressive disease	0	0	2	1	3
Not evaluable	4	3	0	1	8
Response rate (%)	100 (5/5)	100 (3/3)	67 (4/6)	80 (4/5)	84 (16/19)

two cases of diarrhea, one in level 1 and one in level 4 and one febrile neutropenia in level 4 matched the dose-limiting toxicity criteria (d) and (b). Other than these toxicities, alopecia was the most frequently observed toxicity: 85% (23/27) of patients developed grade 2 alopecia during all courses of chemotherapy. Grade 2/3 hypersensitivity and any grade renal toxicity (rise of serum creatinine) were not observed during the study. It was noteworthy that grade 2/3 sensory neuropathy was not observed during the first course of chemotherapy and only one patient [4% (1/27)] developed grade 2 sensory neuropathy during all courses of chemotherapy.

Clinical Response

Eight patients had no measurable disease at entry. In the other 19 patients with two-dimensionally measurable disease, the response to chemotherapy was evaluated (Table 4). Twelve patients achieved complete response and four achieved partial response. The overall response rate was 84% (16/19) among patients with measurable disease. The remaining three patients had progressive disease. The response rate at dose levels 1–4 was 100, 100, 67 and 80%, respectively, suggesting no correlation between the dose level and response rate.

Recommended Dose

Table 5 summarizes the characteristics of chemotherapy at each level. In level 4, the majority of cycles [91% (30/33)] required G-CSF support and more than 30% of chemotherapy cycles required some modification in the dose or starting date of chemotherapy. However, chemotherapy could be continued until the planned cycle was completed or disease progression in most cases [83% (5/6)]. Moreover, 93.4% of the planned doses of agents could be administered at level 4. Considering all the factors, such as hematological and non-hematological toxicities, clinical responses and actual dose deliveries at dose level 4, RD for further study was decided as dose level 4 consisting of 110 mg/m^2 of PTX, 50 mg/m^2 of DOX and 75 mg/m^2 of CDDP.

DISCUSSION

In this study, we evaluated the safety and efficacy of a combination regimen of PTX, DOX and CDDP (TAP) as first-line chemotherapy for AOC. Because of the bone marrow toxicity of both CBDCA and DOX, CDDP seems to be safer than CBDCA to combine with DOX as a platinum analog. On the other hand, the combination of CDDP and PTX may produce severe and irreversible neurotoxicity (2,16,17). To avoid this adverse effect and to reduce cardiac toxicity, PTX was administered in a 24 h continuous infusion (18). The PTX dose was set at 110 mg/m^2 as the minimum dose at which sufficient response could be expected, because there is no dose–response relationship in a range of 110 mg/m^2 or more (19). The dose of CDDP was decided as the standard dose of 75 mg/m^2 (20). The DOX dose was increased from 20 to 50 mg/m^2 and was expected to improve efficacy over the standard combination of PTX and platinum. To avoid excessive toxicity, PTX was administered following DOX (21,22) and CDDP was administered following PTX (23). The regimen therefore consisted of 20–50 mg/m^2 increasing doses of DOX followed by 24 h infusion of 110 mg/m^2 of PTX followed by 75 mg/m^2 of CDDP.

Concerning the safety of the regimen, the three-drug combination regimen seemed to be sufficiently safe to use as first-line chemotherapy for patients with ovarian cancer. The major toxicities observed in our study were neutropenia and leukopenia. Grade 4 neutropenia and leukopenia were observed in 85% (23/27) and 44% (12/27) in the first course of chemotherapy. However, these toxicities rarely lasted long enough to be counted as DLT and were not cumulative in the 2nd to 6th courses of chemotherapy. Thus, these hematological toxicities seemed manageable. Moreover, non-hematological toxicities were generally mild or moderate. The grade 3 toxicities observed were nausea and vomiting in 11% (3/27), diarrhea in 11% (3/27) and febrile neutropenia in 22% (6/27), during all courses

Table 5. Summary of chemotherapies

	Level 1	Level 2	Level 3	Level 4
No. of cycles administered	48	36	32	33
Percentage of cycles required				
G-CSF use	60 (29/48)	72 (26/36)	66 (21/32)	91 (30/33)
Dose reduction	13 (5/39)	7 (2/30)	15 (4/26)	33 (9/27)
Treatment delay	21 (8/39)	20 (6/30)	8 (2/26)	30 (8/27)
Percentage of patients who completed chemotherapy*	67 (6/9)	100 (6/6)	100 (6/6)	83 (5/6)
Average drug administration				
PTX(mg/m^2)	106	108	107	103
DOX(mg/m^2)	19	29	39	47
CDDP(mg/m^2)	72	74	73	70
Percentage of actual/planned doses	96.4	98.2	97.2	93.4

*All six cycles of chemotherapy were completed or chemotherapy was discontinued because of disease progression.

of chemotherapy. Grade 3 sensory neuropathy was not observed during all courses of chemotherapy. To our knowledge, seven phase I or I/II studies (10,24–29), evaluating the value of anthracyclines in a taxane and platinum-based regimen for previously untreated AOC, have been published. The major toxicities observed throughout the studies were hematological toxicities, such as neutropenia, leukopenia and thrombocytopenia. In particular, neutropenia was reported in 100% in some studies (25,27,28). However, the toxicity was readily managed using G-CSF and was rarely complicated with serious infection or sepsis. Non-hematological toxicities, excluding nausea, vomiting and alopecia, were generally mild and manageable. No severe cardiac toxicity or neuropathy was observed throughout the previous studies.

As for the efficacy of the triplet combination in our study, a response rate (RR) of 84% (16/19), including 63% (12/19) complete response (CR), was observed. Even in level 1, 100% RR was achieved and there was no correlation between the dose level and response rate. In the previous studies, that using docetaxel (DOC) as the taxane (28) showed a relatively lower response rate of 36%, but studies using PTX as the taxane showed a higher response rate of 83–100%. In studies using PTX, there were no apparent differences in the response rate between studies using CDDP (86–100%) (25,26) and those using CBDCA (83–100%) (10,24,29) as platinum compound and between studies using DOX (100%) (24,25) and those using EpiDOX (83–86%) (10,26,29) as anthracycline.

In summary, the combination regimen of DOX with PTX and CDDP is highly active and hematological toxicities are readily manageable and non-hematological toxicities, including cardiac toxicity and sensory neuropathy, were mild or moderate. From our study and previous studies, we conclude that the addition of anthracyclines to PTX plus a platinum-based regimen may provide an effective and safe regimen for patients with untreated ovarian cancer. However, the hematological toxicities seem to be relatively severe compared with those reported with a PTX/CBDCA combination (3,30,31). At present, AGO–GINECO (Arbeitsgemeinschaft Gynäkologische Onkologie–Groupe d'Investigateurs Nationaux pour l'Etude des Cancers Ovariens) (32) and NSGO–EORTC–NCIC CTG (Nordic Society of Gynecological Oncology–European Organization for Research and Treatment of Cancer–National Cancer Institute of Canada Clinical Trials Group) (33) are conducting phase III studies comparing epirubicin/paclitaxel/carboplatin vs. paclitaxel/carboplatin. To assess the usefulness of anthracyclines, the results of these studies are awaited.

Acknowledgments

We thank Dr Nagahiro Saijo for his support and suggestions as a member of Data and Safety Monitoring Committee of this study.

References

1. McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *N Engl J Med* 1996;334:1–6.
2. Piccart MJ, Bertelsen K, James K, Cassidy J, Mangioni C, Simonsen E, et al. Randomized intergroup trial of cisplatin–paclitaxel versus cisplatin–cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. *J Natl Cancer Inst* 2000;92:699–708.
3. Ozols RF, Bundy BN, Greer BE, Fowler JM, Clarke-Pearson D, Burger RA, et al. Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. *J Clin Oncol* 2003;21:3194–200.
4. Nguyen HN, Averette HE, Hoskins W, Sevin BU, Penalver M, Steren A. National survey of ovarian carcinoma. VI. Critical assessment of current International Federation of Gynecology and Obstetrics staging system. *Cancer* 1993;72:3007–11.
5. Omura GA, Bundy BN, Berek JS, Curry S, Delgado G, Mortel R. Randomized trial of cyclophosphamide plus cisplatin with or without doxorubicin in ovarian carcinoma: a Gynecologic Oncology Group study. *J Clin Oncol* 1989;7:457–65.
6. Cyclophosphamide plus cisplatin versus cyclophosphamide, doxorubicin and cisplatin chemotherapy of ovarian carcinoma: a meta-analysis. The Ovarian Cancer Meta-Analysis Project. *J Clin Oncol* 1991;9:1668–74.
7. Fanning J, Bennett TZ, Hilgers RD. Meta-analysis of cisplatin, doxorubicin and cyclophosphamide versus cisplatin and cyclophosphamide chemotherapy of ovarian carcinoma. *Obstet Gynecol* 1992;80:954–60.
8. A'Hern RP, Gore ME. Impact of doxorubicin on survival in advanced ovarian cancer. *J Clin Oncol* 1995;13:726–32.
9. Tobinai K, Kohno A, Shimada Y, Watanabe T, Tamura T, Takeyama K, et al. Toxicity grading criteria of the Japan Clinical Oncology Group. The Clinical Trial Review Committee of the Japan Clinical Oncology Group. *Jpn J Clin Oncol* 1993;23:250–7.
10. du Bois A, Luck HJ, Bauknecht T, Meier W, Richter B, Kuhn W, et al. First-line chemotherapy with epirubicin, paclitaxel and carboplatin for advanced ovarian cancer: a phase I/II study of the Arbeitsgemeinschaft Gynäkologische Onkologie Ovarian Cancer Study Group. *J Clin Oncol* 1999;17:46–51.
11. Siddiqui N, Boddy AV, Thomas HD, Bailey NP, Robson L, Lind MJ, et al. A clinical and pharmacokinetic study of the combination of carboplatin and paclitaxel for epithelial ovarian cancer. *Br J Cancer* 1997;75:287–94.
12. Bolis G, Scarfone G, Villa A, Acerboni S, Siliprandi V, Guarnerio P. A phase I trial with fixed-dose carboplatin and escalating doses of paclitaxel in advanced ovarian cancer. *Semin Oncol* 1997;24, No 1, Suppl 2:23–5.
13. Huizing MT, van Warmerdam LJ, Rosing H, Schaefers MC, Lai A, Helmerhorst TJ, et al. Phase I and pharmacologic study of the combination paclitaxel and carboplatin as first-line chemotherapy in stage III and IV ovarian cancer. *J Clin Oncol* 1997;15:1953–64.
14. ten Bokkel Huinink WW, Veenhof C, Huizing M, Rodenhuis S, Helmerhorst T, Dubbelman R, et al. Carboplatin and paclitaxel in patients with advanced ovarian cancer: a dose-finding study. *Semin Oncol* 1997;24, No 1, Suppl 2:31–3.
15. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. *Cancer* 1981;47:207–14.
16. Rowinsky EK, Chaudhry V, Forastiere AA, Sartorius SE, Ettinger DS, Grochow LB, et al. Phase I and pharmacologic study of paclitaxel and cisplatin with granulocyte colony-stimulating factor: neuromuscular toxicity is dose-limiting. *J Clin Oncol* 1993;11:2010–20.
17. Chaudhry V, Rowinsky EK, Sartorius SE, Donehower RC, Cornblath DR. Peripheral neuropathy from taxol and cisplatin combination chemotherapy: clinical and electrophysiological studies. *Ann Neurol* 1994;35:304–11.
18. Smith RE, Brown AM, Mamounas EP, Anderson SJ, Lembersky BC, Atkins JH, et al. Randomized trial of 3-hour versus 24-hour infusion of high-dose paclitaxel in patients with metastatic or locally advanced breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-26. *J Clin Oncol* 1999;17:3403–11.
19. Rowinsky EK, Mackey MK, Goodman SN. Meta analysis of paclitaxel dose–response and dose–intensity in recurrent or refractory ovarian cancer. *Proc Am Soc Clin Oncol* 1996;15:Meeting Abstract.

20. Kaye SB, Paul J, Cassidy J, Lewis CR, Duncan ID, Gordon HK, et al. Mature results of a randomized trial of two doses of cisplatin for the treatment of ovarian cancer. Scottish Gynecology Cancer Trials Group. *J Clin Oncol* 1996;14:2113–9.
21. Sledge GW Jr, Robert N, Sparano JA, Cobeligh M, Goldstein LJ, Neuberg D, et al. Paclitaxel (taxol)/doxorubicin combinations in advanced breast cancer: the Eastern Cooperative Oncology Group experience. *Semin Oncol* 1994;21:15–8.
22. Sledge GW Jr, Robert N, Sparano JA, Cogleigh M, Goldstein LJ, Neuberg D, et al. Eastern Cooperative Oncology Group studies of paclitaxel and doxorubicin in advanced breast cancer. *Semin Oncol* 1995;22:105–8.
23. Rowinsky EK, Gilbert MR, McGuire WP, Noe DA, Grochow LB, Forastiere AA, et al. Sequences of taxol and cisplatin: a phase I and pharmacologic study. *J Clin Oncol* 1991;9:1692–703.
24. Hill M, Macfarlane V, Moore J, Gore ME. Taxane/platinum/anthracycline combination therapy in advanced epithelial ovarian cancer. *Semin Oncol* 1997;24, No 1, Suppl 2:34–7.
25. Naumann RW, Alvarez RD, Omura GA, Segars E, Kilgore LC, Partridge EE. A phase I study of paclitaxel, doxorubicin and cisplatin in patients with previously untreated epithelial ovarian cancer. *Gynecol Oncol* 1998;71:450–3.
26. Papadimitriou CA, Moulopoulos LA, Vlahos G, Voulgaris Z, Kiosses E, Georgoulias N, et al. Paclitaxel, cisplatin and epirubicin first-line chemotherapy in stage III and IV ovarian carcinoma: long-term results of a phase II study. *Cancer* 2000;89:1547–54.
27. Gregory RK, Hill ME, Moore J, A'Hern RP, Johnston SR, Blake P, et al. Combining platinum, paclitaxel and anthracycline in patients with advanced gynaecological malignancy. *Eur J Cancer* 2000;36:503–7.
28. O'Neill VJ, Kaye SB, Reed NS, Paul J, Davis JA, Vasey PA. A dose-finding study of carboplatin–epirubicin–docetaxel in advanced epithelial ovarian cancer. *Br J Cancer* 2002;86:1385–90.
29. Romanini A, Tanganelli L, Carnino F, Fanucchi A, Lionetto R, Pastorino S, et al. First-line chemotherapy with epidoxorubicin, paclitaxel and carboplatin for the treatment of advanced epithelial ovarian cancer patients. *Gynecol Oncol* 2003;89:354–9.
30. Neijt JP, Engelholm SA, Witteveen PO, Tuxen MK, Sorensen PG, Hansen M, et al. Paclitaxel (175 mg/m^2 over 3 h) with cisplatin or carboplatin in previously untreated ovarian cancer: an interim analysis. *Semin Oncol* 1997;24, No 5, Suppl 15:36–9.
31. du Bois A, Luck HJ, Meier W, Mobus V, Costa S, Richter B, et al. Carboplatin/paclitaxel versus cisplatin/paclitaxel as first-line chemotherapy in advanced ovarian cancer: an interim analysis of a randomized phase III trial of the Arbeitsgemeinschaft Gynakologische Onkologie Ovarian Cancer Study Group. *Semin Oncol* 1997;24, No 5, Suppl 15: 44–52.
32. du Bois A, Weber B, Pfisterer J, Goupil A, Wagner U, Barats J, et al. Epirubicin/paclitaxel/carboplatin (TEC) vs. paclitaxel/carboplatin (TC) in first-line treatment of ovarian cancer FIGO stages IIb/IV. Interim results of an AGO/GINECO Intergroup Phase III trial. *Proc Am Soc Clin Oncol* 2001;20:Abstract 805.
33. Kristensen G, Vergote I, Stuart G, Izqiuerdo Delso M, Mirza MR, Aavall-Lundquist E, et al. First line treatment of ovarian cancer FIGO stages IIb–IV with paclitaxel/epirubicin/carboplatin (TEC) vs. paclitaxel/carboplatin (TC). Interim results of an NSGO–EORTC–NCIC CTG Gynecological Cancer Intergroup phase III trial. *Proc Am Soc Clin Oncol* 2002;21:Abstract 805.

Pulmonary Metastasectomy for Uterine Cervical Cancer: A Multivariate Analysis

Kaichiro Yamamoto, MD, PhD, Hiroyuki Yoshikawa, MD, PhD, Kenji Shiromizu, MD, PhD, Toshiaki Saito, MD, PhD, Kazuo Kuzuya, MD, PhD, Ryuichiro Tsunematsu, MD, PhD, and Toshiharu Kamura, MD, PhD

Department of Obstetrics and Gynecology, Sakai Hospital, Kinki University School of Medicine, Osaka, and Department of Obstetrics and Gynecology, University of Tsukuba, Ibaraki, Saitama Cancer Center, Saitama, National Kyushu Cancer Center, Fukuoka, Aichi Cancer Center Hospital, Aichi, National Cancer Center, Tokyo, Kurume University Hospital, Fukuoka, Japan

Background. This study evaluated the results of resection of pulmonary metastases from cervical cancer.

Methods. A total of 7,748 patients with primary stage Ib or II cervical cancer underwent curative initial treatment consisting of radical hysterectomy or radiotherapy in 22 hospitals. Of the 7,748 patients, 29 (0.37%) patients had pulmonary metastases, which were detected after a disease-free period after initial treatment (radical hysterectomy or radiotherapy) and were resected with the intention to cure by June 30, 1998.

Results. The 5-year disease-free survival rate after pulmonary metastasectomy for all patients was 32.9%. Patients with one or two pulmonary metastases had a 5-year disease-free survival rate of 42.2% compared with 0% for patients with three or four metastases ($p = 0.0003$). Patients with squamous cell cancers had a 5-year disease-free survival rate of 47.4% compared with 0% for patients with adenosquamous cell cancers or adenocarcinoma ($p = 0.0141$). On multivariate analysis, the significant prognostic variables for disease-free survival were two or fewer metastases ($p = 0.0232$) and squamous cell cancer ($p = 0.0168$).

Conclusions. Cervical cancer patients with pulmonary metastases after initial treatment (radical hysterectomy or radiotherapy) could expect to achieve long-term disease-free survival by pulmonary metastasectomy when there are two or fewer metastases diagnosed as squamous cell cancer.

(Ann Thorac Surg 2004;77:1179–82)

Although the prevalence of invasive cervical cancer among Japanese women has been reported to be gradually decreasing, new cases are still being diagnosed in approximately 7,000 women annually, and 60% of these women have progressive disease [1]. Overall, recurrent disease will develop in 10% to 20% of patients after primary radical surgery. The prognosis of recurrent cervical cancer is dismal. Thus, increasing our understanding of recurrence and treatment remains important.

Material and Methods

The stage of the disease was classified according to the criteria of the International Federation of Gynecology and Obstetrics.

A total of 7,748 patients with primary International Federation of Gynecology and Obstetrics stage Ib or II cervical cancer underwent initial treatment consisting of radical hysterectomy or radiotherapy in 22 hospitals between January 1, 1983, and December 31, 1997. All patients received potentially curative treatment consisting of radical hysterectomy or radiotherapy. Of the 7,748 patients, 29 (0.37%) patients had pulmonary metastases, which were confirmed by chest radiography or computed tomography after a disease-free period after initial treatment (radical hysterectomy or radiotherapy) and were resected with the intention to cure by June 30, 1998. These patients were examined to analyze the prognostic factors for survival after pulmonary metastasectomy. Their metastatic disease was limited to the lungs.

All thoracotomy specimens were processed according to standard procedures for hematoxylin and eosin–stained histologic preparation and were histologically confirmed to contain cancer consistent with cervical cancer origin. Pulmonary metastases were completely resected in all patients. There were no operative or hospital deaths. Of the 29 patients, 15 patients received cisplatin-based chemotherapy as adjuvant treatment after pulmonary metastasectomy whereas the remainder had no other therapy. The median follow-up period of all patients was 40.1 months, and the median follow-up period of living patients was 51 months (range, 1.4 to 122.3 months).

Clinical data and follow-up information were obtained from the medical records and were further complemented using telephone contacts with patients, family members, and physicians. Disease-free survival (DFS) was defined as the elapsed time from thoracotomy to disease recurrence or death. Death from disease or any recurrent disease, local or distant, was considered an event in DFS calculation. Actuarial survival curves were calculated according to the Kaplan-Meier method [2], and comparisons were made

Accepted for publication June 13, 2003.

Address reprint requests to Dr Yamamoto, Department of Obstetrics and Gynecology, Sakai Hospital, Kinki University School of Medicine, 2-7-1, Harayamadai, Sakai, Osaka 590-0132, Japan; e-mail: yamamoto@sakai.med.kindai.ac.jp.

Published by Elsevier Inc

0003-4975/04/$30.00
doi:10.1016/j.authoracsur.2003.06.023

with the log rank test [3]. For multivariate analysis, we used the Cox proportional hazards model. A p value of less than 0.05 was considered significant.

Results

Patients' Characteristics of Primary Cervical Cancer

The median age was 57 years, with a range of 31 to 76 years. There were 12 patients with stage Ib, 5 with stage IIa, and 12 with stage IIb disease. The histologic classification was made in accordance with the World Health Organization classification. Twenty patients had squamous cell cancers, 3 had adenosquamous cell cancers, and 6 had adenocarcinoma. All patients received potentially curative treatment of radical hysterectomy or radiotherapy. As initial treatment, 25 patients underwent radical hysterectomy and the remainder received radiotherapy. Of the 25 patients who underwent surgery, 8 patients had pelvic lymph node involvement. Four patients who had radiotherapy were not assessable.

Patients' Characteristics of Pulmonary Metastatic Lesions

The median age was 60 years, with a range of 32 to 77 years. The median disease-free interval (DFI, interval between initial treatment and onset of pulmonary metastasis) was 42 months (range, 11 to 97 months). A solitary metastatic lesion was found in 17 patients. Multiple metastases were found in the other 12 patients, two metastatic lesions in 6 patients, three metastatic lesions in 3 patients, and four metastatic lesions in 3 patients. In patients with a solitary metastasis, left lung metastasis was found in 5 patients, and right lung metastasis in 12 patients. In patients with multiple metastases, left lung metastases were found in 2 patients, right lung metastases in 3 patients, and metastases to both lungs in 7 patients. Wedge resection was performed in 8 patients (3 with a solitary lesion, 1 with two lesions, 2 with three lesions, and 2 with four lesions), segmentectomy in 2 patients (1 with a solitary lesion and 1 with four lesions), and lobectomy in 19 patients (13 with a solitary lesion, 5 with two lesions, 1 with three lesions). Median sternotomy was performed in 6 patients (3 with two lesions, 1 with three lesions, and 2 with four lesions), and lateral thoracotomy in 23 patients (17 with a solitary lesion, 3 with two lesions, 2 with three lesions, and 1 with four lesions). Sixteen patients underwent either hilar or mediastinal lymph node dissection. Of 11 patients who showed no evidence of any lymph node metastasis, 5 (45.5%) patients had postthoracotomy recurrence. However, of 5 patients who had hilar or mediastinal lymph node metastasis, 4 (80.0%) patients had postthoracotomy recurrence. Pulmonary metastatic tumor size was obtained in only 18 patients. Of 11 patients with pulmonary metastatic lesions less than 3 cm, 5 (45.5%) patients had postthoracotomy recurrence, and of 7 patients with pulmonary metastatic lesions more than 3 cm, 4 (57.1%) patients had postthoracotomy recurrence.

Table 1. Prognostic Factors: Estimation by Univariate Analysis

Factor	Number	5-year DFS Rate (%)	p Value
Stage			
Ib, IIa	17	34.1	0.9379
IIb	12	30.0	
Histology			
Squamous	20	47.4	0.0141
Adenosquamous + adeno	9	0	
Lymph node metastasis			
Positive	8	37.5	0.9414
Negative	17	25.3	
Age			
<60	13	15.4	0.0071
≧60	16	50.3	
DFI			
<36 months	10	30.0	0.3728
≧36 months	19	33.9	
Number of metastases			
1, 2	23	42.2	0.0003
3, 4	6	0	
Postthoracotomy chemotherapy			
Done	15	28.6	0.8146
None	14	38.1	

adeno = adenocarcinoma; DFI = disease-free interval; DFS = disease-free survival.

Univariate Analysis

The 5-year DFS rate after pulmonary metastasectomy for all patients was 32.9%. Table 1 summarizes the 5-year DFS rate and the results of the univariate analysis of the clinical and pathologic factors using the log rank test. Significant prognostic factors affecting DFS were histology (p = 0.0141), age (p = 0.0071), and number of metastases (p = 0.0003).

Multivariate Analysis

We performed multivariate analysis to identify independent factors affecting DFS. Number of metastases, age, histology, and DFI were included in the model. The results showed that the number of metastases (p = 0.0232) and the histology (p = 0.0168) were the only independent factors affecting DFS (Table 2). None of the other characteristics were significant on multivariate analysis. The actuarial 5-year DFS rates were 42.2% and 0% for patients with two or fewer metastases and those with three or four, respectively (Fig 1). Regarding histology, the actuarial 5-year DFS rates were 47.4% and 0% for patients with squamous cell cancers and adenosquamous cell cancers or adenocarcinoma, respectively (Fig 2).

Comment

Historically, patients who developed distant metastases from cervical cancer had a poor prognosis and were not considered for resection. Systemic treatment with chemo-

Table 2. Univariate and Multivariate Analysis

Factor	Univariate *p* Value	Multivariate		
		Hazard Rate	95% CI	*p* Value
Number of metastasis, 1, 2; 3, 4	0.0003	4.102	1.213–13.869	0.0232
Age, <60 y; ≧60 y	0.0071	0.382	0.126–1.163	0.0903
Histology, squamous; adsq + adeno	0.0141	3.775	1.271–11.212	0.0168
DFI, <36 months; ≧36 months	0.3728	0.662	0.232–1.891	0.4416

adeno = adenocarcinoma; adsq = adenosquamous cell cancer; CI = confidence interval; DFI = disease-free interval.

therapy is the mainstay of treatment for metastatic pulmonary tumors. Various chemotherapy regimens have been used to date. Imachi and associates [4] showed a 45% response rate in patients treated with two or more courses of chemotherapy; however, the mean interval from diagnosis of pulmonary metastasis to death was 7 months (median, 3 months; range, 1 to 59 months). The chemotherapy responses increased with the more frequent inclusion of platinum, but none of these regimens has proven to be useful in significantly prolonging survival [4, 5].

The 5-year DFS rate after pulmonary metastasectomy for the cervical cancer patients in our series was 32.9%, supporting the role of pulmonary resection in selected patients with pulmonary metastases from cervical cancer. The modified indications for pulmonary metastasectomy, ie, (1) the ability to tolerate the procedure, (2) sufficient pulmonary reserve to compensate for the loss of lung capacity, (3) the site of primary must be controlled or controllable, (4) no evidence of extrapulmonary disease, and (5) no better therapy available, are almost universally accepted [6–9]. We also have conformed to these criteria. The reported incidence of pulmonary metastasis from cervical cancer ranges from 2.1% to 9.1% [10–13]. Our 0.37% rate of lung involvement is lower than rates reported previously. This difference may be because we selected patients with stage Ib or II cervical cancer in whom pulmonary metastasis was detected after the disease-free period after initial treatment and surgery was performed in accordance with the indications for surgery described above. Our 65.5% incidence of lobectomy is higher than most pulmonary metastasectomy series. This was for anatomic reasons, because there were many patients whose pulmonary metastatic lesions were near the hilum of the lung.

Five-year survival after pulmonary metastasectomy for cervical cancer varies greatly, ranging from 0% to 60% in some reports [6, 8, 14–17], because the indications and surgical methods differed. Some authors also have reported various factors affecting the survival after thoracotomy.

In this study, we investigated the stage, histologic type, presence or absence of pelvic lymph node metastasis, age, interval between initial treatment and pulmonary metastasis (DFI), number of metastatic pulmonary foci, and presence or absence of chemotherapy after resection as prognostic factors after resection of metastatic pulmonary foci. Univariate analysis showed that significant prognostic factors included the histologic type, age, and number of metastatic foci. On multivariate analysis, significant prognostic factors included the number of metastatic foci and histologic type.

Concerning histology, squamous cell carcinoma showed a better prognosis than adenosquamous cell carcinoma and adenocarcinoma on both univariate and multivariate analysis in our series. Imachi and colleagues [4, 18] reported that in patients with adenocarcinoma, the incidence of pulmonary metastasis and positive peritoneal cytology were higher than those in patients with squamous cell carcinoma, and that these findings were related to a poor prognosis.

With respect to pelvic lymph node metastasis, in our results, the presence or absence of pelvic lymph node

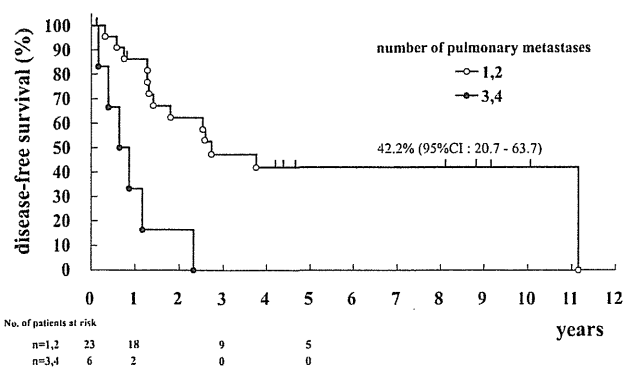

Fig 1. Disease-free survival, patients with one or two pulmonary metastases compared with those with three or four. (CI = confidence interval.)

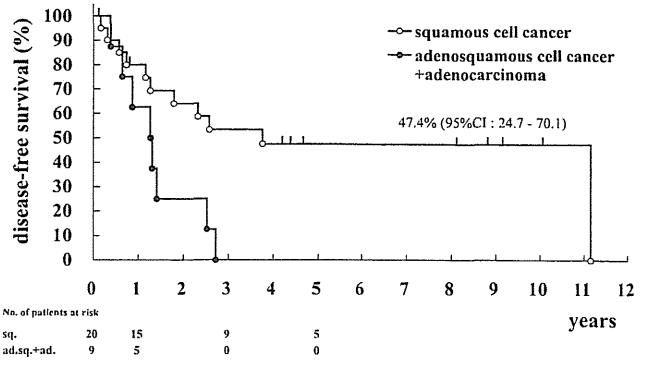

Fig 2. Disease-free survival, patients with squamous cell cancer compared with those with adenosquamous cell cancer or adenocarcinoma. (ad.sq.+ad. = adenosquamous cell cancer + adenocarcinoma; CI = confidence interval; Sq. = squamous cell cancer.)