platin with etoposide plus cisplatin in patients with extensive-stage SCLC.[2] This trial was terminated early, because of a highly statistically significant difference in survival between the two arms. The median overall survival was 12.8 months in the irinotecan/cisplatin arm and 9.4 months in the etoposide/cisplatin arm ($p = 0.002$). In Japan, the combination of irinotecan and cisplatin is recognized as a standard treatment for extensive-stage SCLC.

Amrubicin, a novel 9-aminoanthracycline, inhibits topoisomerase II[3] and also shows strong antitumor effect for SCLC. The West Japan Oncology Group, formerly named the West Japan Thoracic Oncology Group (WJTOG), conducted a phase II study of amrubicin in previously untreated patients with extensive-stage SCLC.[4] In 35 patients treated, a response rate of 76% and a median overall survival of 11.7 months were shown. These figures compare favorably with standard doublet chemotherapy.

Some preclinical studies reported that a combination of topoisomerase I and II inhibitors shows a synergistic cytotoxicity.[5] For SCLC, a combination of this type, irinotecan and etoposide (a topoisomerase II inhibitor), was investigated clinically and showed promising results.[6,7] The similar combination of irinotecan and amrubicin is worthwhile to investigate.

Concurrent administration of a triplet combination requires dose reduction of each drug because of toxicities, especially myelosuppression. A sequential chemotherapy, i.e., a doublet followed by the other drug, can be used to avoid the need for dose reduction. In addition, Norton and Simon[8] presented a theoretical model describing the possibility of a sequential chemotherapy.

Therefore, we investigated a sequential triplet chemotherapy consisting of irinotecan and cisplatin followed by amrubicin in patients with extensive-stage SCLC (WJTOG 0301). The purpose of this study was to evaluate the efficacy and safety of this treatment.

PATIENTS AND METHODS

Patient Selection

Eligible patients were aged 20 to 70 years, had histologically or cytologically proven SCLC, extensive-stage disease, Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, no prior chemotherapy, neither palliative radiation nor surgery of 14 days, measurable lesions, life expectancy of at least 2 months, and adequate organ functions (white blood cell [WBC] ≥4000/μL, neutrophil ≥2000/μL, platelet ≥100,000/μL, hemoglobin ≥10 g/dL, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] ≤2 × upper limit of normal [ULN], total bilirubin ≤1.5 × ULN, creatinine ≤ULN, arterial partial pressure of oxygen ≥60 mm Hg, no abnormality requiring treatment on electrocardiogram, and left ventricular ejection fraction on echocardiogram ≥60%). Patients with any of the following conditions were excluded: symptomatic brain metastases, pleural or pericardial effusion requiring drainage, interstitial pneumonitis, active infection, watery diarrhea or ileus, active gastroduodenal ulcer, continuous administration of steroid or nonsteroidal anti-inflammatory drug, uncontrolled diabetes mellitus or angina pectoris, other active malignancy, and pregnancy or lactation.

All patients gave written informed consent. This study was approved by the institutional review boards at each participating institution.

Treatment Schedule

Chemotherapy consisted of irinotecan 60 mg/m^2 on days 1 and 8 plus cisplatin 60 mg/m^2 on day 1 every 3 weeks for 3 cycles and then amrubicin 40 mg/m^2 alone on days 1 to 3 every 3 weeks for three cycles. Irinotecan was administered as a 90-minute intravenous infusion, cisplatin as a 90-minute intravenous infusion with adequate hydration, and amrubicin as a 5-minute intravenous injection. Prophylactic administration of granulocyte colony-stimulating factor (G-CSF) was allowed at the discretion of the treating physician.

The minimum requirements for the administration of irinotecan and cisplatin were as follows: WBC ≥3000/μL, neutrophil ≥1500/μL, platelet ≥100,000/μL, AST and ALT ≤2.5 × ULN, total bilirubin ≤1.5 × ULN, creatinine ≤ULN, PS of 0 to 2, body temperature ≤37.5°C, no diarrhea, no interstitial pneumonitis, and other nonhematological toxicity ≤grade 2. The minimum requirements for administration of day-8 irinotecan were as follows: WBC ≥3000/μL, platelet ≥100,000/μL, body temperature ≤37.5°C, no diarrhea, no interstitial pneumonitis, and other nonhematological toxicity ≤grade 2. The minimum requirements for administration of amrubicin were as follows: WBC ≥3000/μL, neutrophil ≥1500/μL, platelet ≥100,000/μL, AST and ALT ≤2.5 × ULN, total bilirubin ≤1.5 × ULN, creatinine ≤1.5 × ULN, PS of 0 to 2, body temperature ≤37.5°C, no interstitial pneumonitis, and other nonhematological toxicity ≤grade 2.

If any of the following toxicities was observed, the doses of irinotecan, cisplatin, and amrubicin were reduced to 50, 50, and 35 mg/m^2, respectively: WBC <1000/μL, febrile neutropenia (neutrophil <1000 /μL), platelet <25,000 /μL, or grade 3 nonhematologic toxicity. If creatinine >ULN, the dose of cisplatin was reduced to 50 mg/m^2. If creatinine >2.0 mg/dL, the administration of cisplatin was discontinued. If grade 4 nonhematological toxicity or pneumonitis ≥grade 2 was observed, the study treatment was stopped.

Response and Toxicity Evaluation

Before treatment, a complete medical history was obtained, and physical examination was performed. The following examinations were carried out: complete blood count (CBC) with differential WBC count, blood chemistry, arterial blood gas analysis, urinalysis, electrocardiography, and echocardiography. Staging procedures consisted of chest radiograph, computed tomography (CT) of chest and upper abdomen, magnetic resonance imaging (MRI) or CT of brain, bone scintigraphy, and bone marrow aspiration. During treatment, CBC with differential WBC count, blood chemistry, and chest radiograph were examined at least once a week, and electrocardiography and CT and/or MRI for response evaluation were examined once a month. After treatment, chest radiograph was performed once a month, and CT and/or MRI were performed every 3 months.

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Response was evaluated according to the Response Evaluation Criteria in Solid Tumors.[9] Extramural review of eligibility and response of all patients were performed. Toxicity was evaluated in accordance with the Common Terminology Criteria for Adverse Events, Version 3.0.[10]

Statistical Analysis

The primary end point of this study was response rate. Secondary end points were progression-free survival (PFS), overall survival, and toxicity. Survival curves were drawn using the Kaplan-Meier method.[11]

Assuming that a response rate of 90% would indicate potential usefulness, whereas a rate of 75% would be the lower limit of interest, with $\alpha = 0.05$ (one side) and $\beta = 0.20$, 38 patients were required. Allowing for a 15% loss to follow-up, enrollment of a total of 45 patients was planned.

RESULTS

Patient Characteristics

From September 2004 to September 2006, 45 patients were enrolled in this study. Two patients had limited-stage disease. One patient, who was able to receive thoracic radiation, was excluded from all analyses. The other patient, who was not able to receive thoracic radiation due to pleural dissemination, was included in analysis of toxicity and excluded from analysis of response and survival. Therefore, 43 patients were evaluable for response and survival, and 44 were evaluable for toxicity.

Patient characteristics are shown in Table 1. The median age was 63 years, 37 patients (84%) were men, and 31 patients (70%) had PS of 1. Thirty-nine patients (89%) had distant metastases. Frequent sites of distant metastases were brain, liver, bone, and adrenal gland. Of five patients without distant metastases, four had contralateral hilar lymph node involvement and one had pleural dissemination. No patient received prior treatment, including surgery and radiation.

TABLE 1. Patient Characteristics (n = 44)

Characteristic	n (%)
Sex	
Male	37 (84)
Female	7 (16)
Age (yr)	
Median (range)	63 (47–70)
ECOG performance status	
0	13 (30)
1	31 (70)
Distant metastases	
Present	39 (89)
Absent	5 (11)
Sites of distant metastasis	
Brain	10 (23)
Liver	10 (23)
Bone	10 (23)
Adrenal gland	10 (23)
Lymph node	7 (16)
Lung	6 (14)
Bone marrow	3 (7)
Other	3 (7)
Prior therapy	
None	44 (100)

ECOG, Eastern Cooperative Oncology Group.

TABLE 2. Treatment Delivery (n = 44)

Treatment Cycle	n (%)
Irinotecan/cisplatin	
Cycle 1	44 (100)
Cycle 2	40 (91)
Cycle 3	37 (84)
Amrubicin	
Cycle 1	33 (75)
Cycle 2	30 (68)
Cycle 3	28 (64)

TABLE 3. Tumor Response (n = 43)

	n (%)
Complete response	1 (2)
Partial response	33 (77)
Stable disease	1 (2)
Progressive disease	3 (7)
Not evaluable	5 (12)
Overall response	34 (79) (95% CI, 64–90)

CI, confidence interval.

Treatment Delivery

Of 44 patients, 37 patients (84%) received three cycles irinotecan/cisplatin and 28 patients (64%) completed the full planned chemotherapy, i.e., three cycles irinotecan/cisplatin followed by three cycles amrubicin (Table 2). Dose reduction of irinotecan/cisplatin and amrubicin was necessary in six and seven patients, respectively.

Response and Survival

Of 43 patients, 1 achieved complete response and 33 had partial response, for an overall response rate of 79% (95% confidence interval, 64–90%) (Table 3). Of the 33 partial responders, tumor shrinkage met partial response criteria during an irinotecan/cisplatin cycle in 30 patients and during an amrubicin cycle in 3. In the complete responder, tumor disappearance was achieved during an irinotecan/cisplatin cycle.

The survival curves are shown in Figure 1. The median PFS was 6.5 months (95% confidence interval, 4.9–7.4 months), with a 1-year survival rate of 8%. The median overall survival was 15.4 months (95% confidence interval, 11.7–18.0 months), with a 1-year survival rate of 61%.

Chemotherapy After Progression (Second-Line Treatment)

Thirty-five patients received chemotherapy after progression as follows: etoposide plus carboplatin in 10 patients;

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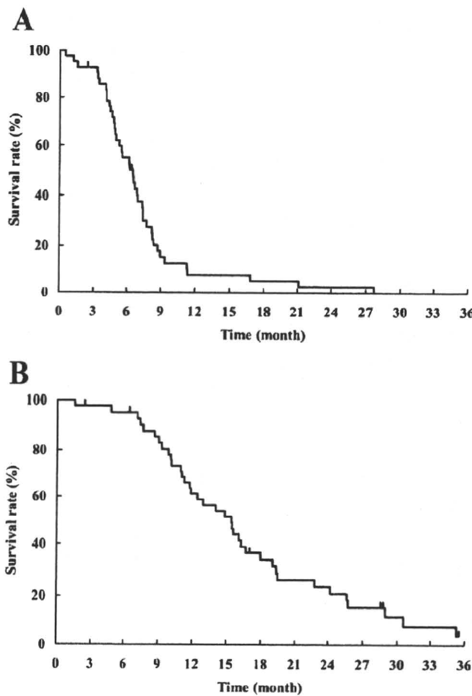

FIGURE 1. Survival curves (n = 43). *A*, Progression-free survival, median 6.5 months (95% confidence interval, 4.9–7.4 months), with a 1-year survival rate of 8%. *B*, Overall survival, median 15.4 months (95% confidence interval, 11.7–18.0 months), with a 1-year survival rate of 61%.

irinotecan plus cisplatin in 6; amrubicin in 5; topotecan plus carboplatin in 4; irinotecan plus amrubicin in 2; irinotecan in 2; and irinotecan plus etoposide, irinotecan plus carboplatin, etoposide plus cisplatin, etoposide, topotecan, and cyclophosphamide plus doxorubicin plus vincristine in 1 patient each.

Toxicity

Toxicities during irinotecan/cisplatin cycles are listed in Table 4. Of 44 patients, grade 3 or 4 leukopenia, neutropenia, anemia, thrombocytopenia, and febrile neutropenia occurred in 6 (14%), 25 (57%), 3 (7%), 0 (0%), and 3 patients (7%), respectively. G-CSF was administered in 12 patients (27%). One patient received transfusion of red blood cell concentrates. One patient (2%) developed grade 3 diarrhea. Grade 3 anorexia was observed in seven patients (16%).

Toxicities during amrubicin cycles are listed in Table 5. Of 33 patients, grade 3 or 4 leukopenia, neutropenia, anemia, thrombocytopenia, and febrile neutropenia occurred in 15 (45%), 30 (91%), 9 (27%), 3 (9%), and 5 patients (15%), respectively. G-CSF was administered in 20 patients (61%). One patient received transfusion of red blood cell concentrates and platelet concentrates, and two other patients received transfusion of red blood cell concentrates. Nonhematological toxicity was not common. One patient (3%) developed grade 3 pneumonitis. This patient was treated with steroid pulse therapy and recovered soon thereafter. No treatment-related death was observed.

TABLE 4. Toxicities During the Irinotecan/Cisplatin Cycle (*n* = 44)

	Grade					
	0	1	2	3	4	≥3
WBC	11	15	12	4	2	6 (14%)
Neutrophil	9	1	9	20	5	25 (57%)
Hemoglobin	3	23	15	3	0	3 (7%)
Platelet	24	19	1	0	0	0 (0%)
Febrile neutropenia	41	0	0	3	0	3 (7%)
AST/ALT	24	15	3	2	0	2 (5%)
Creatinine	35	7	2	0	0	0 (0%)
Nausea	14	14	12	4	0	4 (9%)
Vomiting	24	11	7	2	0	2 (5%)
Anorexia	11	19	7	7	0	7 (16%)
Fatigue	13	21	8	2	0	2 (5%)
Diarrhea	28	10	5	1	0	1 (2%)
Pneumonitis	44	0	0	0	0	0 (0%)
Infection	39	0	3	2	0	2 (5%)
Rash	37	6	0	1	0	1 (2%)

WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

TABLE 5. Toxicities During the Amrubicin Cycle (*n* = 33)

	Grade					
	0	1	2	3	4	≥3
WBC	0	3	15	12	3	15 (45%)
Neutrophil	1	0	2	18	12	30 (91%)
Hemoglobin	0	5	19	5	4	9 (27%)
Platelet	13	13	4	0	3	3 (9%)
Febrile neutropenia	28	0	0	5	0	5 (15%)
AST/ALT	25	8	0	0	0	0 (0%)
Creatinine	30	3	0	0	0	0 (0%)
Nausea	18	12	3	0	0	0 (0%)
Vomiting	31	2	0	0	0	0 (0%)
Anorexia	17	12	3	1	0	1 (3%)
Fatigue	10	18	4	1	0	1 (3%)
Diarrhea	31	1	1	0	0	0 (0%)
Pneumonitis	31	1	0	1	0	1 (3%)
Infection	29	0	2	2	0	2 (6%)
Rash	30	2	1	0	0	0 (0%)

WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

DISCUSSION

We performed a phase II study of sequential triplet chemotherapy consisting of irinotecan and cisplatin followed by amrubicin in patients with extensive-stage SCLC and demonstrated a response rate, median PFS, and median overall survival of 79%, 6.5 months, and 15.4 months, respectively. The primary end point of this study was response rate, and the expected and the threshold rates were set 90% and 75%, respectively. The actual response rate in this study (79%) was lower than the expected rate but higher than the threshold. JCOG 9511 reported a response rate, median PFS, and median overall survival of

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irinotecan/cisplatin arm of 84%, 6.9 months, and 12.8 months, respectively.[2] Comparing this study with JCOG 9511, the response rate and PFS were similar, whereas overall survival was longer in this study. Taking the longer overall survival into consideration, the results of this study were regarded as promising. There is a possibility that the exclusion of PS 2 patients in this study, which were included in JCOG 9511, could have resulted in the longer overall survival. In addition, we could not find any specific trend that would show prolonged overall survival among second-line treatments.

Two randomized trials that compared irinotecan/cisplatin with etoposide/cisplatin were conducted mainly in North America as confirmation studies of JCOG 9511. One was reported by Hanna et al.[12] and the other was conducted by the Southwest Oncology Group (S0124).[13] Although JCOG 9511 showed survival advantage in the irinotecan/cisplatin arm over the etoposide/cisplatin arm, these North American trials did not show significant difference between the two arms. Irinotecan/cisplatin is a standard chemotherapy for SCLC in Japan, whereas etoposide/cisplatin remains standard in North America. It was reported that the response rate, median PFS, and median overall survival of irinotecan/cisplatin arm were 48%, 4.1 months, and 9.3 months in the trial by Hanna et al. and 60%, 5.7 months, and 9.9 months in S0124, respectively. This study showed better survival than the North American trials. However, great caution is needed when comparing this study with the North American trials. S0124 reported the possibility that inherent genetic differences might exist between the study populations, resulting in divergent outcomes with the same cytotoxic agents.[13] A similar suggestion was made for non-small cell lung cancer.[14] Population-related pharmacogenomics is important because the varied results for the same treatment could be attributed to ethnic differences.

Clinical studies of amrubicin for SCLC had been performed, in both first-line and second-line treatment, entirely in Japan.[15] The WJTOG study in first-line treatment reported a response rate of 76% and median overall survival of 11.7 months.[4] These figures compare favorably with standard doublet chemotherapy. Onoda et al.[16] conducted a phase II study of amrubicin in second-line treatment. They treated 16 patients with refractory disease and 44 patients with sensitive relapsed disease and demonstrated a response rate and median overall survival of 50% and 10.3 months in the refractory group and 52% and 11.6 months in the sensitive group, respectively. Furthermore, the North Japan Lung Cancer Study Group conducted a randomized phase II trial of amrubicin in comparison with topotecan in second-line treatment.[17] That trial showed a response rate and median PFS of 38% and 3.5 months for the amrubicin arm and 13% and 2.2 months for the topotecan arm, respectively. Multivariate analysis revealed that amrubicin has more influence than topotecan on overall survival. Amrubicin is one of the most promising new drugs for the treatment of SCLC.

The ECOG reported a phase III trial of topotecan versus observations after cisplatin and etoposide in extensive-stage SCLC.[18] They showed that four cycles of cisplatin/etoposide induction therapy followed by four cycles of topotecan improved PFS but failed to improve overall survival or quality of life in extensive-stage SCLC. Results of the North Japan Lung Cancer Study Group trial suggested that amrubicin is more effective than topotecan for SCLC. The ECOG trial failed to show survival benefit; however, it did show that amrubicin, instead of topotecan, has potential to lead to better survival in extensive-stage SCLC.

Bozcuk et al.[19] reported a meta-analysis of maintenance/consolidation chemotherapy in the management of SCLC. They analyzed 14 randomized trials, encompassing 2550 patients, and concluded that maintenance/consolidation chemotherapy improves survival in SCLC. Sequential amrubicin was stopped for three cycles in this study. If further cycles of amrubicin as maintenance treatment are given, PFS might be further prolonged.

The major toxicity of sequential amrubicin was myelosuppression, whereas nonhematological toxicity was not common. In the above-mentioned WJTOG study, amrubicin was administered at 45 mg/m^2 on days 1 to 3 as monotherapy.[4] To avoid severe myelosuppression in this study, amrubicin was decreased to 40 mg/m^2 on days 1 to 3 as sequential chemotherapy. This study confirmed that this dose of sequential amrubicin was feasible.

Kaneda et al.[20] reported a phase I study of irinotecan and amrubicin. They administered irinotecan on days 1 and 8 and amrubicin on days 1 to 3. They concluded that this combination was not tolerated because of severe myelosuppression. Although concurrent combination of irinotecan and amrubicin is not tolerable, this study showed that sequential combination of these drugs is tolerable. Irinotecan and amrubicin were administered without G-CSF support in both this study and the study by Kaneda et al.

In conclusion, the sequential triplet chemotherapy of irinotecan and cisplatin followed by amrubicin is an effective and well-tolerated treatment in patients with extensive-stage SCLC. Further investigation of this treatment is warranted.

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REFERENCES

1. Sher T, Dy GK, Adjei AA. Small cell lung cancer. *Mayo Clin Proc* 2008;83:355–367.
2. Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002;346:85–91.
3. Hanada M, Mizuno S, Fukushima A, et al. A new antitumor agent amrubicin induces cell growth inhibition by stabilizing topoisomerase II-DNA complex. *Jpn J Cancer Res* 1998;89:1229–1238.
4. Yana T, Negoro S, Takada M, et al. Phase II study of amrubicin in previously untreated patients with extensive-disease small cell lung cancer: West Japan Thoracic Oncology Group (WJTOG) study. *Invest New Drugs* 2007;25:253–258.
5. Vasey PA, Kaye SB. Combined inhibition of topoisomerases I and II—is this a worthwhile/feasible strategy? *Br J Cancer* 1997;76:1395–1397.
6. Masuda N, Matsui K, Negoro S, et al. Combination of irinotecan and etoposide for treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1998;16:3329–3334.
7. Kudoh S, Nakamura S, Nakano T, et al. Irinotecan and etoposide for previously untreated extensive-disease small cell lung cancer: a phase II

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trial of West Japan Thoracic Oncology Group. *Lung Cancer* 2005;49: 263–269.
8. Norton L, Simon R. The Norton-Simon hypothesis revisited. *Cancer Treat Rep* 1986;70:163–169.
9. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–216.
10. Common Terminology Criteria for Adverse Events, Version 3.0. August 9, 2006. Available at: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf. Accessed October 5, 2009.
11. Kaplan EL, Meier P. Nonparametric estimation for incomplete observations. *J Am Stat Assoc* 1958;53:457–481.
12. Hanna N, Bunn PA Jr, Langer C, et al. Randomized phase III trial comparing irinotecan/cisplatin with etoposide/cisplatin in patients with previously untreated extensive-stage disease small-cell lung cancer. *J Clin Oncol* 2006;24:2038–2043.
13. Lara PN Jr, Natale R, Crowley J, et al. Phase III trial of irinotecan/cisplatin compared with etoposide/cisplatin in extensive-stage small-cell lung cancer: clinical and pharmacogenomic results from SWOG S0124. *J Clin Oncol* 2009;27:2530–2535.
14. Gandara DR, Kawaguchi T, Crowley J, et al. Japanese-US common-arm analysis of paclitaxel plus carboplatin in advanced non-small-cell lung cancer: a model for assessing population-related pharmacogenomics. *J Clin Oncol* 2009;27:3540–3546.
15. Ettinger DS. Amrubicin for the treatment of small cell lung cancer: does effectiveness cross the Pacific? *J Thorac Oncol* 2007;2:160–165.
16. Onoda S, Masuda N, Seto T, et al. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. *J Clin Oncol* 2006;24:5448–5453.
17. Inoue A, Sugawara S, Yamazaki K, et al. Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. *J Clin Oncol* 2008;26:5401–5406.
18. Schiller JH, Adak S, Cella D, et al. Topotecan versus observation after cisplatin plus etoposide in extensive-stage small-cell lung cancer: E7593—a phase III trial of the Eastern Cooperative Oncology Group. *J Clin Oncol* 2001;19:2114–2122.
19. Bozcuk H, Artac M, Ozdogan M, et al. Does maintenance/consolidation chemotherapy have a role in the management of small cell lung cancer? A metaanalysis of the published controlled trials. *Cancer* 2005;104: 2650–2657.
20. Kaneda H, Kurata T, Tamura K, et al. A phase I study of irinotecan in combination with amrubicin for advanced lung cancer patients. *Anticancer Res* 2006;26:2479–2485.

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ORIGINAL PAPER

Prognosis of small-cell lung cancer since the introduction of amrubicin

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Abstract Several studies have demonstrated the effectiveness of amrubicin (AMR) in small-cell lung cancer (SCLC). This study aimed to assess the change in the prognosis of SCLC before and after the commercial availability of AMR. We retrospectively analyzed data from 243 patients with newly diagnosed SCLC. Patients diagnosed before the start of the sale of AMR (January 1997–May 2002) constituted Group A, and patients diagnosed after its introduction (December 2002–December 2006), constituted Group B. The overall survival and demographic factors of the 2 groups were compared. Similar comparisons were also performed on subsets. Median survival time (MST) was 313 days for Group A and 388 days for Group B ($P = 0.031$). Group B with limited disease (LD) demonstrated a significantly longer median survival time (321 vs. 506 days; $P = 0.022$) than Group A, whereas no significant difference was noted between the groups of patients with extensive disease (ED) (296 vs. 280 days; $P = 0.895$). In the subset of refractory relapse of LD, the MST was clearly longer in Group B than in Group A (220 vs. 321 days; $P < 0.001$). Multivariate analysis for LD patients indicated that performance status (hazard ratio 2.072; $P = 0.003$) and commercial availability of AMR (0.596; $P = 0.022$) are significant factors. The present study has demonstrated prolonged survival times for LD patients since the start of the sale of AMR. The use of AMR in ED patients requires further investigations.

Keywords Amrubicin · Limited disease · Prognosis · Refractory relapse · Retrospective study · Small-cell lung cancer

Introduction

Lung cancer ranks high among the causes of cancer death in developed countries. Small-cell lung cancer (SCLC) accounts for approximately 13% of all lung cancers [1], and 5-year survival rates of SCLC remain low. The first-line therapy is cisplatin (CDDP) + etoposide (ETP) + concurrent radiotherapy in the case of the limited disease (LD) type of SCLC [2]; CDDP + ETP or CDDP + irinotecan (CPT) in the case of the extensive disease (ED) type of SCLC [3]. This disease recurs in the vast majority of patients.

Many agents have been tried in second-line treatments, although results have shown limited effectiveness. At present, topotecan (TOP) is widely used as a second-line chemotherapy in European and North American countries. However, TOP as second-line chemotherapy is not satisfactory, as it resulted in a response rate of 7% and an MST of 25.9 weeks [4]. The response rate was 26% in a Japanese phase II study in which TOP was used for patients with recurrent SCLC [5].

As a new agent for SCLC, amrubicin (AMR) became commercially available in Japan in December 2002, earlier than in the rest of the world. AMR is an anthracycline derivative and seems to exert its anti-tumor effect primarily by acting on DNA topoisomerase II to stabilize a cleavable complex [6].

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The effectiveness of AMR in SCLC has been established. Onoda et al. conducted a phase II study of single-agent AMR as second-line therapy and reported that the response rate was 50% for patients with refractory relapse and 52% for those with sensitive relapse [7]. In addition, Inoue et al. performed a randomized phase II study to compare TOP with AMR as second-line therapy, and reported that in 60 patients included in the study, the response rate was 38 versus 13%, and the median progression-free survival (PFS) was 3.5 versus 2.2 months [8].

In Japan, at present, AMR is widely used in practice, and several years have passed since the start of its sale. It is expected that the commercial availability and increasing popularity of this promising agent as a second-line therapy will contribute to the improvement of overall survival in patients with SCLC, although this has not yet been confirmed yet. Under these circumstances, we performed a retrospective analysis to compare survival between before and after AMR sale and to identify factors that influence AMR-related changes in overall survival in patients with SCLC.

Patients and methods

Patient selection

Two hundred and eighty-three patients who were newly diagnosed with SCLC at our hospital between January 1997 and December 2006 and who received a first-line therapy were retrospectively analyzed on the basis of their medical records. Patients diagnosed and treated between January 1997 and May 2002 were assigned to the pre-AMR sale group (Group A), and those who were treated and diagnosed between December 2002 and the end of December 2006 formed the post-AMR sale group (Group B). Patients with PS 4 were excluded from the analysis, as were those diagnosed and treated within 6 months prior to the start of the AMR sale (i.e., between June and November 2002), since these patients were considered to fall into the category of patients in a transient period. Patients who underwent radical surgery were also excluded.

LD was defined as a tumor confined to one-hemithorax, including bilateral mediastinal lymphnodes, and supraclavicular lymph nodes. Any involvement beyond that mentioned above was defined as ED. Information on survival was, as a rule, calculated from the date of initiation of treatment with an anti-cancer agent. When the starting date of treatment was unknown, survival time was calculated from the date of diagnosis. The survival time represented the number of days from either starting point to death. Those patients who were lost due to change of hospital or those who survived until the end of the follow-up period were regarded as censored cases. The maximum follow-up period was set at 3 years.

Therapy and relapse type

A 3-day consecutive administration of AMR represented 1 cycle; these cycles were repeated every 3 weeks from the starting date. Those patients for whom information available for efficacy evaluation was insufficient or those for whom treatment was withdrawn because of adverse reactions were regarded as "not evaluable" cases. As a rule, the anti-tumor effect was based on the Response Evaluation Criteria In Solid Tumors (RECIST). For relapse typing, refractory relapse was defined as failure to achieve partial remission (PR) or greater response to first-line therapy, or relapse within 90 days after the last administration of anti-cancer therapy. Sensitive relapse was defined as relapse $\geq$90 days after first-line therapy and the last administration of anti-cancer therapy. For chest irradiation therapy, implementation of radical irradiation was defined as "irradiation performed", regardless of whether it was carried out consecutively or simultaneously. In addition, we investigated whether prophylactic cranial irradiation (PCI) was performed. When any change was made to a first-line treatment, including treatment discontinuation because of an adverse reaction, the post-change regimen was defined as second-line regimen. For the category of sensitive relapse, repeated administration of the same regimen was also defined as second-line therapy.

Statistical analysis

Statistical analysis was performed using software R (version 2.10.0) [9]. Kaplan–Meier survival analysis was performed, and log-rank tests were applied. Multivariate analysis using the Cox proportional hazard model was performed on the survival time data, for which gender, age, PS, as well as pre- and post-AMR sale were used as variables. Demographic factors were statistically analyzed using the chi-square test.

Results

On screening medical records, it was revealed that 283 patients started to receive chemotherapy at our hospital during the specified period. Of these, 243 patients, consisting of 134 in the pre-AMR sale group i.e., those diagnosed and treated between January 1997 and June 2002 (Group A), and 109 in the post-sale group i.e., those diagnosed between December 2002 and December 2006 (Group B), were included in the analysis. Eleven patients with PS 4, 21 patients who were diagnosed and treated in

the transient period, and 8 patients who underwent radical surgery were excluded from the analysis. Table 1 shows the demographic factors. No significant differences were noted between the 2 groups in age, gender, disease stage, or PS. For LD patients (Table 2), the 2 groups did not significantly differ in the proportion of patients who presented with sensitive relapse, those who experienced refractory relapse, or those who underwent radical radiotherapy or PCI, whereas a significant difference was found in the rate of implementation of second-line therapy. For ED patients, the number of women was significantly smaller in Group B (Table 3).

Figure 1 shows the overall survival curves. As for overall survival, the MST was 313 days (95% confidence interval (CI): 257–348 days) for Group A and 388 days (95% CI: 324–486 days) for Group B, showing a significant prolongation in Group B ($P = 0.031$). Evaluation by disease stage revealed that for the survival curves of LD patients (Fig. 2a), the MST was 321 days (95% CI: 248–456 days) for Group A and 506 days (95% CI: 472–1087 days) for Group B, indicating a significant improvement in Group B ($P = 0.022$). The survival curves of ED patients (Fig. 2b) indicated that the MST was 296 days (95% CI: 241–348 days) for Group A and 280 days (95% CI: 262–367 days) for Group B, showing no significant difference ($P = 0.895$).

Comparison of the 2 groups by relapse type for LD patients revealed that for the survival curves of patients with refractory relapse (Fig. 3a), the MST was 220 days (95% CI: 184–276 days) for Group A and 321 days (95% CI: 282–503) for Group B, showing a significant improvement in Group B ($P < 0.001$). The survival curves of those patients with sensitive relapse (Fig. 3b) indicated an MST of 528 days (95% CI: 434–INF) for Group A and of 894 days (95% CI: 498—INF) for Group B ($P = 0.325$).

Table 1 Baseline characteristics of all patients

	Group A	Group B	*P*-value
No. of patients	134	109	
Median age	69	68	0.600
(Range)	(42–87)	(44–81)	
Gender			
Male	113	98	0.278
Female	21	11	
PS			
0,1	84	75	0.834
2,3	42	34	
Stage			
LD	64	58	0.474
ED	70	51	

Group A, January, 1997–May, 2002; Group B, December, 2002–December, 2006; *N* number, *PS* performance status, *LD* limited disease, *ED* extensive disease

Table 2 Therapy-related background of limited disease (LD) patients

	Group A	Group B	*P*-value
N of patients	64	58	
Median age	69	69	0.600
(Range)	(42–87)	(44–81)	
Gender			
Male	53	49	0.803
Female	11	9	
PS			
0,1	51	41	0.249
2,3	13	17	
PCI	8	15	0.184
Thoracic RT	27	31	0.561
Response rate	78.1%	89.7%	0.705
Relapse type			
Sensitive	22	27	0.849
Refractory	32	25	0.765
Unknown	3	0	
No relapse	7	6	
Second-line Cx	21	44	0.013
CPT	12	28	0.001
AMR	2	28	<0.001

Group A January, 1997–May, 2002, *Group B* December, 2002–December, 2006, *N* number, *PS* performance status, *PCI* prophylactic cranial irradiation, *RT* radiation therapy, *Cx* chemotherapy, *CPT* irinotecan, *AMR* amrubicin

Multivariate analysis

For LD patients, multivariate analysis using the Cox proportional hazard model was performed (Table 4), using the following variables: age (above and below 70 years), PS, gender, AMR sale, and whether PCI was implemented. The resulting hazard ratio was 2.072 (95% CI: 1.278–3.357) for PS ($P = 0.003$) and 0.596 (95% CI: 0.384–0.929) for AMR sale ($P = 0.0220$), indicating that these 2 variables are significant factors. Age, gender, and PCI were not significant factors for overall survival.

Discussion

Our study demonstrated an improvement in the overall survival time of patients with SCLC after the commercial availability of AMR. The analysis by disease stage revealed that this effect of prolonging the survival time was observed for LD patients, whereas the survival of ED patients was not prolonged. In particular, it is noteworthy

Table 3 Therapy-related background of extensive disease (ED) patients

	Group A	Group B	*P*-value
N of patients	70	51	
Median age	69	67	0.913
(Range)	(42–83)	(48–81)	
Gender			
Male	60	49	<0.001
Female	10	2	
PS			
0,1	41	34	0.365
2,3	29	17	
Response rate	77.1%	74.5%	0.987
Relapse type			
Sensitive	16	11	0.935
Refractory	50	39	0.912
Unknown	4	0	
No relapse	0	1	
Second-line Cx	25	41	<0.001
CPT	18	32	<0.001
AMR	6	29	<0.001

Group A January, 1997–May, 2002, *Group B* December, 2002–December, 2006, *N* number, *PS* performance status, *Cx* chemotherapy, *CPT* irinotecan, *AMR* amrubicin

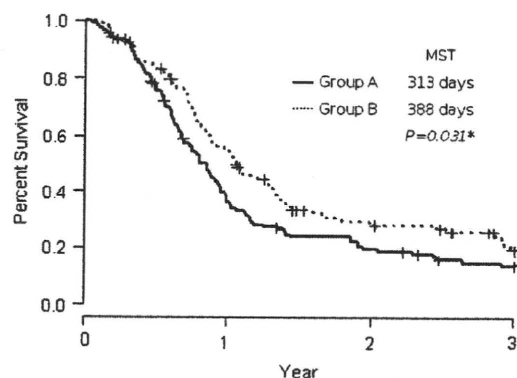

Fig. 1 Overall survival curves. Group A (*solid line*) represents the patients before the commercial availability of AMR and Group B (*broken line*) after the marketing of AMR. *AMR* amrubicin hydrochloride, *MST* median survival time

that the survival time was longer in patients with refractory relapse, who have been highly resistant to therapy. This finding achieved with AMR might have substantially contributed to the improvement in overall survival.

Patients with sensitive relapse typically respond well to chemotherapy, and second-line therapy achieves high response rates. For example, even TOP resulted in a response rate of 24.3% when administered as second-line therapy to patients with sensitive relapse [10]. As such, these patients seem to easily benefit from anti-cancer agents. In our present study, if the prolonged survival time in patients with sensitive relapse had been related to the prolongation of overall survival of recurrent SCLC, it might have merely resulted from continuing administration of anti-cancer therapy. In the present study, however, we could not make a decisive judgment on how effective AMR is in LD patients with sensitive relapse. On the other hand, we found that the survival time of LD patients with refractory relapse was prolonged, which then contributed to prolongation of the overall survival of patients with recurrent SCLC, and this finding seems to be highly important. In other words, for LD patients with sensitive relapse, any type of selected drug contributes to prolongation of the survival time in its own way. For LD patients with refractory relapse, AMR is expected to be effective as single-agent second-line therapy.

A limitation of the present study is that there might be other possible reasons for the prolonged prognosis, e.g., a possible influence of PCI, positive implementation of a second-line therapy in which CPT was used as a key agent, and other unknown reasons. For the LD patients with sensitive relapse included in our analysis, no conclusive decision can be made at present because the follow-up period was too short, and the number of patients was too few. For PCI, a meta-analysis of LD patients who attained complete response (CR) and underwent PCI revealed improvement of the 3-year survival rate by 5.4% [11]. Even ED patients who underwent PCI presented significantly longer survival times [12]. Among the subsets of LD patients in our present study, the number of patients who underwent PCI tended to be greater in Group B, and the contribution of PCI to the prolonged survival time was therefore assumed. However, this finding is inconclusive. The effects of stage migration by PET/CT and progress of the palliative care are thought as other factors. Furthermore, the reason why the median survival time for Group A was less than other reports might be related to low response rate, the progression of radiation technique and the aggressive re-treatment. Database in the US has revealed that the percentage of patients with SCLC has gradually decreased and that the overall survival time of patients with SCLC has been longer in recent years, although only slightly [1]. For example, the 5-year survival rate for LD patients was 4.9% in 1973, compared to 10% in 1998. This increase may be explained by the reduced smoking rates and the increased proportion of women among LD patients. In the present study, no significant differences were noted between Groups A and B in the ratio of men to women or the proportion of LD patients, although other possible causes besides administration of AMR may exist.

For the ED patients included in our analysis, in spite of the fact that more than half of them were treated with AMR, after its commercial availability, prolongation of

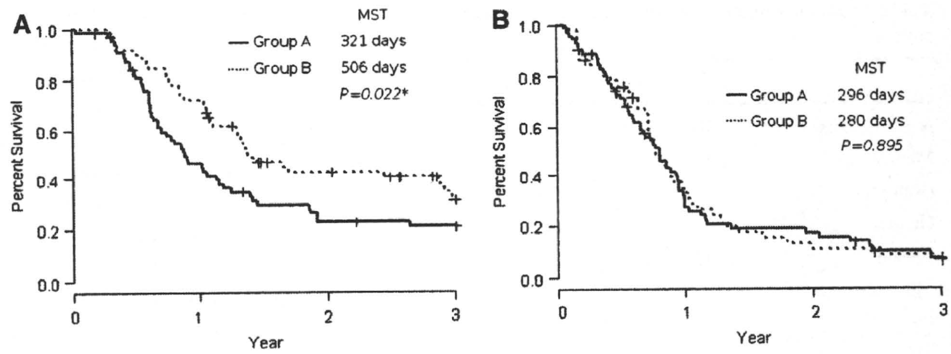

Fig. 2 **a** Survival curves for patients with limited disease (LD). **b** Survival curves for patients with extensive disease (ED). *MST* median survival time

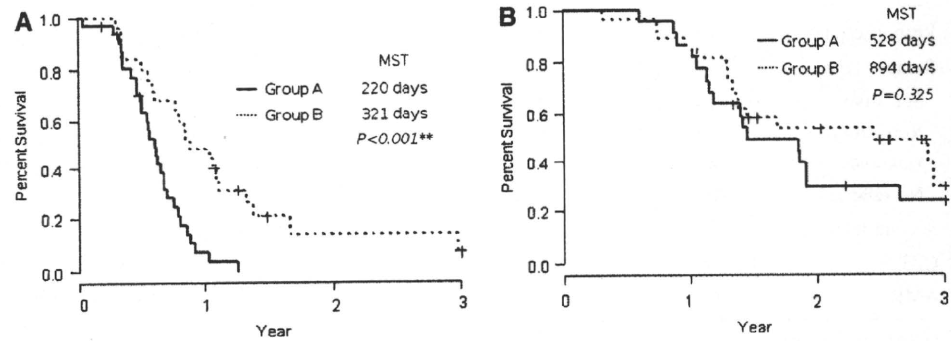

Fig. 3 **a** Survival curves for LD patients with refractory relapse. **b** Survival curves for LD patients with sensitive relapse. *MST* median survival time

Table 4 Cox proportional hazard model analysis for limited disease patients

Factor	Hazard ratio	95% CI	*P* value
Gender	1.428	(0.783–2.605)	0.250
PS	2.072	(1.278–3.357)	0.003
Age	1.240	(0.799–1.924)	0.340
AMR sale	0.596	(0.384–0.927)	0.022
PCI	0.602	(0.322–1.125)	0.110

CI confidential interval, *PS* performance status, *AMR* amrubicin, *PCI* prophylactic cranial irradiation

survival was not observed. When all patients included in the present analysis were evaluated, the frequency of AMR administration was significantly lower in the subset of patients whose PS was poorer upon initial therapy (Table 5a). The reason for the observed improvements in LD patients was because their PS was maintained in second-line therapy and even third-line therapy, and therefore AMR was able to exert its effect. On the other hand, the PS of ED patients worsen frequently on relapse. In addition, their organ functions, including the hemopoietic function, tend to impair. For these reasons, it is considered that AMR was not able to exert its effect in ED patients. AMR should be positively used at relatively early time points when the bone marrow function is preserved. In our present study, AMR was administered as second- or third-line therapy in the vast majority of patients (Table 5b). In the future, an optimal timing for introduction of AMR so as to contribute

Table 5 (A) Amrubicin (AMR) administration by performance status (PS). (B) AMR administration timing in all therapeutic courses

AMR	Yes	No
(A)		
PS 0,1	30	45
PS 2,3	22	12
		$P = 0.029$

Course	N of patients
(B)	
First	5
Second	34
Third	22
Fourth ~	4

AMR amrubicin, *PS* performance status, *N* number

to prolongation of survival in ED patients needs to be investigated. Candidate strategies may include administration of AMR to ED patients with maintained PS, that is, AMR should be used in a first- or second-line therapy, or in maintenance therapies for which an appropriate dosage needs to be determined.

References

1. Govindan R, et al. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the

surveillance, epidemiologic, and end results database. J Clin Oncol. 2006;24:4539–44.
2. Turrisi AT, et al. Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. N Engl J Med. 1999;340: 265–71.
3. Noda K, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med. 2002;346:85–91.
4. O'Brien ME, et al. Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer. J Clin Oncol. 2006;24:5441–7.
5. Takeda K, et al. A phase II study of topotecan in patients with relapsed small-cell lung cancer. Clin Lung Cancer. 2003;4:224–8.
6. Hanada M, et al. A new antitumor agent amrubicin induces cell growth inhibition by stabilizing topoisomerase II-DNA complex. Jpn J Cancer Res. 1998;89:1229–38.
7. Onoda S, et al. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. J Clin Oncol. 2006;24:5448–53.
8. Inoue A, et al. Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. J Clin Oncol. 2008;26:5401–6.
9. R[computer program] (2009) R version 2.10.0. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-progject.org.
10. von Pawel J, et al. Topotecan versus cyclophosphamide, doxorubicin, and vincristine for the treatment of recurrent small-cell lung cancer. J Clin Oncol. 1999;17:658–67.
11. Auperin A, et al. Prophylactic cranial irradiation for patients with small-cell lung cancer in complete remission. Prophylactic Cranial Irradiation Overview Collaborative Group. N Engl J Med. 1999;341:476–84.
12. Slotman B, et al. Prophylactic cranial irradiation in extensive small-cell lung cancer. N Engl J Med. 2007;357:664–72.

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Disturbance of the Growth Hormone–Insulin-like Growth Factor-1 Axis Associated with Poor Performance Status in Patients with Solid Tumors

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Objective: Hormonal imbalance characterized by excessive production of growth hormone (GH) and a low circulating concentration of insulin-like growth factor (IGF)-1 has been demonstrated in individuals with various serious conditions. However, little is known about changes in the GH–IGF-1 axis in cancer patients.
Methods: We prospectively examined the circulating levels of several hormones in 58 patients with solid tumors who were classified according to Eastern Cooperative Oncology Group performance status (PS): PS 0–1, $n = 15$; PS 2, $n = 15$; PS 3, $n = 15$; and PS 4, $n = 13$. The relations of hormone concentrations, with a focus on the GH–IGF-1 system, to PS were evaluated by Spearman's rank correlation test and regression analysis.
Results: The circulating levels of IGF-1, IGF-binding protein-3 and thyroid hormones (total T_3 and T_4) were inversely correlated with PS score. The concentration of GH was increased irrespective of PS but not statistically significant. The ratio of IGF-I to GH was inversely correlated with PS. The levels of GH and IGF-1 in all patients were also inversely correlated.
Conclusions: The present study suggests that the GH–IGF-1 axis is disturbed in patients with cancer.

Key words: growth hormone – insulin-like growth factor-1 – performance status

INTRODUCTION

Medical oncology has made substantial advances with the development of new treatment strategies based on a better understanding of cancer biology. Despite such progress, however, a large proportion of individuals with advanced cancer still experience a fatal outcome (1).

Performance status (PS) refers to the level of activity that cancer patients are capable of achieving and is an important prognostic factor independent of the anatomic extent or histological characteristics of cancer (2). After cancer diagnosis, patients will be exposed to the detrimental consequences not only of the cancer itself but also of anticancer treatment. Most patients with advanced cancer thus exhibit a deterioration in PS at some point during the course of their disease. Such a decreased PS is associated with a substantial impairment in quality of life, reduced responsiveness to anticancer therapies and increased mortality. To date, however, an effective treatment for the cancer-related deterioration in PS has not been developed, largely as a result of its multifactorial pathogenesis. New insights into the underlying pathophysiological mechanisms are likely to provide a basis for the development of effective therapeutic strategies to improve the PS of cancer patients.

Hormonal aberrations characterized by excessive production of growth hormone (GH) and a low circulating concentration of insulin-like growth factor (IGF)-1 have been

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detected in patients with diverse conditions including sepsis, burns, renal failure, AIDS and anorexia nervosa as well as in individuals who have undergone surgery (3,4). Such perturbation of the GH–IGF-1 system may contribute adversely to the condition of critically ill patients, and treatments to correct the hormonal imbalance, by administration of GH or IGF-1, have been explored (4,5). Although circulating GH levels have also been found to be increased in individuals with various types of cancers, including those of the colon, lung, breast, liver and endometrium as well as lymphoma (6–12), the influence of cancer on the GH–IGF-1 axis has not been defined. We have now prospectively examined the circulating levels of several hormones in cancer patients with different PS scores and have investigated the relation of changes in hormonal profile, with a focus on the GH–IGF-1 system, to PS.

PATIENTS AND METHODS

Patients with histologically proven cancer were eligible for the study. Other inclusion criteria were an age of at least 20 years and a projected life expectancy of at least 1 month. The main exclusion criteria were blood malignancies or use of corticosteroids. The study subjects were sequentially enrolled in each institution and divided into four groups on the basis of Eastern Cooperative Oncology Group (ECOG) PS score (0–1, 2, 3 or 4), with a targeted accrual of 15 patients in each group. The number of patients enrolled in each group was counted by the patient registration office and feedback to each institution to enroll planned number of patients. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional ethics committee of each of the participating institutions.

Blood samples were once collected for each patient in the early morning before the subjects had had breakfast and after they had fasted overnight or in the morning excluding 1 h after breakfast and 1 h before lunch. This was planned to avoid possible peaks of GH value in the circadian rhythm. Serum and plasma samples were obtained by centrifugation and stored at $-20^{\circ}C$ until assay. Serum GH and IGF-I levels were determined by solid-phase radioimmunoassay and immune radiometric assay, respectively. Serum triiodothyronine (total T_3), thyroxine (total T_4) and thyroid-stimulating hormone (TSH) levels were determined by electro chemiluminescent immunoassay. Serum concentrations of IGF-binding protein-3 (IGFBP-3) and thyroxine-binding globulin (TBG) were measured by competitive radioimmunoassay. All assays were performed in a blinded manner in the outside laboratory. Other laboratory variables such as total protein, albumin, cholesterol, triglyceride, C-reactive protein, creatinine and hemoglobin as well as markers of liver function were measured in routine hospital tests. Height, weight, body mass index (BMI) and food intake were also recorded for all patients. Primary endpoint of this study was defined as the relation between serum GH levels, IGF-I levels and PS.

Data are presented as means ± SD, and Spearman's rank correlation test was applied to assess the correlation between two variables. A P value of <0.05 was considered statistically significant.

RESULTS

Patient Characteristics

A total of 58 patients (34 men and 24 women) were enrolled in the study at five centers in Japan between January 2005 and March 2006. Median age at enrollment was 64 years (range, 28–81 years). The most frequent principal diagnoses were lung cancer (33%, $n = 19$), gastric cancer (22%, $n = 13$) and colorectal cancer (19%, $n = 11$). The numbers of patients in each PS group at study entry were 15, 15, 15 and 13 for PS 0–1, 2, 3 and 4, respectively. The baseline clinical characteristics of the patients according to the PS group are shown in Table 1. Complete blood test data were available for all patients.

Food Intake, BMI and Laboratory Variables

The Spearman test revealed that PS score was inversely correlated with weight ($r = -0.54$, $P < 0.001$), BMI ($r = -0.53$, $P < 0.001$) and food intake ($r = -0.73$, $P < 0.001$) (Table 2). Inverse correlations were also apparent between PS and circulating levels of total protein ($r = -0.59$, $P < 0.001$), albumin ($r = -0.66$, $P < 0.001$), total cholesterol ($r = -0.33$, $P = 0.014$), choline esterase ($r = -0.61$, $P < 0.001$) and hemoglobin ($r = -0.37$, $P = 0.004$). PS also tended to be positively correlated with levels of alkaline phosphatase ($r = 0.23$) and lactate dehydrogenase ($r = 0.09$), but these relations did not achieve statistical significance. Significant positive correlations were detected between PS and the concentration of C-reactive protein ($r = 0.59$, $P < 0.001$) and the number of white blood cells ($r = 0.42$, $P = 0.001$).

Hormone Levels

The plasma concentration of GH was not significantly correlated with PS ($r = 0.15$, $P = 0.25$), whereas that of IGF-1 was inversely correlated with PS ($r = -0.44$, $P = 0.001$) (Table 3). An inverse correlation was also apparent between PS and the concentration of IGFBP-3 ($r = -0.39$, $P = 0.002$), the major carrier protein for IGF-1 in the circulation. The concentration of GH was inversely correlated with that of IGF-1 ($r = -0.314$, $P = 0.018$). Whereas TSH level was not correlated with PS ($r = 0.04$, $P = 0.76$), the concentrations of total T_3 (-0.57, $P < 0.001$), total T_4 (-0.38, $P = 0.003$) and TBG (-0.44, $P = 0.001$) were inversely correlated with PS. The ratio of IGF-I to GH (IGF-I/GH), a

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combined indicator of GH and IGF-I, also showed correlation with PS ($r = 0.262$, $P = 0.049$) (Table 4).

Table 1. Patient characteristics

	Performance status			
	0–1	2	3	4
Assessable patients	15	15	15	13
Median age (range)	64 (49–73)	66 (50–81)	60 (28–77)	69 (54–81)
Sex (male/female)	11/4	7/8	9/6	7/6
Principal diagnosis				
Lung cancer	9	5	3	2
Gastric cancer	1	3	4	5
Colorectal cancer	2	5	4	0
Esophageal cancer	0	1	1	1
Pancreatic cancer	1	0	0	2
Breast cancer	0	0	1	1
Sarcoma	1	1	0	0
Renal cancer	1	0	0	0
Adenoid cystic cancer	0	0	1	0
Biliary tract cancer	0	0	1	0
Head and neck cancer	0	0	0	1
Cervical cancer	0	0	0	1

DISCUSSION

In this prospective evaluation of hormonal status in cancer patients, we have shown that the circulating levels of thyroid hormones (T_3 and T_4) and of components of the IGF system (IGF-1 and IGFBP-3) were inversely correlated with PS score. Given that the GH concentration also tended to be increased in patients with a high PS score, our results are indicative of an imbalance between GH and the IGF system in such patients.

Increased interpulse levels of GH have been described in critically ill patients including those with several types of cancer (4,13–15). Fasting levels of GH were also found to be significantly greater in patients with colon cancer than in control subjects (2.9 ± 3.1 versus 0.5 ± 0.2 ng/ml) (11). Our data now show a similarly high plasma concentration of GH (3.0 ± 3.7 ng/ml) in cancer patients irrespective of PS, although we did not determine values for matched controls.

Most circulating IGF-1 and IGFBP-3 are synthesized in the liver, where expression of each is increased by GH (Fig. 1). IGF-1 has a long half-life in plasma (up to 12 h), and its circulating level is highly correlated with that of GH. IGFBP-3 binds >95% of plasma IGF-1 and influences cell proliferation by controlling the access of IGF-1 to IGF receptors (16,17). In most instances, the circulating level of IGFBP-3 has been found to correlate with that of IGF-1 and is thought to reflect the status of IGF-1 in plasma. Our prospective data now show that the circulating

Table 2. Laboratory variables stratified by performance status

	Performance status				*P* value
	0–1	2	3	4	
Height (cm)	162 ± 7	158 ± 10	163 ± 9	156 ± 8	NS
Weight (kg)	58 ± 10	53 ± 15	49 ± 10	40 ± 4	<0.001
BMI (kg/m^2)	22 ± 3	21 ± 4	19 ± 4	16 ± 2	<0.001
Food intake (%)	82 ± 25	62 ± 27	27 ± 29	15 ± 19	<0.001
TP (g/dl)	7.1 ± 0.4	6.5 ± 0.4	6.2 ± 0.7	5.8 ± 0.9	<0.001
Albumin (g/dl)	3.9 ± 0.3	3.4 ± 0.5	3.0 ± 0.6	2.4 ± 0.7	<0.001
TC (mg/dl)	180 ± 32	186 ± 53	169 ± 48	125 ± 54	0.014
TG (mg/dl)	126 ± 76	113 ± 51	122 ± 80	88 ± 27	NS
ChE (IU/l)	258 ± 54	178 ± 77	174 ± 82	105 ± 48	<0.001
ALP (IU/l)	450 ± 353	480 ± 344	540 ± 446	741 ± 477	NS
LDH (IU/l)	250 ± 103	256 ± 119	323 ± 265	371 ± 434	NS
Cre (mg/dl)	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 1.2	0.7 ± 0.5	NS
CRP (mg/dl)	0.8 ± 1.0	2.1 ± 2.1	4.6 ± 5.2	10.6 ± 10.5	<0.001
WBC ($10^3/\mu l$)	5.9 ± 1.9	5.3 ± 2.4	8.3 ± 4.8	11.7 ± 6.7	0.001
Hb (g/dl)	12.0 ± 1.6	10.3 ± 1.4	11.5 ± 2.4	9.3 ± 1.7	0.004
Platelets ($10^4/\mu l$)	27.9 ± 7.7	30.6 ± 22.7	25.6 ± 9.5	29.9 ± 12.6	NS

Data are means ± SD. *P* values were determined by Spearman's rank correlation test. NS, not significant; BMI, body mass index; TP, total protein; TC, total cholesterol; TG, triglyceride; ChE, choline esterase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; Cre, creatinine; CRP, C-reactive protein; WBC, white blood cells; Hb, hemoglobin.

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Table 3. Circulating hormone levels according to performance status

	Performance status				*P* value
	0–1	2	3	4	
GH (ng/ml)	2.5 ± 2.4	2.5 ± 3.1	3.1 ± 3.7	4.1 ± 5.5	NS
IGF-1 (ng/ml)	149 ± 49	96 ± 60	135 ± 102	64 ± 42	0.001
IGFBP-3 (μg/ml)	1.9 ± 0.5	1.9 ± 0.8	1.9 ± 0.8	1.0 ± 0.6	0.002
TSH (μIU/ml)	2.6 ± 1.9	2.3 ± 1.8	2.2 ± 1.0	2.5 ± 1.6	NS
Total T_3 (ng/ml)	1.1 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	<0.001
Total T_4 (μg/dl)	9.8 ± 1.8	9.8 ± 1.9	9.6 ± 1.6	7.1 ± 2.4	0.003
TBG (μg/ml)	22.3 ± 3.9	24.4 ± 7.0	20.8 ± 4.3	15.7 ± 4.3	0.001

Data are means ± SD. *P* values were determined by Spearman's rank correlation test. GH, growth hormone; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor-binding protein-3; TSH, thyroid-stimulating hormone; TBG, thyroxine-binding globulin.

Table 4. Ratio of IGF-1 to GH according to performance status

	Performance status				*P* value
	0–1	2	3	4	
IGF-1/GH (ng/ml)	366 ± 645	201 ± 405	185 ± 229	62 ± 98	0.049

Data are means ± SD. *P* values were determined by regression analysis.

concentration of IGF-1 and IGFBP-3 was negatively correlated with PS score but GH did not show clear correlation with PS score. The ratio of IGF-I/GH also showed correlation with PS. These results thus suggest that the relation between GH secretion and circulating IGF-1 levels is disturbed in cancer patients.

Under normal conditions, GH secreted by the pituitary gland induces hepatic IGF-1 production, which in turn exerts feedback suppression of GH secretion (Fig. 1). Acquired GH resistance characterized by the combination of high levels of GH and low levels of IGF-1 has been demonstrated to varying extents in patients with a wide range of conditions including sepsis, trauma, burns, renal failure and AIDS (3,4,18,19). The primary defect in acquired GH resistance is a reduction in IGF-1 concentration which then leads to increased GH concentration; however, low levels of IGF-1 are not improved, despite increased or normal levels of GH (Fig. 1) (3,20). In the present study, a significant inverse correlation was apparent between circulating GH and IGF-1 levels in the entire cohort, consistent with the pattern of acquired GH resistance.

It remains unclear, however, whether the disturbance of the GH–IGF-1 axis is merely a non-specific consequence of cancer or whether it contributes adversely to the complex pathophysiology of cancer. Nutritional state is known to affect the function of the GH–IGF-1 axis. Acute dietary restriction and chronic malnutrition, especially accompanied by severe protein deficiency, have been shown to lead to

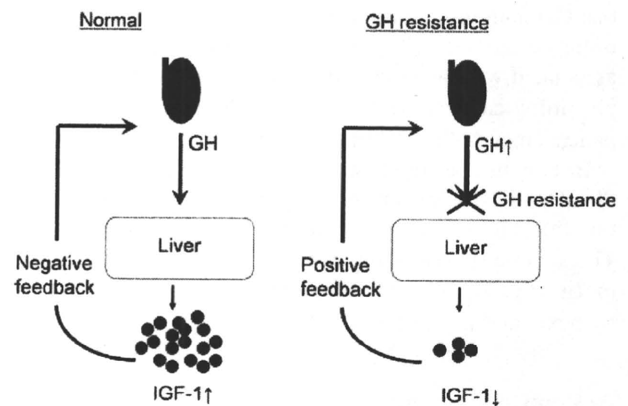

Figure 1. Diagram of the growth hormone (GH) and insulin-like growth factor I (IGF-I) axis in healthy persons (left) and those with acquired GH resistance. Under normal conditions, GH secreted by the pituitary gland stimulates production of IGF-1 by liver. IGF-1 exerts feedback suppression. The primary defect in acquired GH resistance is a reduction in IGF-1 concentration which then leads to increased GH concentration; however, low levels of IGF-1 are not improved, despite increased or normal levels of GH.

increased levels of GH and reduced levels of IGF-1 (21,22). In the present study, the circulating concentration of IGF-1 was significantly correlated with BMI ($r = 0.46$, $P < 0.001$), albumin level ($r = 0.41$, $P = 0.002$) and total protein level ($r = 0.36$, $P = 0.007$). The circulating level of GH was negatively correlated with BMI ($r = -0.40$, $P = 0.003$) but was not related to total protein and albumin levels. Patients with lung cancer were previously shown to have a reduced IGF-1 concentration and an increased GH pulse frequency before the development of malnutrition (23). Furthermore, acquired GH resistance in cachectic patients with colorectal cancer has been proposed not to be an adaptation to malnutrition but to be caused by the tumor itself (24). Together, these various observations suggest that although inadequate nutrition is likely to contribute to the altered GH/IGF-I axis in cancer patients, other factors also play a role. It has been shown that in rat hepatocytes in primary culture cytokines, interleukin-1β

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and tumor necrosis factor-α inhibit GH-stimulated IGF-1 synthesis at least partly due to suppression of hepatic GH receptor synthesis (25). Given the significant positive correlation between PS and C-reactive protein observed in the present study, inflammatory cytokine deregulation in cancer patients can participate in the development of acquired GH resistance. Further studies to investigate the mechanism for acquired GH resistance in cancer patients are warranted.

A wide range of conditions sharing the common feature of catabolism exhibit a similar pattern of disturbance of the GH–IGF-1 axis characterized by high GH and low IGF-1 levels (4). Treatment to reverse this defect by restoring IGF-1 levels through administration of GH has been shown to result in improvement in metabolic parameters and to provide clinical benefit in well-defined groups of patients, such as those with AIDS or anorexia nervosa (4,26). On the other hand, a small pilot study with 10 terminally ill cancer patients showed that GH administration for 3 days had limited effects on metabolic parameters (23). Given that the various conditions associated with acquired GH resistance have different pathophysiological mechanisms, GH administration in cancer patients may not necessarily be clinically beneficial.

In conclusion, the results of the present study show that the GH–IGF-1 system is disturbed in cancer patients and that this anomaly may play a role in the deterioration of PS. Therapeutic strategies for correcting this hormonal imbalance merit investigation. Such approaches may alleviate the cachexia and malaise associated with cancer.

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Conflict of interest statement

None declared.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57:43–66.
2. Hauser CA, Stockler MR, Tattersall MH. Prognostic factors in patients with recently diagnosed incurable cancer: a systematic review. *Support Care Cancer* 2006;14:999–1011.
3. Ross RJ, Chew SL. Acquired growth hormone resistance. *Eur J Endocrinol* 1995;132:655–60.
4. Jenkins RC, Ross RJ. Acquired growth hormone resistance in adults. *Baillieres Clin Endocrinol Metab* 1998;12:315–29.
5. Vance ML, Mauras N. Growth hormone therapy in adults and children. *N Engl J Med* 1999;341:1206–16.
6. Benjamin F. Growth hormone response to insulin hypoglycemia in endometrial carcinoma. *Obstet Gynecol* 1974;43:257–61.
7. Pavelic K, Pekic B, Gabrilovac J, Bratic-Mikes V, Boranic M. Hormonal changes in patients with haematological malignancies. *Biomedicine* 1981;35:181–4.
8. Scheuer A, Grun R, Lehmann FG. Peptide hormones in liver cirrhosis and hepatocellular carcinoma. *Oncodev Biol Med* 1981;2:1–10.
9. Axelrod L, Halter JB, Cooper DS, Aoki TT, Roussell AM, Bagshaw SL. Hormone levels and fuel flow in patients with weight loss and lung cancer. Evidence for excessive metabolic expenditure and for an adaptive response mediated by a reduced level of 3,5,3′-triiodothyronine. *Metabolism* 1983;32:924–37.
10. Emerman JT, Leahy M, Gout PW, Bruchovsky N. Elevated growth hormone levels in sera from breast cancer patients. *Horm Metab Res* 1985;17:421–4.
11. Tayek JA, Bulcavage L, Chlebowski RT. Relationship of hepatic glucose production to growth hormone and severity of malnutrition in a population with colorectal carcinoma. *Cancer Res* 1990;50:2119–22.
12. Mazzoccoli G, Giuliani A, Bianco G, De Cata A, Balzanelli M, Carella AM, et al. Decreased serum levels of insulin-like growth factor (IGF)-I in patients with lung cancer: temporal relationship with growth hormone (GH) levels. *Anticancer Res* 1999;19:1397–9.
13. Voerman HJ, Strack van Schijndel RJ, Groeneveld AB, de Boer H, Nauta JP, Thijs LG. Pulsatile hormone secretion during severe sepsis: accuracy of different blood sampling regimens. *Metabolism* 1992;41:934–40.
14. Van den Berghe G, de Zegher F, Lauwers P, Veldhuis JD. Growth hormone secretion in critical illness: effect of dopamine. *J Clin Endocrinol Metab* 1994;79:1141–6.
15. Rondanelli M, Solerte SB, Fioravanti M, Scevola D, Locatelli M, Minoli L, et al. Circadian secretory pattern of growth hormone, insulin-like growth factor type I, cortisol, adrenocorticotropic hormone, thyroid-stimulating hormone, and prolactin during HIV infection. *AIDS Res Hum Retroviruses* 1997;13:1243–9.
16. Michell NP, Dent S, Langman MJ, Eggo MC. Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation. *Growth Factors* 1997;14:269–77.
17. Le Roith D. Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N Engl J Med* 1997;336:633–40.
18. Laurence J. Growth hormone in HIV/AIDS: current uses and future prospects. *Pediatr AIDS HIV Infect* 1995;6:281–91.
19. Anker SD, Volterrani M, Pflaum CD, Strasburger CJ, Osterziel KJ, Doehner W, et al. Acquired growth hormone resistance in patients with chronic heart failure: implications for therapy with growth hormone. *J Am Coll Cardiol* 2001;38:443–52.
20. Bentham J, Rodriguez-Arnao J, Ross RJ. Acquired growth hormone resistance in patients with hypercatabolism. *Horm Res* 1993;40:87–91.
21. Clemmons DR, Klibanski A, Underwood LE, McArthur JW, Ridgway EC, Beitins IZ, et al. Reduction of plasma immunoreactive somatomedin C during fasting in humans. *J Clin Endocrinol Metab* 1981;53:1247–50.
22. Hartman ML, Veldhuis JD, Johnson ML, Lee MM, Alberti KG, Samojlik E, et al. Augmented growth hormone (GH) secretory burst frequency and amplitude mediate enhanced GH secretion during a two-day fast in normal men. *J Clin Endocrinol Metab* 1992;74:757–65.
23. Tayek JA, Brasel JA. Failure of anabolism in malnourished cancer patients receiving growth hormone: a clinical research center study. *J Clin Endocrinol Metab* 1995;80:2082–7.
24. Huang Q, Nai YJ, Jiang ZW, Li JS. Change of the growth hormone-insulin-like growth factor-I axis in patients with gastrointestinal cancer: related to tumour type and nutritional status. *Br J Nutr* 2005;93:853–8.
25. Wolf M, Bohm S, Brand M, Kreymann G. Proinflammatory cytokines interleukin 1β and tumor necrosis factor α inhibit growth hormone stimulation of insulin-like growth factor I synthesis and growth hormone receptor mRNA levels in cultured rat liver cells. *Eur J Endocrinol* 1996;135:729–37.
26. Hashizume K, Suzuki S, Komatsu A, Hiramatsu K, Mori J, Yamazaki M, et al. Administration of recombinant human growth hormone normalizes GH-IGF1 axis and improves malnutrition-related disorders in patients with anorexia nervosa. *Endocr J* 2007;54:319–27.

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Tumor and Stem Cell Biology

Cancer Research

FOXQ1 Is Overexpressed in Colorectal Cancer and Enhances Tumorigenicity and Tumor Growth

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Abstract

Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family, and it has recently been proposed to participate in gastric acid secretion and mucin gene expression in mice. However, the role of FOXQ1 in humans and especially in cancer cells remains unknown. We found that *FOXQ1* mRNA is overexpressed in clinical specimens of colorectal cancer (CRC; 28-fold/colonic mucosa). A microarray analysis revealed that the knockdown of *FOXQ1* using small interfering RNA resulted in a decrease in $p21^{CIP1/WAF1}$ expression, and a reporter assay and a chromatin immunoprecipitation assay showed that *p21* was one of the target genes of FOXQ1. Stable FOXQ1-overexpressing cells (H1299/FOXQ1) exhibited elevated levels of p21 expression and inhibition of apoptosis induced by doxorubicin or camptothecin. Although cellular proliferation was decreased in H1299/FOXQ1 cells *in vitro*, H1299/FOXQ1 cells significantly increased tumorigenicity [enhanced green fluorescent protein (EGFP): 2/15, FOXQ1: 7/15] and enhanced tumor growth (437 ± 301 versus 1735 ± 769 mm^3, $P < 0.001$) *in vivo*. Meanwhile, stable p21 knockdown of H1299/FOXQ1 cells increased tumor growth, suggesting that FOXQ1 promotes tumor growth independent of p21. Microarray analysis of H1299/EGFP and H1299/FOXQ1 revealed that FOXQ1 overexpression upregulated several genes that have positive roles for tumor growth, including *VEGFA, WNT3A, RSPO2*, and *BCL11A*. CD31 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of the tumor specimens showed that FOXQ1 overexpression mediated the angiogenic and antiapoptotic effect *in vivo*. In conclusion, FOXQ1 is overexpressed in CRC and enhances tumorigenicity and tumor growth presumably through its angiogenic and antiapoptotic effects. Our findings show that FOXQ1 is a new member of the cancer-related FOX family. *Cancer Res; 70(5); 2053–63. ©2010 AACR.*

Introduction

The forkhead box (*Fox*) gene family is a large and diverse group of transcription factors that share certain characteristics of a conserved, ~100 amino acid DNA-binding motif known as the forkhead or winged helix domain; over 100 proteins with forkhead domains have been identified, comprising at least 17 subclasses to date (1). The Fox gene family plays various important roles, not only in biological processes including development, metabolism, immunology, and senescence but also in cancer development (2, 3).

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Forkhead box Q1 (*FOXQ1*, also known as HFH1) is a member of the FOX gene family and contains the core DNA binding domain, whereas the flanking wings of FOXQ1 contribute to its sequence specificity (4). As a transcription factor, FOXQ1 is known to repress the promoter activity of smooth muscle–specific genes, such as telokin and SM22α, in A10 vascular muscle cells (5), and FOXQ1 expression is regulated by Hoxa1 in embryonic stem cells (6). The biological function of *Foxq1* has been clearly identified in hair follicle differentiation in satin (sa) homozygous mice (7); interestingly, satin mice also exhibit suppressed natural killer cell function and T-cell function, suggesting a relation with immunology. Satin mice have provided evidence that Hoxc13 regulates foxq1 expression and that "cross-talk" occurs between Homeobox and Fox (8). Foxq1 mRNA is widely expressed in murine tissues, with particularly high expression levels in the stomach and bladder (5). Recently, two important findings have been reported regarding its involvement in stomach surface cells. Foxq1-deficient mice exhibit a lack of gastric acid secretion in response to various secretagogue stimuli (9). On the other hand, Foxq1 regulates gastric MUC5AC synthesis, providing clues as to the lineage-specific cell differentiation in gastric surface epithelia (10). Despite accumulating evidence supporting the biological function of the murine foxq1 gene in hair follicle

morphogenesis and gastric epithelial cells, no data regarding the cellular and biological functions of human *FOXQ1*, especially in cancer cells, are available.

$p21^{CIP1/WAF1}$ (hereafter called p21) is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have shown that p21 functions as a G_1 cyclin kinase inhibitor (11, 12) and a downstream molecule of p53 (13). p21 possesses a variety of cellular functions, including the negative modulation of cell cycle progression (14), cellular differentiation (15), and the regulation of p53-dependent antiapoptosis (reviewed in ref. 16). The expression of p21 is regulated by both p53-dependent and p53-independent mechanisms at the transcriptional level. Other regulatory mechanisms of p21 expression involve proteasome-mediated degradation, mRNA stability, alterations in the epigenetic silencing of the p21 promoter, and secondary decreases resulting from viral activity targeting p53, such as the activities of human papilloma virus and hepatitis C virus (17). However, its expression is considered to be regulated mainly at the transcriptional level (18). Accumulating data indicate that many molecules from diverse signaling pathways can activate or repress the p21 promoter, including p53, transforming growth factor-β (TGF-β), c-jun, Myc, Sp1/Sp3, signal transducers and activators of transcriptions, CAAT/enhancer binding protein-α (C/EBP-α), C/EBP-β, basic helix-loop-helix proteins, and myogenic differentiation 1 (reviewed in ref. 19). Thus, p21 is integrally involved in both cell cycle and apoptosis; therefore, identifying its regulatory molecules is of great importance.

We performed a microarray analysis of clinical samples of paired colorectal cancer (CRC) specimens and normal colonic mucosa specimens to identify genes that were overexpressed in CRC. Our results revealed that *FOXQ1* gene expression was ~28-fold higher in CRC than in normal colonic mucosa, and we hypothesized that FOXQ1 may play a role in CRC. In the present study, we investigated the biological function of FOXQ1.

Materials and Methods

Antibodies. The following antibodies were used: anti-p21, anti-p53, anti-cdk2, anti-cdk4, anti-cyclin D, anti-phosphorylated Rb, anti-poly(ADP-ribose) polymerase (PARP), anti-cleaved PARP, anti-caspase-3, anti-cleaved caspase-3, secondary antibodies, and Myc-tag mouse antibody (Cell Signaling), as well as anti-β-actin (Santa Cruz Biotechnology). A mouse anti-CD31 monoclonal antibody was purchased from BD Biosciences.

Cell lines and cultures. The DLD-1, MKN74, H1299, SBC3, and U251 cell lines were cultured in RPMI 1640 (Sigma). The WiDr, CoLo320DM, and human embryonic kidney cell line 293 (HEK293) cell lines were cultured in DMEM (Sigma), and the LoVo cell line was cultured in Ham/F12 medium [Life Technologies Bethesda Research Laboratories (BRL)]. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Patients and samples. Paired CRC and noncancerous colonic mucosa samples were evaluated using a microarray analysis in the first consecutive 10 patients. These samples and another 36 CRC samples were analyzed using real-time reverse transcription–PCR (RT-PCR). The RNA extraction method and the quality check protocol have been previously described (20). This study was approved by the institutional review board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants. The cDNA fragment encoding human full-length FOXQ1 was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa) with 5′-GGG AAT TCG CGG CCA TGA AGT TGG AGG TCT TCG TC-3′ and 5′-CCC TCG AGC GCT ACT CAG GCT AGG AGC GTC TCC AC-3′ sense and antisense primers, respectively. The methods used in this section have been previously described (21). Short hairpin RNA (shRNA) targeting p21 was constructed using oligonucleotides encoding small interfering RNA (siRNA) directed against p21 and a nonspecific target as follows: 5′-CTA AGA GTG CTG GGC ATT TTT-3′ for p21 shRNA and 5′-TGT TCG CAG TAC GGT AAT GTT-3′ for control shRNA. They were cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech) according to manufacturer's protocol. The stable transfectants expressing enhanced green fluorescent protein (EGFP) or FOXQ1 or FOXQ1 with shRNA targeting p21 for each cell line were designated as HEK293/EGFP, HEK293/FOXQ1, CoLo320/EGFP, CoLo320/FOXQ1, H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21. The FOXQ1 human cDNA was tagged at the NH_2 terminus with the myc epitope using the pCMV-Myc vector (Clontech) for chromatin immunoprecipitation (ChIP) assay.

SiRNA transfection. Two different sequences of siRNA targeting human FOXQ1 and negative control siRNA were purchased from QIAGEN. The sequences of FOXQ1 and control siRNA were as follows: FOXQ1#1 sense, 5′-CCA UCA AAC GUG CCU UAA A-3′ and antisense, 5′-UUU AAG GCA CGU UUG AUG G-3′; FOXQ1#4 sense, 5′-CGC GGA CUU UGC ACU UUG A-3′ and antisense, 5′-UCA AAG UGC AAA GUC CGC G-3′; control siRNA (scramble) sense, 5′-UUC UCC GAA CGU GUC ACG U-3′ and antisense, 5′-ACG UGA CAC GUU CGG AGA A-3′; control siRNA (GFP) sense, 5′-GCA AGC UGA CCC UGA AGU UCA U-3′ and antisense, 5′-GAA CUU CAG GGU CAG CUU GCC G-3′. The methods of transfection have been previously described (22).

Real-time RT-PCR and Western blot analysis. The methods used in this section have been previously described (21). The primers used for real-time RT-PCR were purchased from Takara as follows: FOXQ1 forward, 5′-CGC GGA CTT TGC ACT TTG AA-3′ and reverse, 5′-AGC TTT AAG GCA CGT TTG ATG GAG-3′; p21 forward, 5′-TCC AGC GAC CTT CCT CAT CCA C-3′ and reverse, 5′-TCC ATA GCC TCT ACT GCC ACC ATC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD) forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-ATG GTG GTG AAG ACG CCA GT-3′. The experiment was performed in triplicate.

Luciferase reporter assay. The human p21 promoter reporter vector was constructed according to a previously described method (13). The p21 promoter fragment was cut between the *Kpn*I and *Xho*I restriction sites and was transferred into the luciferase reporter vector pGL4.14 (Promega). All sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. All the samples were examined in triplicate.

ChIP. ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to manufacturer's protocol. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. The putative region of the p21 promoter (−2264 to −1971) was amplified with the following primers: 5′-TTG AGC TCT GGC ATA GAA GA-3′ (forward) and 5′-TAC CCA GAC ACA CTC TAA GG-3′ (reverse). As a negative control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) second intron promoter was amplified with the following primers: 5′-AAT GAA TGG GCA GCC GTT AG-3′ (forward) and 5′-AGC TAG CCT CGC TCC ACCTGA C-3′ (reverse).

Xenograft studies. Two separate xenograft studies were performed independently. Nude mice (*BALB/c nu/nu*; 6-week-old females; CLEA Japan, Inc.) were used for the *in vivo* studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (23). To assess tumorigenicity, suspensions of 1×10^6 H1299/EGFP or H1299/FOXQ1 cells (in 0.1 mL PBS) were s.c. injected into the left or right flanks of nude mice (n = 15), respectively. To evaluate tumor growth, a suspension of 6×10^6 H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21 cells (in 0.1 mL PBS) were s.c. inoculated (n = 10) into nude mice. The tumor volume was calculated as length × width^2 × 0.5. The tumor formation was assessed every 2 to 3 d. At the end of the experiment, the mice were sacrificed and the xenografts were resected, fixed in 10% buffered formalin for 6 to 10 h, and processed for histologic analysis.

Immunohistochemical and immunofluorescence staining. The methods used in this section have been previously described (24, 25).

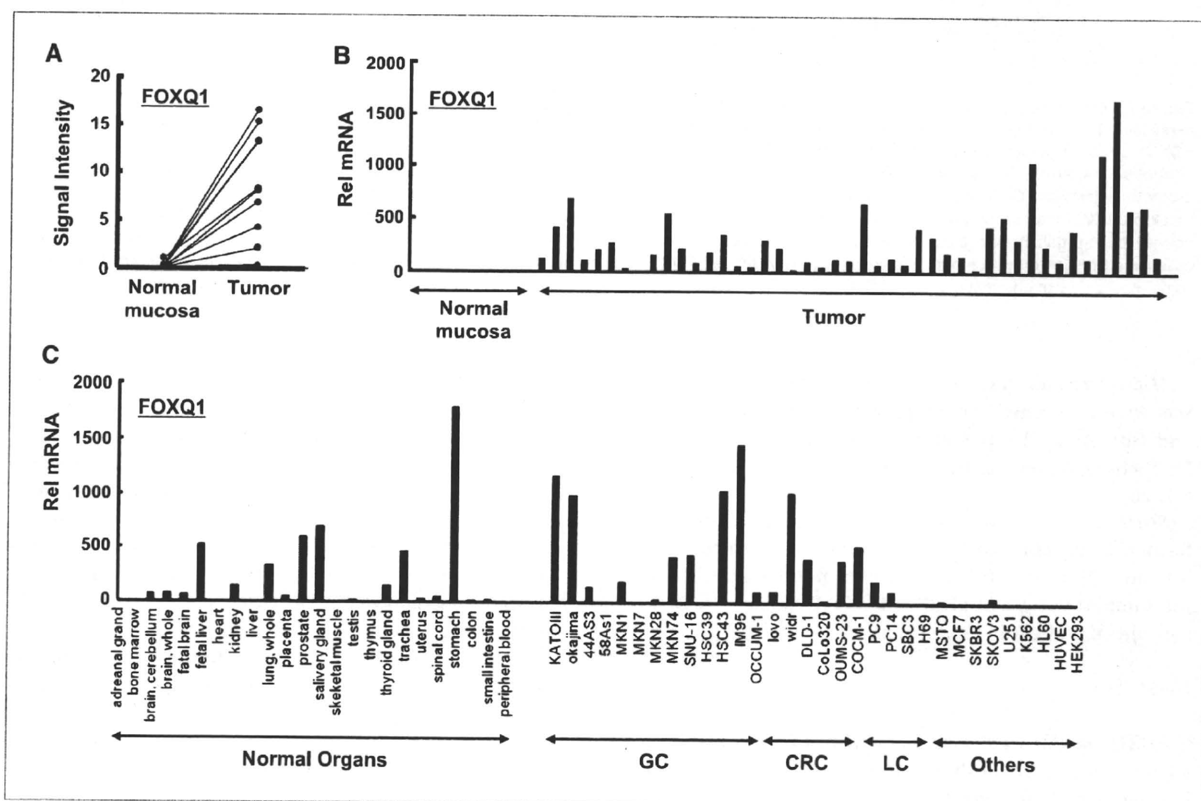
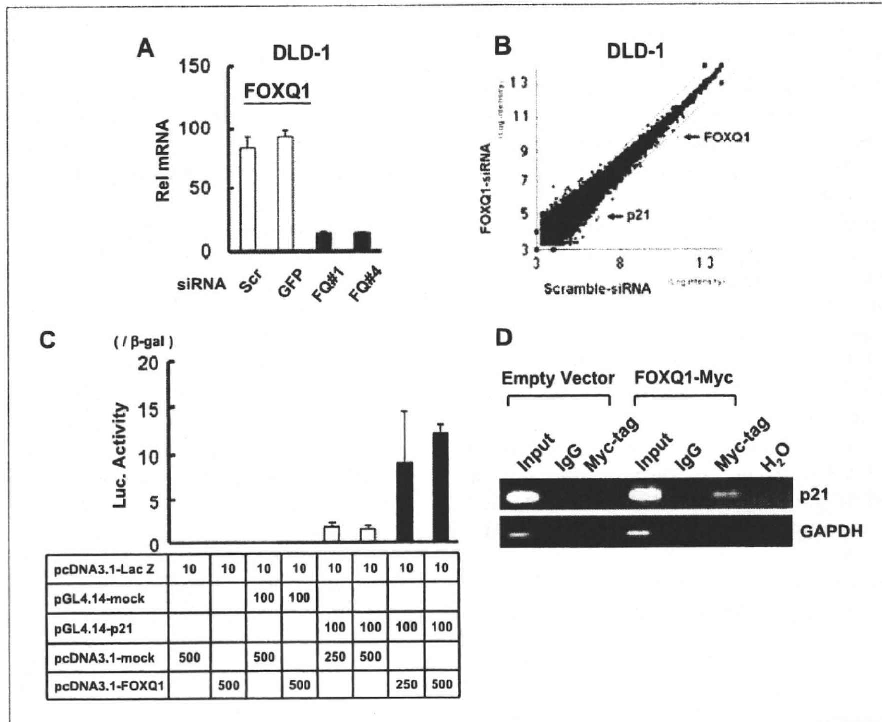


Figure 1. *FOXQ1* expression in CRC. A, mRNA expression of *FOXQ1* obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR for 10 paired and an additional 36 CRC samples. C, the mRNA expression levels of *FOXQ1* were determined using a real-time RT-PCR analysis of human normal tissue (left) and 30 human cancer cell lines, HEK293, and human umbilical vascular endothelial cell (HUVEC) cell lines (right). GC, gastric cancer; LC, lung cancer; Rel mRNA, normalized mRNA expression levels (*FOXQ1*/*GAPD* × 10^4).



pcDNA3.1-Lac Z	10	10	10	10	10	10	10	10
pGL4.14-mock			100	100				
pGL4.14-p21					100	100	100	100
pcDNA3.1-mock	500		500		250	500		
pcDNA3.1-FOXQ1		500		500			250	500

Figure 2. FOXQ directly regulates *p21* transcription. A, FOXQ1-targeting siRNA (FQ#1 and FQ#4) suppressed FOXQ1 expression in DLD-1 cells. The mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR. B, microarray analysis of DLD-1 cells transfected with control-siRNA or FOXQ1-siRNA. The longitudinal axis indicates the mRNA expression of FOXQ1-siRNA transfected cells and the horizontal axis indicates that of control-siRNA. Arrow, FOXQ1 or p21 expression. Each point indicates the normalized and log base 2 transformed microarray data. C, induction of p21 promoter activity by FOXQ1. Luciferase vectors with either an empty or p21 promoter (pGL4.14-mock or pGL4.14-p21) were transiently cotransfected with a mock or FOXQ1 expression plasmid (pcDNA3.1-mock or pcDNA3.1-FOXQ1) expressing β-galactosidase as an internal control. The results were normalized to β-galactosidase activity and are representative of at least three independent experiments. D, ChIP of FOXQ1 on the promoter of *p21*. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. Agarose gel shows PCR amplification (35 cycles) of the *p21* promoter using inputs (1% of chromatin used for ChIP) or ChIPs as templates. Primers to the *GAPDH* promoter were used as the negative control.

Microarray analysis. The microarray procedure and analysis were performed according to the Affymetrix protocols and BRB Array Tools software, Ver. 3.3.0,[4] developed by Dr. Richard Simon and Dr. Amy Peng, as reported previously (21, 26).

Statistical analysis. The statistical analyses were performed using Microsoft Excel (Microsoft) to calculate the SD and to test for statistically significant differences between the samples using a Student's t test. A P value of <0.05 was considered statistically significant.

Results

FOXQ1 mRNA was overexpressed in CRCs. A microarray analysis for 10 paired CRC samples identified 30 genes as being significantly upregulated by >10-fold in CRC ($P < 0.001$; Supplementary Table S1). *FOXQ1*, an uncharacterized transcription factor, was upregulated by 28-fold in the CRC specimens (Fig. 1A), exhibiting the fourth highest level of upregulation [after interleukin-8, matrix metalloproteinase-1 (MMP), and MMP-3]. Real-time RT-PCR for the 10 paired samples and an additional 36 CRC samples showed that *FOXQ1* mRNA was markedly overexpressed in the CRC samples but was only expressed at a very low level in noncancerous colonic mucosa ($P < 0.001$; Fig. 1B). The average levels of *FOXQ1* expression were 299 ± 326 and 4.0 ± 5.0 ($\times 10^4$/GAPD), respectively.

FOXQ1 expression in normal tissues and cancer cell lines. To investigate the expression of *FOXQ1*, we analyzed the mRNA expression levels of *FOXQ1* in panels of human normal tissues and cancer cell lines using real-time RT-PCR. High levels of *FOXQ1* expression were observed in the stomach, salivary gland, prostate, trachea, and fetal liver among the 24 normal tissues that were examined (Fig. 1C, left). Relatively weak expression levels were detected in brain-derived tissues, kidney, lung, placenta, and thyroid gland. These results were consistent with those of a previous report (27).

[4] http://linus.nci.nih.gov/BRB-ArrayTools.html