Circulating Endothelial Cells in Non-small Cell Lung Cancer Patients Treated with Carboplatin and Paclitaxel

Makoto Kawaishi, MD, Yutaka Fujiwara, MD,† Tomoya Fukui, MD,* Terufumi Kato, MD,* Kazuhiko Yamada, MD,† Yuichiro Ohe, MD, PhD,† Hideo Kunitoh, MD, PhD,† Ikuo Sekine, MD, PhD,† Noboru Yamamoto, MD, PhD,† Hiroshi Nokihara, MD, PhD,† Takeshi Watabe, PhD,‡ Yuji Shimoda, PhD,‡ Tokuzo Arao, MD, PhD,§ Kazuto Nishio, MD, PhD,§ Tomohide Tamura, MD† and Fumiaki Koizumi, MD, PhD**

Introduction: Circulating endothelial cells (CECs) increase in cancer patients and play an important role in tumor neovascularization.
Methods: This study was designed to investigate the role of CEC as a marker for predicting the effectiveness of a carboplatin plus paclitaxel based first line chemotherapy in advanced non-small cell lung cancer (NSCLC).
Results: The CEC count in 4 ml of peripheral blood before starting chemotherapy (baseline value) was significantly higher in NSCLC patients, ranging from 32 to 4501/4 ml (n = 31, mean ± SD = 595 ± 832), than in healthy volunteers (n = 53, 46.2 ± 86.3). We did not detect a significant correlation between the CEC count and estimated tumor volume. CECs were significantly decreased by chemotherapy as compared with pretreatment values (175.6 ± 24 and 173.0 ± 24, day +8, +22, respectively). We investigated the correlation between baseline CEC and the clinical effectiveness of chemotherapy. CEC values are significantly higher in patients with clinical benefit (partial response and stable disease, 516 ± 458, 870.8 ± 1215, respectively) than in progressive disease patients (211 ± 150). Furthermore, a statistically significant decrease in CECs, on day 22, was observed only in patients with partial response. Patients who had a baseline CEC count greater than 400/4 ml showed a longer progression-free survival (>400, 271 days [range: 181–361] versus <400, 34 [range: 81–186], p = 0.019).
Conclusion: CEC is suggested to be a promising predictive marker of the clinical efficacy of the CBDCA plus paclitaxel regimen in patients with NSCLC.

Key Words: Circulating endothelial cell, NSCLC, Chemotherapy.

(*J Thorac Oncol.* 2009;4: 208–213)

*Shien-Lab; †Medical Oncology, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan; ‡Center for Molecular Biology and Cytogenetics, SRL Inc., Shinmachi, Hino-shi, Tokyo; and §Department of Genome Biology, Kinki University School of Medicine, Osaka-Sayama-shi, Osaka, Japan.
Disclosure: The authors declare no conflicts of interest.
Address for correspondence: Fumiaki Koizumi, MD, PhD, Shien-Lab, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan. E-mail: fkoizumi@gan2.res.ncc.go.jp

ISSN: 1556-0864/09/0402-0208

Angiogenesis plays a critical role in the growth and metastasis of solid tumors.[1] The clinical importance of angiogenesis in human tumors has been demonstrated by several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis, i.e., survival, in patients with various types of cancers including non-small cell lung cancer (NSCLC).[2–6] Furthermore, Natsume et al.[7] reported the antitumor activities of anticancer agents to be less active against vascular endothelial growth factor-secreting cells (SBC-3/VEGF), in vivo as compared with its mock transfectant (SBC-3/Neo). In recent years, antiangiogenic agents have also been demonstrated to be active against a variety of malignancies, including lung, colorectal, and renal cancer.[8–10] Thus, angiogenesis is a promising target for cancer treatment and is related to the prognosis and efficacy of these drugs, though the tumor vessel biomarkers which predict the effectiveness of antiangiogenic agents and other anticancer agents are not always useful and have not become well-established.

Circulating endothelial cells (CECs) have been recognized as a useful biomarker for vascular damage. CECs are increased in cardiovascular disease, vasculitis, infectious disease, and various cancers.[11–14] Recently, CECs were found to be more numerous and viable in cancer patients than in healthy subjects.[14,15] Furthermore, elevated CECs in cancer patients were found to be nearly normalized when the tumor was removed surgically or with chemotherapy.[15] Therefore, most CECs are considered to be disseminated tissue endothelial cells in the tumors and the CEC number may reflect the extent of tumor angiogenesis. Indeed, the CEC level has been demonstrated to correlate with the plasma level of VEGF, one of the pivotal factors promoting tumor angiogenesis.[15] Mancuso et al. reported that CEC kinetics and viability are promising predictors of the response to chemotherapy with antiangiogenic activity in patients with advanced breast cancer.[16] Thus, CEC is likely to be a useful marker for predicting the effectiveness of chemotherapy as a noninvasive angiogenesis marker.

NSCLC is the leading cause of cancer-related death worldwide. NSCLC accounts for approximately 50% of patients presenting with unresectable advanced stage,[17] and platinum-based chemotherapy offers only a small improve-

ment in survival with advanced NSCLC.[18,19] Over the past decade, several new agents against NSCLC have become available, including the taxanes, gemcitabine, vinorelbine, and irinotecan. The combination of platinum and these new agents has resulted in a high response rate and prolonged survival compared with older chemotherapy regimens (e.g., vindesine, mitomycin, ifosfamide, with cisplatin). Therefore, these regimens are considered standard chemotherapy for advanced NSCLC.[20–26] Although new agents have different mechanisms of action, these combination regimens have not been administered based on the biologic characteristics of each tumor.

Paclitaxel inhibits several endothelial cell functions in vitro such as proliferation, migration, morphogenesis, and metalloprotease production.[27–29] These activities result in antiangiogenic activity in in vivo xenograft models.[27,30] Interestingly, human endothelial cells are more sensitive to paclitaxel than other cellular types.[29] We hypothesized that the CEC value is associated with tumor neovascularization, which is one of the targets of paclitaxel. In the present study, we investigated whether the CEC count at baseline is associated with the effectiveness of the CDDP plus paclitaxel regimen in patients with advanced-stage NSCLC.

MATERIALS AND METHODS

Patients

Patients with histologically or cytologically documented advanced NSCLC were eligible for this study. Each patient was required to meet the following criteria: (1) no prior treatment including chemotherapy, surgery, irradiation, or any fluid drainage; (2) no prior general anesthesia for diagnostic procedures including mediastinoscopy or thoracoscopy; (3) no concomitant diseases including ischemic heart diseases, systemic vasculitis, pulmonary hypertension, or serious complications including infectious disease or diabetes; (4) written informed consent. The trial document was approved by the institutional review board. The clinical characteristics of the patients are shown in Table 1.

TABLE 1. Baseline Characteristics of the Patients

Characteristic	$N = 31$ No. (%)
Gender	
Male	17 (55)
Female	14 (45)
Median age (yr)	60
Range	43–71
ECOG performance status	
0	18 (58)
1	13 (42)
Stage	
IIIA	2 (6)
IIIB	7 (23)
IV	22 (71)
Histology	
Adenocarcinoma	23 (74)
Squamous cell carcinoma	4 (13)
Others	4 (13)

Treatment Schedule and Response Evaluation

All patients were treated according to the following chemotherapeutic regimen: paclitaxel at 200 mg/m^2 over a 3-hour period followed by carboplatin at a dose with an area under the curve of 6 on day 1, repeated every 3 weeks. The treatment was repeated for three or more cycles unless the patients met the criteria for progressive disease (PD) or experienced unacceptable toxicity.

The major axis (a) and minor axis (b) of the tumor mass in each patient were measured with computed tomography. Estimated tumor volume (ETV) was calculated using the following formula; ETV = $4/3 \times \pi$ (a/2 × b/2) × (a/2 + b/2)/2. Computed tomography examinations were performed before treatment and with every one or two cycles of chemotherapy. Response was evaluated according to the RECIST, and tumor markers were excluded from the criteria.[31]

Assay for CEC

Blood samples from NSCLC patients and healthy volunteers were drawn into a 10-ml Cellsave Preservative Tube (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. The CEC protocol used was approved by the Institutional Review Board and written informed consent was obtained from each subject. Samples from NSCLC were obtained before (baseline) and 8 and 22 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 hours after collection. All evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Immunicon Corp) which consists of CellTracks AutoPrep system and the CellSpotter Analyzer system was used for endothelial cell enumeration.[32,33] In this system, CD146+/DAPI+/CD105-PE+/CD45APC- cells are defined as CECs. Briefly, cells which express CD146 were immunomagnetically captured using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4V,6-diamidino-2-phenylindole (DAPI), CD105 antibodies conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 conjugated to allophycocyanin (CD45-APC). In this system, the CD146-enriched, fluorescently labeled cells were identified as CECs when the cells exhibited the DAPI+/CD105+/CD45- phenotype. We performed CEC enumeration twice, using the same sample, and calculated the mean value.

Statistical Analyses

This study was carried out as exploratory research for detecting CECs from NSCLC patients. The number of enrolled patients was therefore not precalculated. Spearman's correlation analysis was performed to investigate the correlation between CEC count and ETV. Between-group comparisons were made using the t test. The association between CEC count and progression free survival (PFS) was estimated using the Kaplan-Meier method. The log-rank test was used to assess the survival difference between strata. Differences were considered statistically significant at $p < 0.05$.

RESULTS

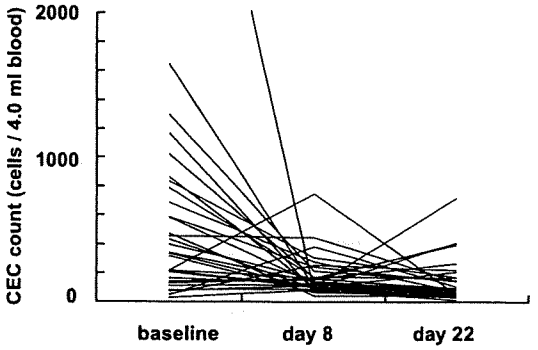
Patient Characteristics

A total of 32 patients were enrolled in the study between August 2005 and March 2006 (Table 1). One patient withdrew consent to participate. Table 1 summarizes the characteristics of the study population. The median age of the patients was 60 years (range, 43–71). The histologic and/or cytologic diagnosis was adenocarcinoma in 23 patients (74.2%), squamous cell carcinoma in 4 (12.9%), and unclassified NSCLC in 4 (12.9%). There were 17 males (54.8%). The clinical stage was IIIA in 2 patients (6.5%), IIIB in 7 (22.6%), and IV in 22 (71.0%).

Ninety-two CEC samples from 31 patients (three samples per patient) were obtained and analyzed. One sample, obtained 22 days after treatment, was not examined because of inadequate collection.

Quantification of CEC

In 31 advanced NSCLC patients, CECs ranged from 32 to 4501 cells/4.0 ml of blood, mean ± SD = 595 ± 832 at baseline. CEC counts were elevated in a large portion of patients with NSCLC as compared with healthy volunteers (n = 53, mean ± SD = 46.2 ± 86.3/4 ml). Case 21 had an exceptionally high CEC count (4501 at baseline). We did not detect a significant correlation between the CEC count and ETV in the 28 assessable patients (p = 0.84, Figure 1). The analysis of CECs during the first course of treatment showed CEC levels to be reduced by CBDCA plus paclitaxel chemotherapy as compared with pretreatment values (176 ± 141 at 8 days and 173 ± 189 at 22 days after treatment) (Figure 2). These reductions were significant (p = 0.011 on day 8 and p = 0.04 on day 22), but there was no significant difference between CEC amounts on day 8 versus day 22 (p = 0.476). There was no difference in the amount of CEC at baseline when patients were subgrouped according to characteristics, such as sex, smoking history, histologic type, and clinical stage. Furthermore, there was no correlation of CEC amounts with the blood examination data (e.g., number of white blood cells, neutrophils, lymphocytes, hemoglobin, platelets, albumin, LDH, CRP, CEA, CYFRA).



	baseline	day 8	day 22
mean ± SD	595± 832	176± 141 *	173 ± 189 *

FIGURE 2. Circulating endothelial cell (CEC) levels during the first course of CDDP plus paclitaxel chemotherapy. *p < 0.05 versus values at baseline.

CEC Amounts and Objective Tumor Response to Chemotherapy

Thirteen (41.9%) of the 31 patients who received carboplatin and paclitaxel therapy showed a partial response (PR) and 12 (38.7%) showed stable disease (SD). The other 6 patients (19.4%) showed PD. The amounts of CEC at baseline in the patients who showed PR and SD were 516 ± 458/4 ml and 871 ± 1215/4 ml, respectively, and these values were significantly higher than in PD patients (211 ± 150/4 ml, p = 0.023 and p = 0.044, respectively) (Figure 3*A*). Although CEC decrements during chemotherapy were observed in all three subgroups, the extent of the decrements tended to be greater in

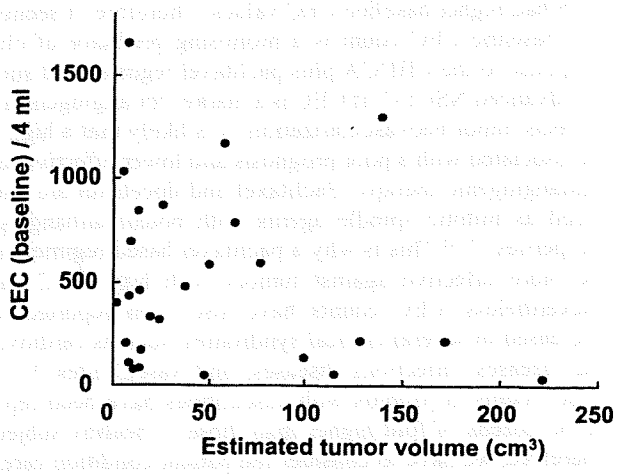


FIGURE 1. Scatter plot analysis to determine the correlation between the number of circulating endothelial cell (CEC) and estimated tumor volume (ETV). ETV is calculated with computed tomography (CT) examination. Case 21 is not included.

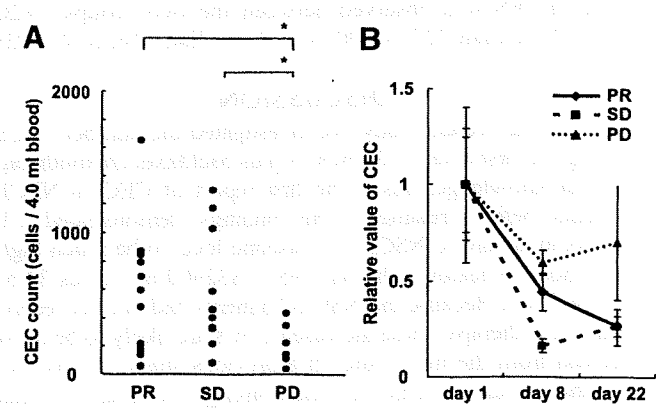


FIGURE 3. *A*, Comparison of circulating endothelial cell (CEC) amount at baseline in non-small cell lung cancer (NSCLC) patients with different clinical responses to CBDCA plus paclitaxel chemotherapy. *p < 0.05 versus values of patients with progressive disease (PD). Case 21 is not included. *B*, Relative change in CEC amount in patients with partial response (PR), stable disease (SD), and PD.

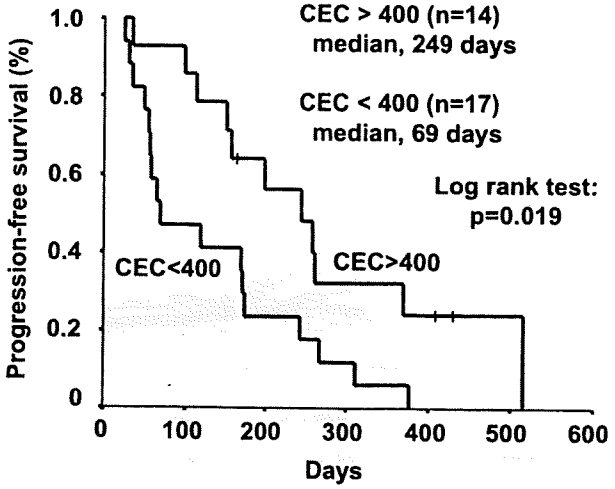


FIGURE 4. Progression-free-survival according to circulating endothelial cell (CEC) count at baseline. The median duration of progression-free survival was greater in patients whose CEC count exceeded 400 (median, 244 days) than in patients whose CEC count was less than 400 (69 days).

patients with PR and SD than in those with PD (Figure 3*B*). In the subgroup analysis, a significant decrease in CECs was observed on day 22 only in PR patients ($p = 0.018$).

CEC Amounts and PFS

For all 31 patients, the median PFS was 154 days (range, 81–361 days). Univariate analysis indicated that patients who had a CEC count of more than 400/4 ml at baseline showed a significantly improved PFS ($n = 14$, median; 244 days) (Log-rank test, $p = 0.019$, Figure 4). A CEC count below 400 at baseline was associated with a poorer PFS ($n = 17$, median; 69 days). The CEC count did not exceed the value of 400/4 ml in any of the healthy volunteers. When we compared the patients whose CEC counts exceeded 200 with those whose counts were less than 200, a consistent difference in PFS was observed between the two groups (>200; $n = 22$, median 227, <200; $n = 9$, median 116, $p < 0.039$).

DISCUSSION

In the present study, we investigated the number of CEC during the first course of CBDCA plus paclitaxel chemotherapy. To our knowledge, this is the first report of CEC in NSCLC patients before treatment. Our findings demonstrated CEC counts in advanced NSCLC at baseline level to be much higher than those in healthy subjects (595 ± 832/4.0 ml versus 32.6 ± 29.5/4.0 ml). Because the NSCLC patients had not yet received anticancer therapy, these increased CECs are likely to be mostly derived from the tumor site. In a previous study, it was found that the amounts of CECs correlate strongly with tumor volume in vivo in an animal model[34]. Nevertheless, we did not find a significant correlation between CECs and ETV. Because the number of CECs could be influenced by many factors related to tumor vasculature, neovascularization, and localization of the tumor, our failure to identify a strong correlation in this study is not surprising. We were also unable to detect a significant direct correlation between CEC amounts and various blood examination data including tumor markers such as CEA and CYFRA. It is unclear at present what biologic characteristics of the tumor or clinical features the CEC number most closely reflects as a biomarker. Mancuso et al. reported that CECs are strongly associated with plasma levels of VCAM-1 and VEGF in breast cancer and lymphoma patients.[15,34] Because VCAM-1 and VEGF are crucial factors for tumor angiogenesis, the variability in CEC values among NSCLC patients might indicate a difference in the neovascularization of each tumor.

We were further able to demonstrate that elevated CECs decreased dramatically after CBDCA plus paclitaxel treatment, but did not reach the level of healthy subjects. Decreased CEC values did not rise again during the first cycle of chemotherapy. Although myelosupression was observed on day 8 and recovered on day 22 in many patients (data not shown), CEC kinetics do not parallel those of WBC, indicating that CEC kinetics might not be influenced by myelopoiesis. Several clinical studies in the field measuring CEC found chemotherapy to be associated with either an increase or a decrease in CECs.[35–39] The different tumor types, stages, prior therapy or not, the anticancer drugs used, measuring points and quantification methods of CEC might have influenced the CEC results after treatment. In the present study, the pretreatment CEC value was much higher than that in lung cancer with metastasis (mean ± SD = 146 ± 270/4 ml), as reported elsewhere.[33] Although the details of the prior therapy in patients with metastatic carcinoma were not provided,[33] chemotherapy can eventually decrease the CEC count.

Schiller et al. compared four standard chemotherapy regimens, cisplatin plus paclitaxel, cisplatin plus gemcitabine, cisplatin plus docetaxel, and carboplatin plus paclitaxel and found no significant difference in survival.[25] Despite the different modes of action of each nonplatinum agent against tumors and different biologic characteristics of each tumor, we could not select the regimen based on these characteristics. In our small study, the patients with PR/SD and longer PFS had higher baseline CEC values. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the CBDCA plus paclitaxel regimen and survival in advanced NSCLC. If CEC is a marker for angiogenesis and reflects tumor neovascularization, it is likely that a high CEC is associated with a poor prognosis and lower effectiveness of antiangiogenic therapy. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties.[27–30] This is why a paclitaxel based regimen might be more effective against tumors with high CEC values. Nevertheless, CEC counts have also been reported to be increased in several clinical syndromes, such as cardiovascular diseases, infectious diseases, and vasculitides.[11–13] The CEC counts in patients with vasculitides have been reported to be dozens of fold higher than those in healthy subjects,[12] therefore, we have to consider the patient condition carefully while interpreting the CEC counts in individual patients, although there were no patients with vasculitis in the present study. Further clinical investigation, with a similar approach, including other nonplatinum anticancer agents, such as

CDDP plus gemcitabine, is essential for the clinical application of CEC for made-to-order chemotherapy in NSCLC.

Antiangiogenic therapy targeting the VEGF pathway such as bevacizumab and VEGFR inhibitors have shown promise in the treatment of solid tumors.[8,39] These agents inhibit endothelial cells through inhibition of the VEGF pathway. It was recently demonstrated that the addition of bevacizumab to CBDCA plus paclitaxel in advanced NSCLC patients produces a significant survival benefit as compared with chemotherapy alone.[40] Considering the outstanding clinical trial and our present study, it would be of great interest to investigate the role of CEC in this regimen.

In conclusion, CECs were measured in NSCLC patients before treatment. Our small clinical study indicates that the CEC count at baseline is a potential biomarker for predicting the response to chemotherapy and PFS, but further clinical evaluation is needed. In the near future, we will start a clinical investigation, using a similar approach, to examine other chemotherapeutic regimens.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for the 3rd Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Welfare and Labour, Japan.

REFERENCES

1. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg* 1972;175:409–416.
2. Gasparini G, Harris AL. Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. *J Clin Oncol* 1995;13:765–782.
3. Dickinson AJ, Fox SB, Persad RA, Hollyer J, Sibley GN, Harris AL. Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. *Br J Urol* 1994;74:762–766.
4. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964–3968.
5. Williams JK, Carlson GW, Cohen C, Derose PB, Hunter S, Jurkiewicz MJ. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994;168:373–380.
6. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000;60:3088–3095.
7. Natsume T, Watanabe J, Koh Y, et al. Antitumor activity of TZT-1027 (Soblidotin) against vascular endothelial growth factor-secreting human lung cancer in vivo. *Cancer Sci* 2003;94:826–833.
8. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–2342.
9. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–434.
10. Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 2004;22:2184–191.
11. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999;93:2951–2958.
12. Woywodt A, Streiber F, De Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA associated small-vessel vasculitis. *Lancet* 2003;361:206–210.
13. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med* 2001;163:195–200.
14. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. *Ann Oncol* 2004;15:139–145.
15. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001;97:3658–3661.
16. Mancuso P, Colleoni M, Calleri A, et al. Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood* 2006;108:452–459.
17. Bülzebruck H, Bopp R, Drings P, et al. New aspects in the staging of lung cancer. Prospective validation of the International Union Against Cancer TNM classification. *Cancer* 1992;70:1102–1110.
18. Grilli R, Oxman AD, Julian JA. Chemotherapy for advanced non-small-cell lung cancer: how much benefit is enough? *J Clin Oncol* 1993;11:1866–1872.
19. Non-small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. *BMJ* 1995;311:899–909.
20. Kubota K, Watanabe K, Kunitoh H, et al. Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. *J Clin Oncol* 2004;22:254–261.
21. Le Chevalier T, Brisgand D, Douillard JY, et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small cell lung cancer: results of a European multicenter trial including 612 patients. *J Clin Oncol* 1994;12:360–367.
22. Belani CP, Lee JS, Socinski MA, et al. Randomized phase III trial comparing cisplatin-etoposide to carboplatin-paclitaxel in advanced or metastatic non-small cell lung cancer. *Ann Oncol* 2005;16:1069–1075.
23. Yana T, Takada M, Origasa H, et al. New chemotherapy agent plus platinum for advanced non-small cell lung cancer: a meta-analysis. *Proc Am Soc Clin Oncol* 2002;21:328a.
24. Baggstrom MQ, Stinchcombe TE, Fried DB, Poole C, Hensing TA, Socinski MA. Third-generation chemotherapy agents in the treatment of advanced non-small cell lung cancer: a meta-analysis. *J Thorac Oncol* 2007;2:845–853.
25. Schiller JH, Harrington D, Belani CP, et al; Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–98.
26. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* 2007;18:317–323.
27. Belotti D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* 1996;2:1843–1849.
28. Hayot C, Farinelle S, De Decker R, et al. In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol* 2002;21:417–425.
29. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs* 2003;14:13–19.
30. Vacca A, Ribatti D, Iurlaro M, et al. Docetaxel versus paclitaxel for antiangiogenesis. *J Hematother Stem Cell Res* 2002;11:103–118.
31. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
32. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res* 2006;66:2918–2922.
33. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A* 2007;71A:105–114.
34. Mancuso P, Calleri A, Cassi C, et al. Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* 2003;522:83–97.

35. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res* 2005;11:3514–3522.
36. Fürstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer* 2006;94:524–531.
37. Rademaker-Lakhai JM, Beerepoot LV, Mehra N, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C beta-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer. *Clin Cancer Res* 2007;13:4474–4481.
38. McAuliffe JC, Trent JC. Biomarkers in gastrointestinal stromal tumor: should we equate blood-based pharmacodynamics with tumor biology and clinical outcomes? *Clin Cancer Res* 2007;13:2643–2650.
39. Hanrahan EO, Heymach JV. Vascular endothelial growth factor receptor tyrosine kinase inhibitors vandetanib (ZD6474) and AZD2171 in lung cancer. *Clin Cancer Res* 2007;13:S4617–S4622.
40. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355: 2542–2550; 2007;356:318.

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 270 (2008) 260–268
CANCER Letters
www.elsevier.com/locate/canlet

Antiangiogenic cancer therapy using tumor vasculature-targeted liposomes encapsulating 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one, SU5416

Yasufumi Katanasaka [a,b], Tomoko Ida [a], Tomohiro Asai [a], Kosuke Shimizu [a], Fumiaki Koizumi [b], Noriyuki Maeda [c], Kazuhiko Baba [d], Naoto Oku [a,*]

[a] *Department of Medical Biochemistry, School of Pharmaceutical Sciences and Global COE, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*
[b] *Shien-Lab Medical Oncology Department, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[c] *Nippon Fine Chemical Co., Ltd., Takasago, Hyogo 676-0074, Japan*
[d] *Taiho Pharmaceutical Co., Ltd., Kawauchi-cho, Tokushima 771-0194, Japan*

Received 21 February 2008; received in revised form 21 February 2008; accepted 8 May 2008

Abstract

Previously, we identified angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG), and showed that APRPG-modified liposomes could selectively target to tumor neovasculature. Here, we designed an APRPG-modified liposome encapsulating SU5416, an angiogenesis inhibitor, to overcome the solubility problem, and to enhance the antiangiogenic activity of SU5416. Liposomal SU5416 appeared to have the appropriate characteristics, such as particle size and stability in serum. It showed a significantly lower hemoglobin release than SU5416 dissolved in a Cremophor EL-containing solvent. Compared with peptide-unmodified liposomal SU5416, the APRPG-modified liposomal SU5416 significantly suppressed tumor growth and with no remarkable side effects. Thus, targeted delivery of antiangiogenic drugs with tumor vasculature-targeted liposomes may be useful for antiangiogenic cancer therapy.

Keywords: Angiogenesis; Drug delivery systems; SU5416; Antiangiogenic therapy; APRPG-modified liposomes

1. Introduction

Angiogenesis is the development of new blood vessels from pre-existing vessels, and is an attractive target for cancer therapy because it is essential for tumor growth and hematogenous metastasis [1]. Vascular targeting therapy is divided into two main types: (i) antiangiogenic approach, which prevents the processes of angiogenesis in tumors, through inhibitors of angiogenic signaling; and (ii) antivascular approach, which impairs the established neovasculature using a vascular disrupt agent [2].

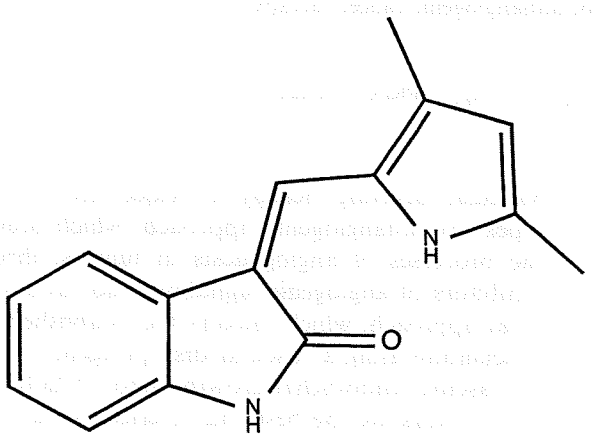
Vascular endothelial growth factor (VEGF) and its receptors are the best-characterized signal pathway in angiogenesis and are regarded as a target molecule for the antiangiogenic approach [3]. In

* Corresponding author. Tel.: +81 54 264 5701; fax: +81 54 264 5705.
E-mail address: oku@u-shizuoka-ken.ac.jp (N. Oku).

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.05.009

fact, several drugs that inhibit VEGF signal transduction have been developed. For example, bevacizumab, a humanized anti-VEGF-A monoclonal antibody, and SU11248, a small molecule inhibitor against receptor tyrosine kinases (RTKs) of VEGF receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), have both been approved for cancer treatment [4].

Z-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone (SU5416) is a potent inhibitor of VEGFR-2 tyrosine kinase [5]. The structure of SU5416 is shown in Fig. 1. This inhibitor has been shown to suppress VEGF-mediated angiogenesis in vitro and in vivo through the inhibition of autophosphorylation of VEGFR-2 by blocking the AMP-binding site within the kinase domain of the receptor [6]. It has been reported that SU5416 has no direct cytotoxic properties to cancer cells but inhibits tumor growth in numerous tumor xenograft models [7]. In Phase I and II trials, the therapeutic efficacy of SU5416 has been shown in combination with certain anticancer drugs. In a Phase III clinical trial, however, SU5416 showed no significant clinical benefit, and some patients showed striking responses induced by the toxicity of the solvent with Cremophor EL (CrEL) that was used to dissolve SU5416 for clinical administration [7–9]. Since CrEL has been known to induce various undesirable effects such as anaphylactic shock or hemolysis [10,11], coadministration with dexamethasone or other steroids is required to prevent hypersensitivity reactions [12]. Therefore, much effort has been devoted to improving the aqueous solubility of some agents to forgo using CrEL. For further enhancement of antiangiogenic effects and reduction of the side effects of SU5416, drug delivery systems (DDS) can be an important factor. However, studying antiangiogenic drugs in the field of DDS is not sufficient.



Molecular Formula: $C_{15}H_{14}N_2O$

Molecular Weight: 238.3

Fig. 1. Structure of 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1, 3-dihydro-indol-2-one, SU5416.

Liposomes are small lipid vesicles and one of the most advanced drug nanocarriers in DDS studies [13]. As drug carriers, liposomes have various favorable characteristics for cancer therapy, such as low toxicity, long-term blood circulation, and accumulation in inflamed tissues and tumors by enhanced permeability and retention (EPR) effect [14,15]. Liposomal formulation of hydrophobic drugs has been shown to overcome the solubility problem and the solvent-induced side effect [16]. In addition, liposomes can be modified with various molecules, such as antibodies, carbohydrates, or peptides, to selectively target several kinds of cells [17]. In our previous studies, we identified angiogenic vessel-homing peptide Ala-Pro-Arg-Pro-Gly (APRPG), and utilized it in liposomal drug delivery. APRPG peptide-modified liposomes directly targeted angiogenic endothelial cells, and doxorubicin-incorporated APRPG-modified liposomes significantly suppressed tumor growth through the disruption of tumor neovasculature [18–20]. These studies raise the possibility that APRPG-modified liposomes are also useful drug carriers for targeted delivery of antiangiogenic drugs.

In this study, to overcome the solubility problem and to enhance the antiangiogenic effect of SU5416, we designed the SU5416-incorporated APRPG-modified liposome. We evaluated the characteristics of liposomal SU5416 as a liposomal drug, such as its encapsulation efficiency, stability in serum, VEGF inhibitory activity, and hemolytic activity in vitro. Subsequently, the therapeutic effect of APRPG-modified liposomal SU5416 in tumor-bearing mice was examined.

2. Materials and methods

2.1. Cell culture and materials

Colon26 NL-17 carcinoma cells were cultured in DMEM/Ham's F12 medium (WAKO, Osaka, Japan) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and 10% heat-inactivated fetal bovine serum (FBS, Japan Bio Serum Co., Ltd., Tokyo, Japan) at 37 °C in a 5% CO_2 atmosphere. Human umbilical vein

endothelial cells (HUVECs, Takara Bio Inc., Otsu, Shiga, Japan) were maintained in endothelial growth medium-2 (EGM-2, Cambrex Corporation, Walkersville, MD, USA) at 37 °C under 5% CO_2 in a humidified chamber. HUVECs used in this study were between passages 4 and 7. The lipids for preparing liposomes were the products of Nippon Fine Chemical, Co., Ltd., (Takasago, Hyogo, Japan).

2.2. Preparation of liposomal SU5416

Liposomes were prepared as described previously [19]. In brief, dipalmitoylphosphatidylcholine (DPPC), palmitoyl-oleoylphosphatidylcholine (POPC), cholesterol, and SU5416 solutions in chloroform were mixed (10:10:5:1 as a molar ratio) and dried under reduced pressure to make a thin lipid film. A distearoylphosphatidylethanolamine polyethyleneglycol (DSPE-PEG) or APRPG peptide-conjugated DSPE-PEG (DSPE-PEG-APRPG) solution was respectively, added to the initial lipid solutions in the proportion of 10-mol % to PC for the modification of the liposomes with PEG or PEG-APRPG. The thin lipid films were hydrated with 20 mM HEPES-buffered saline (pH 7.4), and the liposome solutions were frozen and thawed for three cycles with liquid nitrogen. The liposome size was then adjusted by extrusion through 100 nm-pore sized polycarbonate filters. The particle size and ζ-potential of liposomal SU5416 was measured using ZETASIZER (Malvern Instruments, Worcs, UK).

2.3. Determination of entrapment efficiency of SU5416 into liposomes

Liposomal SU5416 were prepared as described above. The prepared liposomes were fractionated by gel filtration chromatography using PD-10 column (GE Healthcare, UK. Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The turbidity of each fraction was determined by measuring the absorbance at 750 nm to define the liposome fractions. The amount of SU5416 in each fraction was quantified by absorption at 440 nm using high performance liquid chromatography (HPLC, HITACHI, Tokyo, Japan) equipped with ODS-80Ts column (Tosoh Corporation, Tokyo, Japan). The mobile phase for the HPLC analysis was composed of methanol and 35 mM KH_2PO_4 (3:1).

2.4. Stability of liposomal SU5416 in presence of serum

The prepared liposome solutions were incubated in the presence or absence of 50% FBS for 1 h at 37 °C. After that, the liposomes were separated by gel filtration chromatography using Sepharose™ 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) as described previously [21], and the amount of SU5416 in the liposome fractions was determined using HPLC as described above.

2.5. Cell proliferation assay

HUVECs were seeded (7500 cells/well) on a gelatin-coated 96-well plate and incubated overnight. After the change of medium to 0.5% FBS-containing endothelial basal medium-2 (EBM-2, Cambrex Corporation), the cells were treated with SU5416 (dissolved in DMSO), PEG-liposomal SU5416 (PEG-Lip-SU5416), or APRPG-PEG-liposomal SU5416 (APRPG-Lip-SU5416) and incubated for 3 h at 37 °C. Then, recombinant human $VEGF_{165}$ (20 ng/ml as final concentration, BD biosciences, San Diego, CA, USA) was added to the each well, and the cells were further incubated for 48 h. Colon26 NL-17 cells were seeded (3000 cells/well) on a 96-well plate in DMEM/Ham's F12 supplemented with 10% FBS and incubated overnight. Then, the cells were treated with the samples and further incubated for 48 h at 37 °C. The cell viability was measured with TetraColorOne™ (Seikagaku, Tokyo, Japan) according to the manufacturer's instruction.

2.6. Hemolytic assay

Free SU5416 was dissolved in the following components: polyethylene glycol 400; CrEL (Nakalai Tesque, Kyoto, Japan); benzyl alcohol; and dehydrated ethanol (45:31.5:2:21.5 w/w %) as described previously [7], and the SU5416 solution was diluted with 0.45% sodium chloride before treatment. Hemolytic assay was performed as described previously [22] with some modification. In brief, blood was obtained from 6-week-old BALB/c male mice (Japan SLC, Shizuoka, Japan). Red blood cells were collected by centrifugation (2000*g*, 5 min, 4 °C, five times) of the blood. The pellet was resuspended in 20 mM HEPES-buffered saline (pH 7.4) to give a 5% (v/v) solution. The suspension was added to HEPES-buffered saline, free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 and incubated for 30, or 60 min at 37 °C. After centrifugation, the supernatants were transferred to a 96-well plate. Hemolytic activity was determined by measuring the absorption at 570 nm. Control samples of 0% lysis (in HEPES buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment.

2.7. Therapeutic experiment

Colon26 NL-17 carcinoma cells were subcutaneously implanted (1.0×10^6 cells) into the posterior flank of 4-week-old BALB/c male mice. HEPES-buffered saline (Control), free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 was intravenously injected every other day (3 mg/kg/day as SU5416) from day 5 to day 13 after tumor implantation. The tumor size and body weight were monitored daily as described previously [19]. The animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka.

2.8. Statistical analysis

Statistical analysis of the experiments was performed by unpaired Student's *t*-test using KaleidaGraph software (HULINKS, Tokyo, Japan).

3. Results

3.1. Characterization of liposomal SU5416

To investigate whether liposomal SU5416 has appropriate characteristics as a liposome agent, we examined its entrapment efficiency into the liposomes, particle size, ζ-potential, and its stability in the presence of serum. In gel filtration chromatography analysis, liposome fractions were defined by turbidity (absorption at 750 nm). More than 85% of SU5416 was detected in the liposome fractions of Control (non-modification), PEG- and APRPG-modified liposomes (Fig. 2). SU5416-encapsulated liposomes had approximately 130 nm of particle size and −3.0 mV of ζ-potential, respectively (Table 1). The notable change of particle size and the leakage of SU5416 from the liposomes were not observed until 14 days after preparation of the liposomes (Fig. 3). We also examined the stability of liposomal SU5416 in the presence of serum. PEG-Lip-SU5416 and APRPG-Lip-SU5416 were incubated with or without serum, and liposomal SU5416 was fractionated by gel filtration chromatography. After the incubation with serum, more than 85% of SU5416 in comparison with PBS alone were detected in the liposome fractions, fraction 5–10 (Fig. 4). These analyses revealed that SU5416 was effectively and stably encapsulated in the liposomes, and PEG-Lip- and APRPG-Lip-SU5416 stably existed in the presence of serum.

3.2. Growth inhibitory activity of liposomal SU5416

SU5416 has been shown to suppress endothelial cell proliferation through the inhibition of VEGF signal transduction [5]. To confirm that liposomal SU5416 has similar growth inhibitory activity against VEGF-stimulated endothelial cells, we performed a cell proliferation assay. PEG- and APRPG-Lip-SU5416 significantly inhibited endothelial cell proliferation induced by treatment with VEGF in a concentration dependent manner as well as free SU5416 (Fig. 5A). On the contrary, free SU5416 and liposomal SU5416 did not suppress the proliferation of Colon26 NL-17 carcinoma cells (Fig. 5B). These data suggest that encapsulated SU5416 maintains an inhibitory activity against VEGF signal transduction.

3.3. Suppression of hemolysis by liposomalization of SU5416

Since SU5416 is a hydrophobic compound, it is dissolved in the solvent containing CrEL for use in the

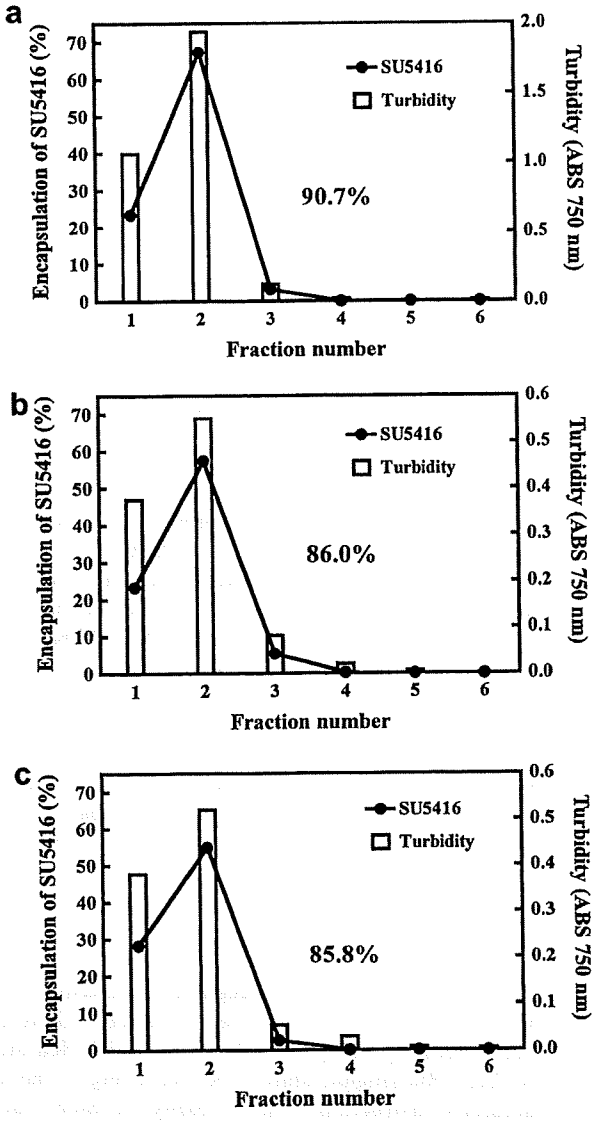


Fig. 2. Entrapment of SU5416 into Control, PEG- or APRPG-modified liposomes. Control liposomal SU5416 (a), PEG-modified liposomal SU5416 (b), and PEG-APRPG-modified liposomal SU5416 (c) were fractionated by gel filtration chromatography with PD-10 column. The turbidity (bar, left *Y* axis) was determined by measurement of the absorption at 750 nm, and the amount of SU5416 (dot, right *Y* axis) was measured using HPLC (absorption at 440 nm). The calculated entrapment efficiency is indicated in each graph.

Table 1
Particle size and ζ-potential of liposomal SU5416

	Particle size (nm)	ZP (mV)
PEG-Lip-SU5416	131.8 ± 14	−3.0 ± 2.2
APRPG-Lip-SU5416	142.6 ± 28	−3.0 ± 1.0

The data indicate the means ± SD.
ZP, ζ-potential.

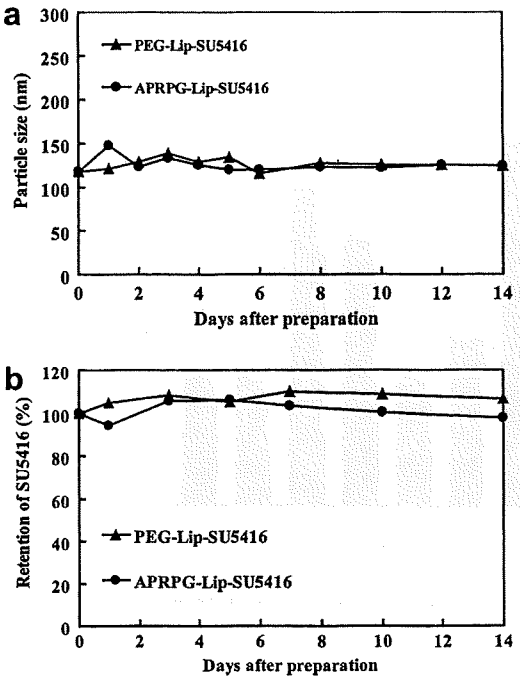


Fig. 3. Stability of liposomal SU5416 in particle size and entrapment efficiency. PEG- and APRPG-modified liposomal SU5416 were incubated until day 14 at 4 °C. The particle size of PEG-Lip-SU5416 (closed triangle) and APRPG-Lip-SU5416 (closed circle) was measured at indicated times (a). The amount of SU5416 into the liposomes was determined after gel filtration chromatography, and the relative entrapment efficiency was calculated as compared to that of the day 0 (b).

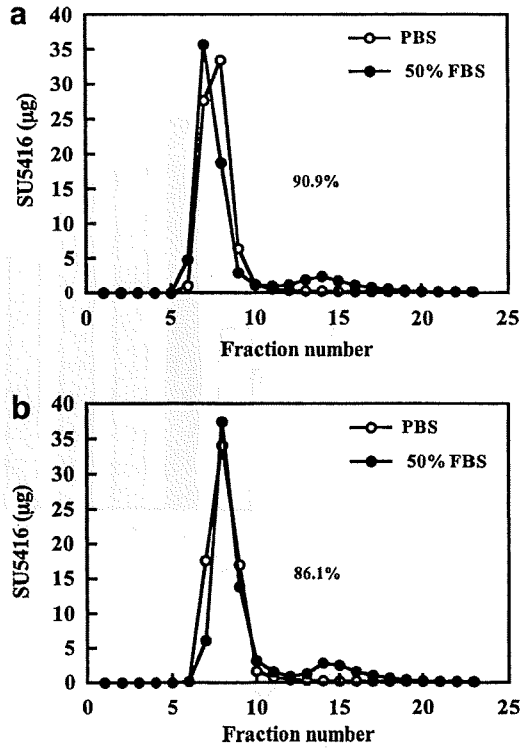


Fig. 4. Retention of SU5416 into liposomes in the presence of serum. PEG- (a) and APRPG-modified liposomal SU5416 (b) were incubated with (open circle) or without 50% fetal bovine serum (closed circle) for 1 h at 37 °C. Liposomal SU5416 was fractionated by gel filtration chromatography. The amount of SU5416 was measured using HPLC. The retention efficiency of SU5416 is indicated in each graph.

clinical studies. CrEL has been shown to induce some undesirable effects such as hemolysis [16]. To determine whether liposomalization of SU5416 precludes these side effects, we examined its hemolytic activity. Free SU5416 dissolved in the solvent induced remarkable hemolysis. In contrast, PEG- and APRPG-Lip-SU5416 showed a significantly low hemolytic activity (Fig. 6).

3.4. Tumor growth suppression by treatment with APRPG-modified liposomal SU5416 in tumor-bearing mice

Finally, the effect of APRPG-Lip-SU5416 in Colon26 NL-17 carcinoma cell-bearing mice was examined. APRPG-Lip-SU5416 significantly suppressed tumor growth compared with control ($p < 0.05$), free SU5416 ($p < 0.05$), and PEG-Lip-SU5416-treatment ($p < 0.01$, Fig. 7a). However, free SU5416 and PEG-Lip-SU5416 showed no tumor growth suppression under the present experimental conditions. SU5416- and liposomal SU5416-treatment did not affect the body weight changes of the mice, an indicator of a side effect (Fig. 7b). Although most of the mice showed shock-like behavior by injection intravenously with SU5416 dissolved in the CrEL-containing solvent, the behavior was not induced by liposomal SU5416 (data not shown).

4. Discussion

In this study, we attempted to develop neovasculature-targeted liposomal SU5416 to overcome the problem of solubility and to enhance the antiangiogenic activity of SU5416 through an active targeting strategy. Liposomal SU5416 has an appropriate particle size and an almost neutral electronic charge. These characteristics have been known to affect liposome distribution. In fact, it has been reported that liposomes having a particle size of approximately 100 nm and a neutral charge accumulate in inflammation region such as tumors through enhanced permeability and retention (EPR) effect [15]. It is also known that hydrophobic agents incorporated into the liposomal membrane transfer to plasma lipoproteins in the bloodstream. Therefore, we examined the stability of liposomal SU5416 in

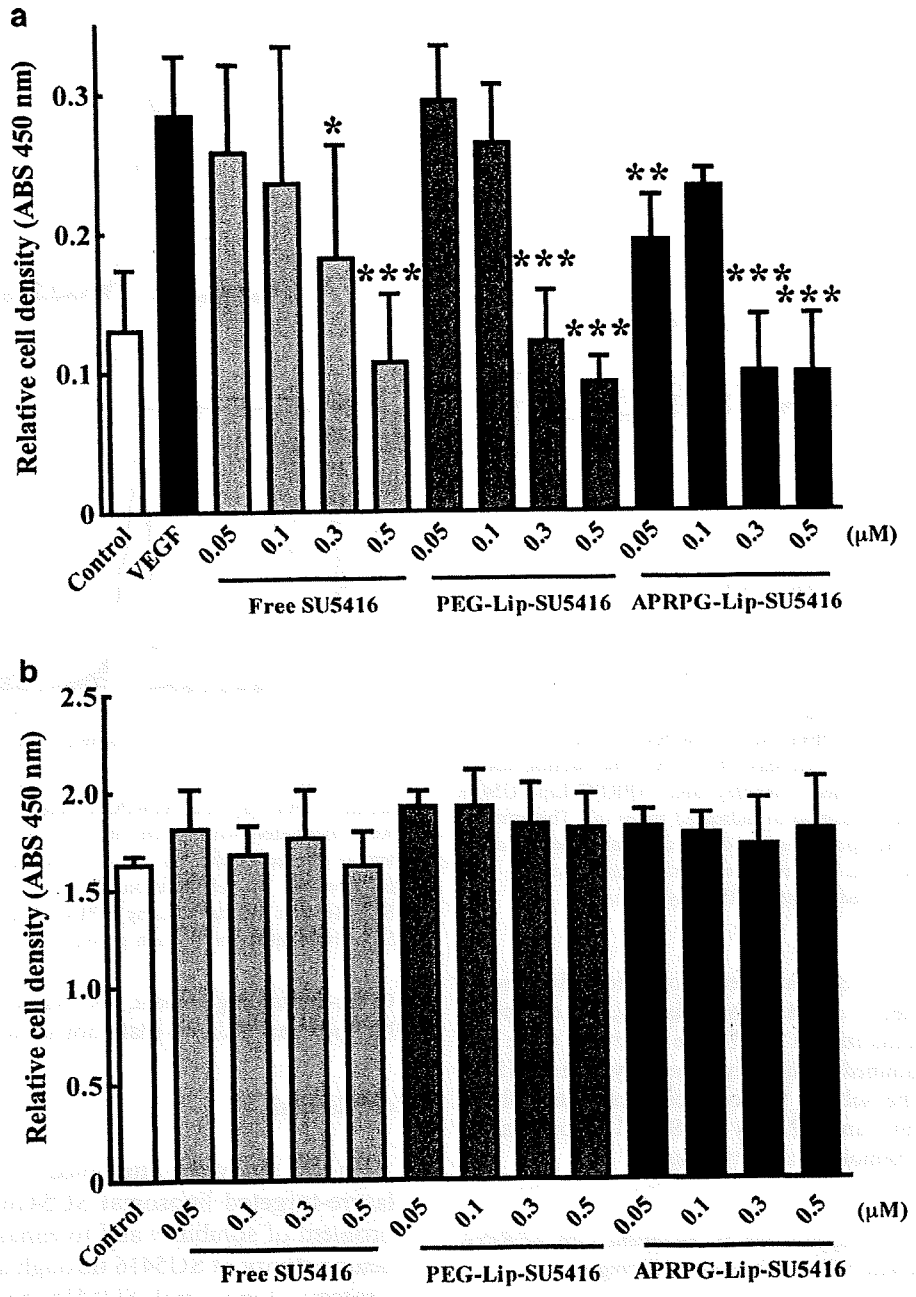


Fig. 5. Inhibited effect of liposomal SU5416 on VEGF-induced endothelial cell growth. (a) HUVECs (7500 cells/well) were seeded on a 96-well plate. The culture medium was changed to EBM-2 containing 0.5% FBS, and the cells were treated with free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 at indicated concentration and incubated for 3 h at 37 °C. Then, the cells were added to rhVEGF$_{165}$ (20 ng/mL as final concentration) and further incubated for 48 h. (b) Colon26 NL-17 cells (3000 cells/well) were also seeded on a 96-well plate and incubated overnight. The cells were treated with these samples and further incubated for 48 h. Finally, cell viability was determined with TetraColor ONE™. The bars indicate the means ± SD. ($n = 4$), and the significant differences are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus VEGF-treated group.

the presence of serum, and observed it to be quite stable there. In addition, liposomalization of SU5416 maintained the antiangiogenic activity of SU5416. These findings suggest that SU5416-incorporated liposomes can adequately function as a liposomal drug.

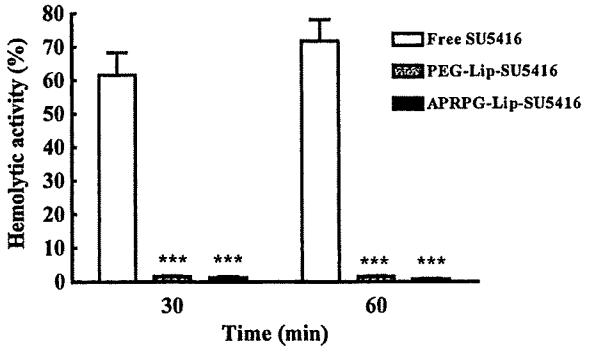


Fig. 6. Reduction of solvent-induced hemolysis by liposomalization of SU5416. Red blood cells were collected by centrifugation of the blood and resuspended in HEPES-buffered saline. The cell suspension was added to HEPES-buffered saline, free SU5416, PEG-Lip-SU5416, or APRPG-Lip-SU5416 and incubated for 30 or 60 min at 37 °C. After centrifugation, hemolytic activity was determined by measuring the absorbance (570 nm) of the supernatant. Control samples of 0% lysis (in HEPES buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment. The bars indicate the means ± SD. ($n = 4$). Significant difference is shown as follow: $^{***}p < 0.001$ versus free SU5416.

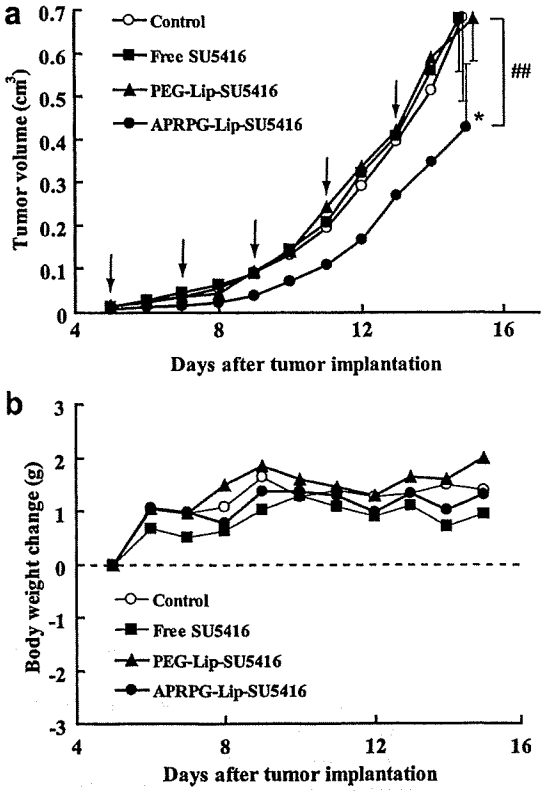


Fig. 7. Suppression of tumor growth by treatment with APRPG-modified liposomal SU5416 in tumor-bearing mice. Colon26 NL-17 carcinoma cells were implanted s.c. into the left posterior flank of 4-week-old BALB/c male mice ($n = 5$–6 per group). The mice were injected i.v. with HEPES buffer (Control, open circle), free SU5416 (3 mg/kg, closed square), PEG- (closed triangle) or APRPG-modified liposomal SU5416 (as SU5416 dosage, 3 mg/kg, closed circle) on days 5, 7, 9, 11, and 13 after tumor implantation. Tumor volume (a) and body weight change (b) were determined as described in the Section 2. Arrows show the days of injection. The data indicate the means ± SD, and the significant differences are indicated as follows: $^{*}p < 0.05$ versus control and free SU5416; $^{\#\#}p < 0.01$ versus PEG-Lip-SU5416.

We found that the liposomal SU5416 did not induce hemolysis in vitro and shock-like behaviors when it was intravenously injected. SU5416 is dissolved in the solvent containing CrEL that has been shown to induce various side effects [11]. Liposomes have also been used to formulate a variety of poorly water soluble drugs [23,24]. For example, by formulation into liposomes, paclitaxel, an anticancer drug used by dissolving in a mixture of 50% ethanol and 50% CrEL, has improved solubility, pharmacokinetics, and antitumor activity yet avoided any solvent-induced side effects [25,26]. Our findings suggest that liposomalization of SU5416 can overcome the solubility problem and decrease the risk of side effects caused by a solvent.

In an in vivo experiment, although APRPG-Lip-SU5416 did not exhibit any dramatic antitumor effect, it showed a statistically significant antitumor activity and without any prominent side effect. These results suggest that APRPG-modified liposomes may enhance antiangiogenic activity through targeted delivery of SU5416 to angiogenic endothelial cells in vivo. The previous study has shown that free SU5416 can suppress tumor growth by frequent injection at a high dose (10–25 mg/kg) [6], and therefore it is not thought to suppress tumor growth under the present treatment conditions (3 mg/kg/day, 5×). In addition, PEG-Lip-SU5416 also did not show the antitumor activity. One of the possible difference between APRPG-Lip-SU5416 and PEG-Lip-SU5416 is whether or not the liposomes directly target tumor endothelial cells [18,19]. PEG or other polymer modification is useful for a drug delivery system by the prolongation of drug circulation in the blood [27,28]. Since PEG liposomes accumulate in tumor tissues through the endothelial cell layer by the EPR effect, PEG-Lip-SU5416 seems to be weakly associated with angiogenic endothelial cells in the tumors. Our data suggest that active targeting to angiogenic endothelial cells may be an useful strategy to enhance the therapeutic effect of angiogenesis inhibitors. To improve the effect, it may be necessary to optimize liposome formulation (ligand

density, lipid composition, etc.) or to modify other ligands (antibodies, peptides, etc.).

In conclusion, we have shown that (i) SU5416 can be formulated in liposomes; (ii) Liposomal SU5416 can be administered without remarkable side effects; and (iii) APRPG-Lip-SU5416 exhibits higher antitumor activity than PEG-Lip-SU5416. Thus, tumor vasculature-targeted liposomes may be useful for drug delivery of antiangiogenic drugs, and the development of such DDS may advance antiangiogenic cancer therapy.

Acknowledgements

This research was supported by the Center of Excellence (COE) program in the 21st Century and Cooperation of Innovative Technology.

References

[1] P. Carmeliet, Angiogenesis in life, disease and medicine, Nature 438 (2005) 932–936.
[2] D. Neri, R. Bicknell, Tumour vascular targeting, Nat. Rev. Cancer 5 (2005) 436–446.
[3] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[4] W.A. Spannuth, A.K. Sood, R.L. Coleman, Angiogenesis as a strategic target for ovarian cancer therapy, Nat. Clin. Pract. Oncol. 5 (2008) 194–204.
[5] T.A. Fong, L.K. Shawver, L. Sun, C. Tang, H. App, T.J. Powell, Y.H. Kim, R. Schreck, X. Wang, W. Risau, A. Ullrich, K.P. Hirth, G. McMahon, SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types, Cancer Res. 59 (1999) 99–106.
[6] D.B. Mendel, R.E. Schreck, D.C. West, G. Li, L.M. Strawn, S.S. Tanciongco, S. Vasile, L.K. Shawver, J.M. Cherrington, The angiogenesis inhibitor SU5416 has long-lasting effects on vascular endothelial growth factor receptor phosphorylation and function, Clin. Cancer Res. 6 (2000) 4848–4858.
[7] M. Zangari, E. Anaissie, A. Stopeck, A. Morimoto, N. Tan, J. Lancet, M. Cooper, A. Hannah, G. Garcia-Manero, S. Faderl, H. Kantarjian, J. Cherrington, M. Albitar, F.J. Giles, Phase II study of SU5416, a small molecule vascular endothelial growth factor tyrosine kinase receptor inhibitor, in patients with refractory multiple myeloma, Clin. Cancer Res. 10 (2004) 88–95.
[8] A. Arora, E.M. Scholar, Role of tyrosine kinase inhibitors in cancer therapy, J. Pharmacol. Exp. Ther. 315 (2005) 971–979.
[9] C. Ye, D. Sweeny, J. Sukbuntherng, Q. Zhang, W. Tan, S. Wong, A. Madan, B. Ogilvie, A. Parkinson, L. Antonian, Distribution, metabolism, and excretion of the anti-angiogenic compound SU5416, Toxicol. In Vitro 20 (2006) 154–162.
[10] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation, Eur. J. Cancer 37 (2001) 1590–1598.
[11] Y. Zou, H. Fu, S. Ghosh, D. Farquhar, J. Klostergaard, Antitumor activity of hydrophilic paclitaxel copolymer prodrug using locoregional delivery in human orthotopic non-small cell lung cancer xenograft models, Clin. Cancer Res. 10 (2004) 7382–7391.
[12] W.M. Stadler, D. Cao, N.J. Vogelzang, C.W. Ryan, K. Hoving, R. Wright, T. Karrison, E.E. Vokes, A randomized Phase II trial of the antiangiogenic agent SU5416 in hormone-refractory prostate cancer, Clin. Cancer Res. 10 (2004) 3365–3370.
[13] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, Science 303 (2004) 1818–1822.
[14] I. Cheong, X. Huang, K. Thornton, L.A. Diaz Jr., S. Zhou, Targeting cancer with bugs and liposomes: ready, aim, fire, Cancer Res. 67 (2007) 9605–9608.
[15] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release 65 (2000) 271–284.
[16] A.O. Nornoo, D.S. Chow, Cremophor-free intravenous microemulsions for paclitaxel II. Stability, in vitro release and pharmacokinetics, Int. J. Pharm. 349 (2008) 117–123.
[17] L. Nobs, F. Buchegger, R. Gurny, E. Allemann, Current methods for attaching targeting ligands to liposomes and nanoparticles, J. Pharm. Sci. 93 (2004) 1980–1992.
[18] N. Maeda, S. Miyazawa, K. Shimizu, T. Asai, S. Yonezawa, S. Kitazawa, Y. Namba, H. Tsukada, N. Oku, Enhancement of anticancer activity in antineovascular therapy is based on the intratumoral distribution of the active targeting carrier for anticancer drugs, Biol. Pharm. Bull. 29 (2006) 1936–1940.
[19] N. Maeda, Y. Takeuchi, M. Takada, Y. Sadzuka, Y. Namba, N. Oku, Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome, J. Control. Release 100 (2004) 41–52.
[20] N. Oku, T. Asai, K. Watanabe, K. Kuromi, M. Nagatsuka, K. Kurohane, H. Kikkawa, K. Ogino, M. Tanaka, D. Ishikawa, H. Tsukada, M. Momose, J. Nakayama, T. Taki, Anti-neovascular therapy using novel peptides homing to angiogenic vessels, Oncogene 21 (2002) 2662–2669.
[21] K. Ichikawa, T. Urakami, S. Yonezawa, H. Miyauchi, K. Shimizu, T. Asai, N. Oku, Enhanced desensitization efficacy by liposomal conjugation of a specific antigen, Int. J. Pharm. 336 (2007) 391–395.
[22] D. Guggi, N. Langoth, M.H. Hoffer, M. Wirth, A. Bernkop-Schnurch, Comparative evaluation of cytotoxicity of a glucosamine-TBA conjugate and a chitosan-TBA conjugate, Int. J. Pharm. 278 (2004) 353–360.
[23] P. Crosasso, M. Ceruti, P. Brusa, S. Arpicco, F. Dosio, L. Cattel, Preparation characterization and properties of sterically stabilized paclitaxel-containing liposomes, J. Control. Release 63 (2000) 19–30.
[24] S. Kim, Liposomes as carriers of cancer chemotherapy, current status and future prospects, Drugs 46 (1993) 618–638.
[25] A. Sharma, E. Mayhew, L. Bolcsak, C. Cavanaugh, P. Harmon, A. Janoff, R.J. Bernacki, Activity of paclitaxel liposome formulations against human ovarian tumor xenografts, Int. J. Cancer 71 (1997) 103–107.
[26] T. Yang, F.D. Cui, M.K. Choi, J.W. Cho, S.J. Chung, C.K. Shim, D.D. Kim, Enhanced solubility and stability of

PEGylated liposomal paclitaxel: in vitro and in vivo evaluation, Int. J. Pharm. 338 (2007) 317–326.
[27] R. Satchi-Fainaro, M. Puder, J.W. Davies, H.T. Tran, D.A. Sampson, A.K. Greene, G. Corfas, J. Folkman, Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470, Nat. Med. 10 (2004) 255–261.
[28] M.J. Vicent, R. Duncan, Polymer conjugates: nanosized medicines for treating cancer, Trends Biotechnol. 24 (2006) 39–47.

Identification of prognostic biomarkers in gastric cancer using endoscopic biopsy samples

Yasuhide Yamada,[1] Tokuzo Arao,[2] Takuji Gotoda,[3] Hirokazu Taniguchi,[4] Ichiro Oda,[3] Kuniaki Shirao,[1] Yasuhiro Shimada,[1] Tetsuya Hamaguchi,[1] Ken Kato,[1] Tetsutaro Hamano,[7] Fumiaki Koizumi,[5] Tomohide Tamura,[1] Daizo Saito,[3] Tadakazu Shimoda,[4] Makoto Saka,[6] Takeo Fukagawa,[6] Hitoshi Katai,[6] Takeshi Sano,[6] Mitsuru Sasako[6] and Kazuto Nishio[2,8]

[1]Medical Oncology, [3]Endoscopic Division, [4]Diagnostic Pathology Division, [5]Shien Lab, and [6]Surgical Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [2]Department of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511; [7]Hamano Statistical Analysis Ltd, 6-7-15 Chuo-cho, Higashikurume, Tokyo 203-0054, Japan

(Received June 22, 2008/Revised July 16, 2008/Accepted July 16, 2008/Online publication September 28, 2008)

Endoscopic biopsy prior to chemotherapy provides an opportunity for studying biomarkers to predict the overall survival in gastric cancer patients. This prospective study was performed to identify prognostic biomarkers in patients with unresected gastric cancer. Fifty-nine cases of chemotherapy-naive metastatic gastric cancer were enrolled in this study. A microarray analysis was performed using 40 biopsy samples to identify candidate genes whose expressions might be correlated with the overall survival. After adjusting for clinical covariates based on a multivariate analysis, the identified genes were validated using real-time reverse transcription polymerase chain reaction (RT-PCR) analysis in 19 independent validation samples. Ninety-eight candidate genes whose expression levels were significantly correlated with the overall survival were identified using a microarray analysis based on a proportional hazards model ($P < 0.005$). Multivariate analysis was performed to assess 10 of these genes, and the results yielded a statistical significance level for *DACH1* and *PDCD6*. We further evaluated these two genes in independent samples using real-time RT-PCR and found that lower mRNA expression levels of *PDCD6* were correlated significantly with a poor overall survival. We identified *PDCD6* as a prognostic biomarker in patients with unresected gastric cancer using endoscopic biopsy samples. Our PCR-based single gene prediction strategy successfully predicted the overall survival and may lead to a better understanding of this disease subgroup. (*Cancer Sci* 2008; 99: 2193–2199)

Over the past two decades, various anticancer agents have been examined for their efficacy against gastric cancer, including 5-fluorouracil (5-FU) and 5-FU-based drugs, taxanes, CPT-11 and cisplatin, all administered either as monotherapy or in combination regimens;[1] however, the median survival time (MST) of these patients remains at only approximately 7 months.[2,3] In a recent randomized phase III trial examining oral S-1 monotherapy and cisplatin plus irinotecan combination therapy, the response rates to both S-1 and to the cisplatin plus irinotecan combination therapy were approximately 50%, indicating that around half of the patients did not respond to chemotherapy,[4–7] and the MST in both the arms was less than 1 year.[8] Thus, the prognosis of patients with gastric cancer remains poor.

The commonly recognized prognostic factors in cases of unresectable gastric cancer are the performance status, presence/absence of liver metastases, presence/absence of peritoneal metastases and the serum levels of alkaline phosphatase.[9] Many molecular biomarkers have been also investigated for their potential to predict the outcome in hypothesis-based studies. Several studies have shown that the mRNA levels and immunohistochemical staining intensity of thymidylate synthase (TS) in gastric cancers treated with fluorouracil are associated with the response and survival; in addition, the excision repair cross-complementing (ERCC)1 gene expression level has been shown to be associated with the clinical outcome in patients treated with cisplatin.[10,11] HER2 expression has also been reported to be a prognostic marker in cases of differentiated gastric cancer.[12,13] Mutation of p53 and high p53 protein expression, and high expression levels of urokinase-plasminogen activator, xanthine oxidoreductase, claudin-4, vascular endothelial growth factor, interleukin-8 and cyclin E have all been correlated with poor survival.[13–19] In terms of epigenetic alterations, reduced expression of acetylated histone H4 or DNA methylation of CDH1 and RAR-β have been shown to be correlated with tumor invasiveness and the tumor metastasizing potential.[20,21]

On the other hand, the recent introduction of the microarray technology has enabled significant genes to be identified almost throughout the genome using a hypothesis-free approach. The possibility of performing genome-wide searches is a major advantage, and such searches may be the only way to discover genes that would otherwise be unlikely to even be suggested as candidates. In gastric cancer, biopsy samples of the primary lesions can be easily obtained by endoscopy prior to treatment; however, few prospective biomarker studies using endoscopic biopsy samples to predict patient outcome have been performed to date. Therefore, we conducted a prospective study to identify biomarkers for predicting survival in patients with unresected metastatic gastric cancer.

Materials and Methods

Patients and samples. The eligible subjects in this study were patients with histologically confirmed, untreated and metastatic stage IV gastric cancer between 20 and 75 years of age. Additional inclusion criteria included an Eastern Cooperative Oncology Group performance status of 0–2. The exclusion criteria included history of prior chemotherapy or major surgery. All patients received chemotherapy using a 5-FU-based regimen (5-FU alone, S1 alone, 5-FU + methotrexate, 5-FU + cisplatin, or S1 + cisplatin) or a CPT-11 plus cisplatin regimen. Sixty-five gastric cancer patients were enrolled in the study. Of these, two were excluded because of insufficient RNA quantities extracted from their biopsy specimens, and four were excluded because of the poor RNA quality. Thus, samples from the remaining 59 patients were analyzed. The survival time was followed after the patients were initiated on chemotherapy. This study was approved

[8]To whom correspondence should be addressed. E-mail: knishio@med.kindai.ac.jp

doi: 10.1111/j.1349-7006.2008.00935.x

by the Institutional Review Board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

The endoscopic biopsy samples collected were immediately placed in an RNA stabilization solution (Isogen; Nippongene, Tokyo, Japan) and stored at −80°C. Other biopsy samples obtained from the same location were reviewed by a pathologist to confirm the presence of tumor cells. The RNA extraction method and the quality check protocol have been described previously.[22]

Study design. This prospective study was started in July 2003 and enrollment was completed in November 2006 at the National Cancer Center Hospital. Fifty-nine gastric cancer samples were evaluated in this study. The samples were divided into a training set ($n = 40$) and a validation set ($n = 19$; 2:1) using computer-generated randomization (Microsoft Office Excel, Microsoft, Redmond, WA, USA). A microarray analysis was performed using the training set of 40 samples, and candidate genes whose expressions were correlated with the overall survival were identified. Multivariate analysis was performed to adjust the expression of 10 of these candidate genes for clinical features. Finally, the significant genes were evaluated in an independent set of 19 samples and survival was predicted using the results of real-time reverse transcription polymerase chain reaction (RT-PCR) analyses.

Real-time RT-PCR. Real-time RT-PCR was performed for 10 genes: *DACH1* (dachshund homolog 1, NM_004392); *EGFR* (epidermal growth factor receptor, NM_005228); *MT1X* (metallothionein 1X, NM_005952); *YWHAE* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide, NM_006761); *GPX3* (glutathione peroxidase 3, NM_002084); *PDCD6* (programmed cell death 6, NM_013232); *WDR33* (WD repeat domain 33, NM_018383); *C14orf43* (chromosome 14 open reading frame 43, NM_194278); *MYLIP* (myosin regulatory light chain interacting protein, NM_013262); and *GKAP1* (G kinase anchoring protein 1, NM_025211). Glyceraldehyde 3 phosphate dehydrogenase (*GAPD*, NM_002046) was used to normalize the expression levels in the subsequent quantitative analyses. RNA was converted to cDNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA). The transcripts were quantified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and 7900HT Fast Real-time PCR system (Applied Biosystems) and reported relative to the *GAPD* expression levels. The PCR conditions were as follows: one cycle of denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. To amplify the target genes, the following primers were purchased from Takara (Yotsukaichi, Japan): DACH1-FW, 5′-AAG GGC TGC TAA AGC AAT CAG G-3′, and DACH1-RW, 5′-CTT TGT GGC AAA GCG ACA TTA GG-3′; EGFR-FW, 5′-GGT GCG AAT GAC AGT AGC ATT ATG A-3′, and EGFR-RW, 5′-AAA TGG GCT CCT AAC TAG CTG AAT C-3′; MT1X-FW, 5′-TTG ATC GGG AAC TCC TGC TTC T-3′, and MT1X-RW, 5′-ACA CTT GGC ACA GCC GAC A-3′; GPX3-FW, 5′-ATG CCT ACA GGT ATG CGT GAT TG-3′, and GPX3-RW, 5′-TGC AGG CAC ACA GAT GGT ACA-3′; PDCD6-FW, 5′-TCA AGG CCA GAC TAG ATC AGC CTA A-3′, and PDCD6-RW, 5′-GCT GGG ATG AGG CAC ATG AC-3′; YWHAE-FW, 5′-GGC AGA ATT TGC CAC AGG AA-3′, and YWHAE-RW, 5′-ACC TAA GCG AAT AGG ATG CGT TG-3′; WDR33-FW, 5′-ATG CAT GGG CTC TGT CAG TTT C-3′, and WDR33-RW, 5′-GGC TGA TAC CGG GAC AAC ACT AC-3′; C14orf43-FW, 5′-CAG ACT GGC AAG CCT AAC TCC ATA-3′, and C14orf43-RW, 5′-CAA GGC TGT TCC TGT GCT CTG-3′; MYLIP-FW, 5′-ACG TCT ATC TGC CAA CGC ACA C-3′, and MYLIP-RW, 5′-CAG TTC ATG GAA ACA TGC CAA GTC-3′; GKAP1-FW, 5′-TTG CGA ATA AGT TTC GGA GCA TC-3′, and GKAP1-RW, 5′-GCC ACT GCC ACT ATC CAC TTG TAA-3′; GAPD-FW, 5′-GCA CCG TCA AGG CTG AGA AC-3′, and GAPD-RW, 5′-ATG GTG GTG AAG ACG CCA GT-3′.

Oligonucleotide microarray study. The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA). In brief, the total RNA extracted from the tumor samples was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) for quality check, and cRNA was synthesized using the GeneChip 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNA were then purified and used for construction of the probes. Hybridization was performed using the Affymetrix GeneChip HG-U133 Plus 2.0 array for 16 h at 45°C. The signal intensities were measured using a GeneChip Scanner3000 (Affymetrix) and converted to numerical data using the GeneChip Operating Software, ver. 1 (Affymetrix).

Statistical analysis. The microarray analysis was performed using the BRB Array Tools software ver. 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr Richard Simon and Dr Amy Peng. In brief, a log base 2 transformation was applied to the raw microarray data, and global normalization was used to calculate the median over the entire array. Genes were excluded if the percentage of data missing or filtered out exceeded 20%. Genes that passed the filtering criteria were then considered for further analysis. We computed a statistical significance level ($P < 0.005$) for each gene based on a univariate proportional hazards model.

To adjust the expression of 10 genes (*DACH1*, *EGFR*, *MT1X*, *YWHAE*, *GPX3*, *PDCD6*, *WDR33*, *C14orf43*, *MYLIP* and *GKAP1*) for clinical features (age, sex, performance status [PS], number of metastatic sites, received chemotherapy), clinical data and the normalized microarray expression data of the 10 genes were imported into SAS software ver. 9.1.3 (SAS Institute, Cary, NC, USA) and a Cox regression model was constructed for multivariate analysis against each of the variables. The study groups were divided into two groups based on each of the clinical features: age (<65 or ≥65 years), sex (male or female), PS (0 or ≥1), number of metastatic sites (<3 or ≥3), chemotherapy (5-FU-based or CPT11 + CDDP) and expression levels of 10 genes). $P < 0.05$ was considered significant.

Results

Identification of 98 candidate prognosis-related genes using a microarray analysis. The univariate analysis of clinical features including age (<65 or ≥65 years), sex, PS (0 or ≥1), number of metastatic sites (1, 2 or ≥3) and received chemotherapy (5-FU-based or CPT11 + CDDP) were performed for 40 microarray samples (Table 1). There were no significant differences between any of the two groups divided according to age, sex, number of metastatic sites or received chemotherapy; however, significant differences were noted between the two groups divided according to PS ($P = 0.048$).

To identify the candidate prognosis-related genes from amongst over 47 000 transcripts, a microarray analysis was performed for a training set of 40 samples. A total of 21 308 genes passed the filtering criteria and were further analyzed. Ninety-eight genes were significantly correlated with survival, according to a Cox proportional hazards model ($P < 0.005$) (Table 2). Fifty-nine genes were protective genes (hazard ratio, <1), and 39 were risk genes (hazard ratio >1).

A heat-map of the expression values of the 98 selected genes comparing the unfavorable prognosis group (survival time, <180 days) and favorable prognosis group (survival time, ≥180 days) is shown in Fig. 1. Genes are plotted via hierarchical clustering.

Multivariate analysis of prognosis-related genes. Of the 98 candidate genes, we prioritized those that: (i) were selected by overlapping probes; (ii) were novel genes; or (iii) had a lower

doi: 10.1111/j.1349-7006.2008.00935.x

Table 1. Univariate analysis of clinical features

Variable	No. of patients	MST (days)	*P*-value (log-rank test)
Age (years)			
≥65	16	235	0.454
<65	24	250	
Sex			
Male	29	243	0.926
Female	11	267	
PS			
≥1	24	182	0.048
0	16	309	
Metastasis			
1, 2	10	137	0.102
≥3	30	261	
Chemotherapy			
5-FU-based	26	245	0.594
CPT11 + CDDP	14	240	

MST, median survival time; PS, performance status.

P-value according to a Cox proportional hazards model. We selected the following 10 genes of interest for real-time RT-PCR analysis: *DACH1*, *EGFR*, *MT1X*, *YWHAE*, *GPX3*, *PDCD6*, *WDR33*, *C14orf43*, *MYLIP* and *GKAP1*.

To adjust for relevant clinical covariates against these 10 genes, we performed a multivariate analysis (Table 3). The results of the multivariate analysis revealed that high *DACH1* expression and high *PDCD6* expression were significantly correlated with the favorable outcome ($P = 0.0134$ and $P = 0.0015$, respectively). We therefore considered that the *DACH1* and *PDCD6* expressions were independent prognostic markers from the results of the multivariate analysis. Results of microarray data and patient survival in the training set of 40 patients are shown in Fig. 2. The Kaplan–Meier method was used for *DACH1* and *PDCD6*. The low *PDCD6* and *DACH1* expression groups had significantly poorer outcomes ($P < 0.0001$ and $P = 0.0045$).

Validation using real-time RT-PCR in independent samples. The mRNA expression levels of *DACH1* and *PDCD6* were quantified using real-time RT-PCR in 19 independent samples to validate the results of the microarray. While the expression levels of *DACH1* were not correlated with survival, those of *PDCD6* in independent samples were significantly correlated with the survival ($P = 0.007$) (Table 4). The Kaplan–Meier method was used to estimate the overall survival using the median value (Fig. 3a). All quantified expression levels of real time RT-PCR data are shown as Fig. 3(b). The mRNA expressions of *PDCD6* varied by approximately 25 fold (range, 0.98–25.1). The low *PDCD6* expression groups had significantly poorer outcomes ($P = 0.0018$). We concluded that *PDCD6* was a valuable gene for predicting the survival in patients with gastric cancer. These results indicate that our PCR-based single gene prediction strategy using endoscopic biopsy samples could successfully predict the overall patient survival.

Discussion

Several studies have identified prognostic biomarkers in cases of gastric cancer using microarray analysis. Hasegawa *et al.* identified 12 genes that were associated with lymph node metastasis.[23] Hippo *et al.* identified several genes associated with lymph node metastasis, including Oct-2, and genes associated with the histological type, including liver-intestine cadherin.[24] These studies introduced a novel direction in which microarray analysis could be used to predict postoperative recurrences. Inoue *et al.* selected 78 genes that were differentially expressed between aggressive and non-aggressive cancers and constructed a prognostic scoring system.[25] Leung *et al.* found that high CCL18 expression levels were associated with prolonged overall and disease-free survival.[26] They also found that phospholipase A2 group IIA expression in gastric adenocarcinoma was associated with prolonged survival and less frequent metastasis.[27] Chen *et al.* demonstrated a survival prediction model consisting of three genes (*CD36*, *SLAM*, *PIM-1*) that was capable of predicting poor or good survival in 23 (76.7%) of 30 newly enrolled patients.[28] Most of these studies used surgical specimens to predict postsurgical survival and were conducted retrospectively. Thus, we think that our present prospective study is unique in that we used endoscopic biopsy samples to predict the survival time in patients with unresectable gastric cancer. In patients with unresectable cancer, endoscopic biopsy samples may be the most appropriate specimens available non-invasively for microarray analysis. Although tumor heterogeneity may pose problems when biopsy samples are used as representative tissue specimens and further investigation is required, we believe that endoscopic biopsy samples should continue to be used for microarray analyses. Current clinical study has been confronted with a number of obstacles. Microarray analysis for clinical studies, in particular, has been hampered with bottlenecks such as RNA quality, the extremely large number of genes to be analyzed, an immature analytical tool or methodology and so on. There are two types of obstacles: controllable obstacles and uncontrollable ones. One uncontrollable obstacle is a complex chemotherapy regimen. It is easy to say that a clinical biomarker study should be performed in one particular regimen. Chemotherapy regimen has, however, progressed and become more sophisticated in a short range of time. This study was prospective clinical study and was largely followed by a guideline, Recommendations for Tumor Marker Prognostic Studies (REMARK). To minimize

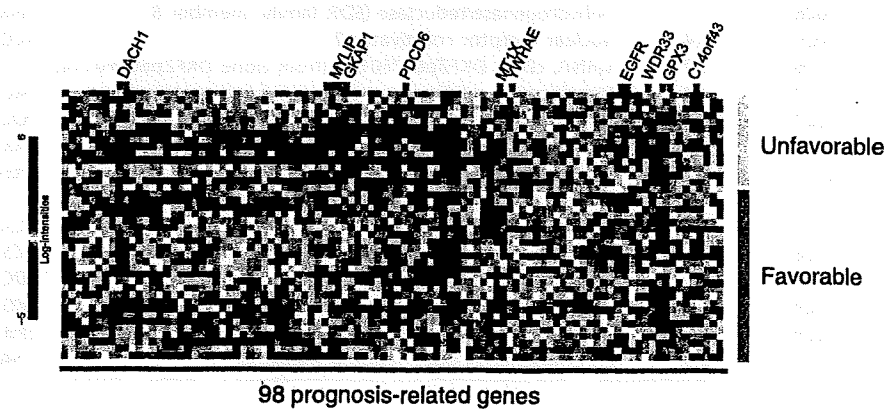


Fig. 1. Heat map of expression values for microarray identifying 98 genes whose expressions were correlated with survival. The hierarchical clustering of the 98 genes comparing the unfavorable prognosis group (survival time, <180 days) and favorable prognosis group (survival time, ≥180 days) is shown. The blue or red colors of each block represent the normalized gene expression levels. Each row represents a sample, and each column represents a gene. The 10 genes included in the multivariate analysis (Table 3) are shown.

Table 2. Prognosis-related genes identified using microarray analysis

P-value	Hazard ratio	Description	Gene	Probe set	Pass	PCR		
0.0002	1.8	Epidermal growth factor receptor	*EGFR*	201984_s_at	2	PCR	1	0.1
0.0005	0.1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	*DDX54*	219111_s_at			2	0.1
0.0005	0.5	Chimerin (chimaerin) 2	*CHN2*	213385_at			3	0.1
0.0005	6.1	Ubiquitin-like domain containing CTD phosphatase 1	*UBLCP1*	227413_at			4	0.2
0.0006	0.5	PTK2 protein tyrosine kinase 2	*PTK2*	241387_at			5	0.2
0.0008	3.4	Der1-like domain family, member 2	*DERL2*	218333_at			6	0.2
0.0008	0.5	Leucine rich repeat containing 14	*LRRC14*	32062_at			7	0.2
0.0009	4.5	WD repeat domain 33	*WDR33*	222763_s_at		PCR	8	0.2
0.0009	0.1	Rhomboid domain containing 3	*RHBDD3*	217622_at			9	0.2
0.001	0.3	Myosin regulatory light chain interacting protein	*MYLIP*	228098_s_at	3	PCR	10	0.2
0.0013	4.7	Chromosome 14 open reading frame 43	*C14orf43*	225980_at		PCR	11	0.2
0.0013	0.2	BCL6 co-repressor	*BCOR*	223915_at			12	0.2
0.0013	0.5	MAD1 mitotic arrest deficient-like 1 (yeast)	*MAD1L1*	233921_s_at			13	0.2
0.0013	4.9	Chromosome 14 open reading frame 109	*C14orf109*	213246_at			14	0.2
0.0014	4.2	Hypothetical protein LOC124512	*LOC124512*	225808_at			15	0.2
0.0014	5.0	Ring finger protein 167	*RNF167*	212047_s_at			16	0.2
0.0014	0.6	Hypothetical LOC25845	*LOC25845*	225457_s_at			17	0.2
0.0014	4.2	General transcription factor II, i	*GTF2I*	232710_at			18	0.3
0.0014	0.2	Rho guanine nucleotide exchange factor (GEF) 10-like	*ARHGEF10L*	1570511_at			19	0.3
0.0014	0.3	G kinase anchoring protein 1	*GKAP1*	229312_s_at		PCR	20	0.3
0.0015	1.9	Glutathione peroxidase 3 (plasma)	*GPX3*	214091_s_at	2	PCR	21	0.3
0.0016	0.5	Dachshund homolog 1 (*Drosophila*)	*DACH1*	1567101_at	2	PCR	22	0.3
0.0016	0.3	Diacylglycerol kinase, theta 110kDa	*DGKQ*	226605_at			23	0.3
0.0017	0.6	Hepatocellular carcinoma-associated antigen 112	*HCA112*	218345_at			24	0.3
0.0018	3.5	Mediator of RNA polymerase II transcription, subunit 31 homolog	*MED31*	222867_s_at			25	0.3
0.0018	6.9	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	*YWHAE*	210317_s_at		PCR	26	0.3
0.0018	0.1	KH domain containing, RNA binding, signal transduction associated 1	*KHDRBS1*	201488_x_at			27	0.3
0.0019	0.3	Solute carrier family 25 (mitochondrial carrier; Graves disease autoantigen), member 16	*SLC25A16*	210686_x_at			28	0.3
0.0019	4.9	Hypothetical protein LOC51255	*LOC51255*	223064_at			29	0.3
0.002	0.2	Cyclin L2 /// similar to Aurora kinase A-interacting protein	*CCNL2 /// LOC643556*	222999_s_at			30	0.3
0.002	7.4	Lectin, mannose-binding, 1	*LMAN1*	224629_at			31	0.3
0.002	0.2	Erythrocyte membrane protein band 4.1 like 4A	*EPB41L4A*	228259_s_at			32	0.3
0.0022	0.2	KIAA0999 protein	*KIAA0999*	204155_s_at			33	0.3
0.0022	0.5	ELOVL family member 7	*ELOVL7*	227180_at			34	0.3
0.0023	4.0	Churchill domain containing 1	*CHURC1*	233268_s_at			35	0.4
0.0024	4.0	Yippee-like 2 (*Drosophila*)	*YPEL2*	227020_at			36	0.4
0.0024	5.9	Hermansky–Pudlak syndrome 1	*HPS1*	210112_at			37	0.4
0.0025	0.3	Hypothetical protein LOC285831	*LOC285831*	228857_at			38	0.4
0.0026	3.5	CDC37 cell division cycle 37 homolog (*Saccharomyces cerevisiae*)-like 1	*CDC37L1*	219343_at			39	0.4
0.0026	2.1	Ankyrin repeat and SOCS box-containing 9	*ASB9*	205673_s_at			40	0.4
0.0026	0.2	Hypothetical gene supported by AK125149	*LOC401577*	239247_at			41	0.5
0.0026	0.3	TBC1 domain family, member 23	*TBC1D23*	236755_at			42	0.5
0.0026	0.3	MRNA full length insert cDNA clone EUROIMAGE 2362292		235505_s_at			43	0.5
0.0026	0.4	Dehydrogenase/reductase (SDR family) member 8	*DHRS8*	217989_at			44	0.5
0.0026	0.4	Nuclear receptor coactivator 2	*NCOA2*	242369_x_at			45	0.5
0.0026	0.2	MRNA; cDNA DKFZp667E0114 (from clone DKFZp667E0114)		235660_at			46	0.5
0.0027	0.4	Transforming, acidic coiled-coil containing protein 1	*TACC1*	242290_at			47	0.5
0.0027	0.2	POU domain, class 2, transcription factor 1	*POU2F1*	1562280_at			48	0.5
0.0027	2.9	p21(CDKN1A)-activated kinase 6	*PAK6*	1555310_a_at				0.5
0.0027	0.5	Mannosyl (alpha-1,3-)-glycoprotein β-1,4-N-acetylglucosaminyltransferase, isozyme A	*MGAT4A*	226039_at			50	0.5
0.0027	5.1	Zinc finger CCCH-type containing 14	*ZC3H14*	204216_s_at			51	0.5
0.0028	0.5	Acyl-CoA synthetase short-chain family member 2	*ACSS2*	235805_at			52	0.5
0.0028	0.3	Programmed cell death 6	*PDCD6*	222380_s_at		PCR	53	0.6
0.0029	3.8	ERGIC and golgi 2	*ERGIC2*	226422_at			54	0.6
0.0029	0.4	Erythrocyte membrane protein band 4.1 like 5	*EPB41L5*	225855_at			55	0.6
0.003	6.5	Chromosome 14 open reading frame 32	*C14orf32*	212644_s_at			56	0.6

doi: 10.1111/j.1349-7006.2008.00935.x

Table 2. (*Continued*)

P-value	Hazard ratio	Description	Gene	Probe set	Pass	PCR		
0.0031	0.2	Transcribed locus		239437_at			57	1.8
0.0031	0.3	DOT1-like, histone H3 methyltransferase (*S. cerevisiae*)	*DOT1L*	231297_at			58	1.9
0.0031	2.2	Transcription elongation factor A (SII)-like 8	*TCEAL8*	224819_at			59	1.9
0.0031	0.3	Laminin, β 1	*LAMB1*	236437_at			60	2.0
0.0032	2.7	FK506 binding protein 5	*FKBP5*	224840_at			61	2.0
0.0033	0.5	Integrin, α 6	*ITGA6*	244665_at			62	2.1
0.0034	2.7	COMM domain containing 9	*COMMD9*	218072_at			63	2.2
0.0034	0.2	Eukaryotic translation initiation factor 4 γ, 3	*EIF4G3*	201936_s_at			64	2.3
0.0035	0.5	235616_at	*235616_at*	235616_at			65	2.6
0.0036	1.9	Metallothionein 1X	*MT1X*	204326_x_at		PCR	66	2.6
0.0036	2.7	Peroxiredoxin 5	*PRDX5*	1560587_s_at			67	2.7
0.0037	0.3	Core-binding factor, runt domain, α subunit 2; translocated to, 2	*CBFA2T2*	207625_s_at			68	2.7
0.0037	0.4	Transcribed locus, moderately similar to XP_531878.2		230168_at			69	2.7
0.0038	0.3	Zinc finger protein 346	*ZNF346*	236267_at			70	2.8
0.0038	2.0	Metallothionein 1H-like protein /// hypothetical protein LOC650610	*LOC645745 /// LOC650610*	211456_x_at			71	2.9
0.0039	0.2	Hypothetical protein DKFZp586I1420	*DKFZp586I1420*	213546_at			72	3.4
0.0039	2.0	Adrenergic, β-2-, receptor, surface	*ADRB2*	206170_at			73	3.5
0.0039	0.3	CTD-binding SR-like protein rA9	*KIAA1542*	234952_s_at			74	3.5
0.0039	2.6	Peroxiredoxin 5	*PRDX5*	222994_at			75	3.6
0.004	0.2	ATPase, H^+ transporting, lysosomal 42kDa, V1 subunit C1	*ATP6V1C1*	226463_at			76	3.8
0.004	8.0	XK, Kell blood group complex subunit-related family, member 8	*XKR8*	218753_at			77	3.8
0.004	0.3	Caspase 6, apoptosis-related cystein peptidase	*CASP6*	242323_at			78	4.0
0.0041	0.4	Coagulation factor XII (Hageman factor)	*F12*	205774_at			79	4.0
0.0041	0.3	Centaurin, γ 2	*CENTG2*	240758_at			80	4.2
0.0042	0.6	LR8 protein	*LR8*	220532_s_at			81	4.2
0.0042	0.2	WD repeat domain 42A	*WDR42A*	243318_at			82	4.5
0.0042	2.6	Potassium channel tetramerisation domain containing 14	*KCTD14*	219545_at			83	4.7
0.0043	2.8	6-Phosphogluconolactonase	*PGLS*	218388_at			84	4.9
0.0044	3.8	Bruno-like 6, RNA binding protein (*Drosophila*)	*BRUNOL6*	227775_at			85	4.9
0.0044	2.3	Zinc finger protein 415	*ZNF415*	205514_at			86	5.0
0.0045	0.5	HIR histone cell cycle regulation defective homolog A (*S. cerevisiae*)	*HIRA*	240451_at			87	5.1
0.0046	0.5	Cardiolipin synthase 1	*CRLS1*	241741_at			88	5.9
0.0046	0.3	c-mer proto-oncogene tyrosine kinase	*MERTK*	233079_at			89	6.1
0.0047	0.2	Additional sex combs like 2 (*Drosophila*)	*ASXL2*	218659_at			90	6.5
0.0047	3.6	Platelet endothelial aggregation receptor 1	*PEAR1*	228618_at			91	6.9
0.0047	0.3	Core-binding factor, runt domain, α subunit 2; translocated to, 2	*CBFA2T2*	238549_at			92	7.4
0.005	0.6	Lysosomal associated protein transmembrane 4 β	*LAPTM4B*	208029_s_at			93	8.0

Pass, number of overlapped probes; PCR, the genes that were subsequently examined using real-time RT-PCR.

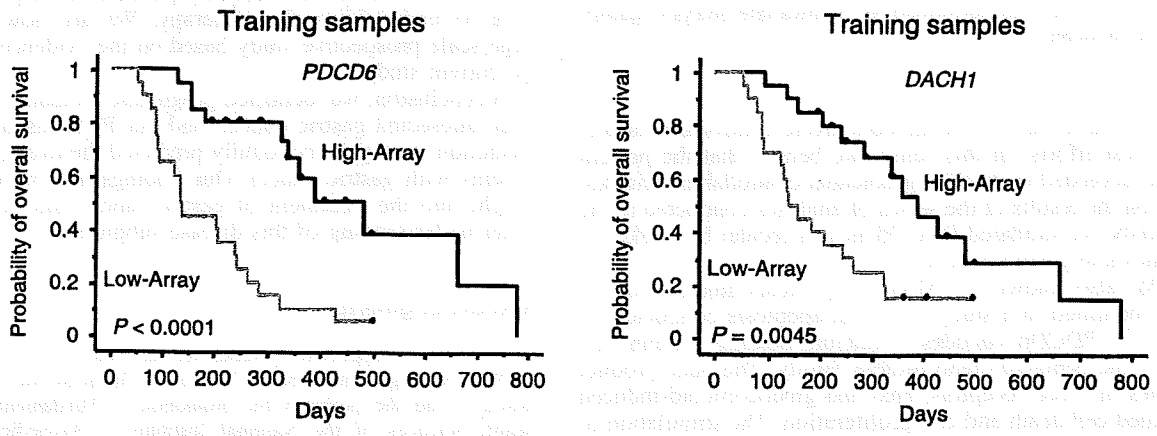


Fig. 2. Results of microarray data and patient survival in the training set of 40 patients. The Kaplan–Meier method was used for *DACH1* and *PDCD6*. The patients were divided into high and low expression groups by median values. The low *PDCD6* and *DACH1* expression groups had significantly poorer outcomes ($P < 0.0001$ and $P = 0.0045$). High-Array, group with high expression levels as determined by signal intensity of microarray data. Low-Array, group with low expression levels as determined by signal intensity of microarray data.