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BRIEF REPORT

EML4-ALK Mutations in Lung Cancer That Confer Resistance to ALK Inhibitors

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SUMMARY

The EML4 (echinoderm microtubule-associated protein-like 4)–ALK (anaplastic lymphoma kinase) fusion-type tyrosine kinase is an oncoprotein found in 4 to 5% of non–small-cell lung cancers, and clinical trials of specific inhibitors of ALK for the treatment of such tumors are currently under way. Here, we report the discovery of two secondary mutations within the kinase domain of EML4-ALK in tumor cells isolated from a patient during the relapse phase of treatment with an ALK inhibitor. Each mutation developed independently in subclones of the tumor and conferred marked resistance to two different ALK inhibitors. (Funded by the Ministry of Health, Labor, and Welfare of Japan, and others.)

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EML4-ALK IS A FUSION-TYPE PROTEIN TYROSINE KINASE THAT IS PRESENT in 4 to 5% of cases of non–small-cell lung cancer and is generated as a result of a small inversion within the short arm of human chromosome 2.[1-3] EML4-ALK undergoes constitutive dimerization through interaction between the coiled-coil domain within the EML4 region of each monomer, thereby activating ALK and generating oncogenic activity. In transgenic mice that express EML4-ALK specifically in lung epithelial cells, hundreds of adenocarcinoma nodules develop in both lungs soon after birth, and oral administration of a specific inhibitor of ALK tyrosine kinase activity rapidly eradicates such nodules from the lungs.[4] These observations reveal the essential role of EML4-ALK in the carcinogenesis of non–small-cell lung cancer harboring this fusion kinase. Furthermore, clinical trials are investigating crizotinib (PF-02341066), an inhibitor of the tyrosine kinase activity of both ALK and the met proto-oncogene (MET), for the treatment of EML4-ALK–positive non–small-cell lung cancer.

In addition to crizotinib, other tyrosine kinase inhibitors have been shown to have pronounced therapeutic activity in patients with cancer. For instance, imatinib mesylate and gefitinib, tyrosine kinase inhibitors for the c-abl oncogene 1 nonreceptor tyrosine kinase (ABL) and epidermal growth factor receptor (EGFR), improve the outcome for patients who have chronic myeloid leukemia that is positive for the BCR (breakpoint cluster region protein)–ABL fusion kinase[5] and patients who have non–small-cell lung cancer that is associated with EGFR activation,[6]

respectively. Unfortunately, however, a fraction of the target tumors are either refractory to corresponding tyrosine kinase inhibitors from the start of treatment or become resistant after an initial response.

In a case of EML4-ALK–positive non–small-cell lung cancer that became resistant to crizotinib after successful treatment for 5 months, we have discovered two de novo mutations in EML4-ALK, each of which confers resistance to the drug.

CASE REPORT

The patient was a 28-year-old man without a history of smoking who had received a diagnosis of lung adenocarcinoma, at a tumor–node–metastasis (TNM) clinical stage of T4N3M1, in April 2008. Given that the tumor did not harbor any EGFR mutations, the patient was treated with conventional chemotherapy. However, his tumor progressed after six cycles of three two-drug combinations. In November 2008, the presence of *EML4-ALK* variant 1 messenger RNA (mRNA)[1] in the tumor was confirmed by means of reverse transcription–polymerase-chain-reaction (PCR) analysis of a sputum sample. At this stage, the patient had large tumor nodules in the hilum of the right lung, multiple enlarged lymph nodes in the mediastinum, atelectasis in the right lung, and a massive effusion in the right pleural cavity (Fig. 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

The patient was enrolled in the A8081001 study of crizotinib (ClinicalTrials.gov number, NCT00585195) on November 28, 2008, with oral administration of the drug at a dose of 250 mg twice per day. Within 1 week after the start of crizotinib treatment, his symptoms improved markedly. Although he had a partial response to the treatment, his pleural effusion was not completely eradicated (Fig. 1 in the Supplementary Appendix). After 5 months of treatment, however, the tumor abruptly started to grow again, resulting in a rapid expansion of the pleural effusion and in the development of tumors in both lungs (Fig. 1 in the Supplementary Appendix). The patient was withdrawn from the trial on May 25, 2009, and a sample of the pleural effusion in the right lung was then obtained for molecular analysis.

METHODS

DNA sequencing and characterization of the EML4-ALK mutants are described in detail in the Supplementary Appendix.

RESULTS

Because our patient's tumor resumed growth despite sustained administration of the ALK inhibitor crizotinib, we speculated that it might have acquired secondary genetic changes that confer resistance to the drug. Furthermore, given that resistance to tyrosine kinase inhibitors often results from acquired mutations within the target kinases,[7-9] we first examined the possibility that EML4-ALK itself had undergone amino acid changes.

Molecular analysis was performed on sputum specimens obtained before crizotinib treatment and pleural-effusion specimens obtained after relapse when treatment was stopped. Given that the proportion of tumor cells in the two types of specimens may have differed, we performed deep (high-coverage) sequencing of *EML4-ALK* complementary DNA (cDNA) derived from the specimens, using a high-throughput sequencer (Genome Analyzer II, Illumina) (Fig. 2 in the Supplementary Appendix). The sensitivity of our sequencing system, examined with the use of cDNA corresponding to the Janus kinase 3 (JAK3) amino acid mutation V674A[10] as a control, revealed that the maximum detection sensitivity was no more than one mismatched read per 6.50×10^5 total reads (Table 1 in the Supplementary Appendix).

Using deep sequencing, we detected a known single-nucleotide polymorphism, rs3795850, in the cDNA from the four specimens that were positive for *EML4-ALK* (Table 2 and Fig. 3 in the Supplementary Appendix). In addition, a T→C change at a position corresponding to nucleotide 4230 of human wild-type *ALK* cDNA (GenBank accession number, NM_004304) was detected at a low frequency (8.9%) in the sputum cDNA from our patient. Furthermore, two new alterations, G→A and C→A changes at positions corresponding to nucleotides 4374 and 4493 of wild-type *ALK* cDNA, were detected at frequencies of 41.8% and 14.0%, respectively, in the patient's pleural-effusion cDNA. There were no other recurrent alterations (present in 5% of reads) in the kinase-domain cDNA derived from any of the specimens.

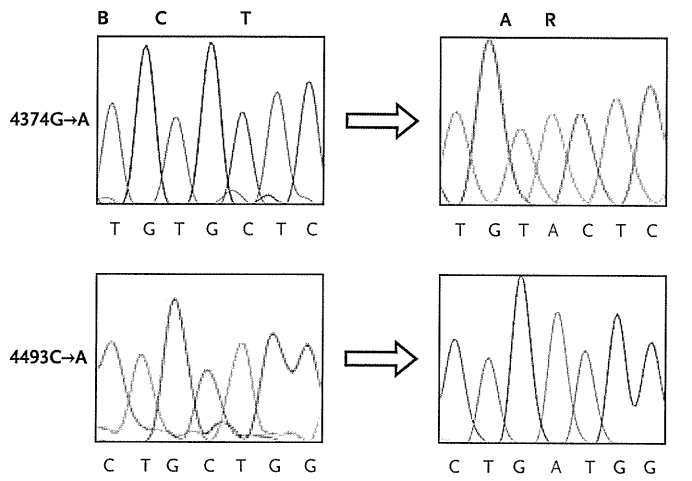

Figure 1. Secondary Mutations within *EML4-ALK*.

Electrophoretograms are shown for *EML4-ALK* cDNA clones prepared from sputum specimens obtained from our patient before crizotinib treatment and from pleural-effusion specimens obtained after relapse. The 4374G→A and 4493C→A mutations are present in the specimens obtained after relapse.

We next attempted to confirm these nucleotide changes by using Sanger sequencing. To rule out the possibility that the mutations had occurred in endogenous wild-type *ALK* rather than in *EML4-ALK*, we performed PCR with a forward primer targeted to *EML4* cDNA so that only the fusion cDNA would be amplified (Fig. 2 in the Supplementary Appendix). We did not detect the 4230T→C change among the 256 fusion cDNA clones derived from the patient's sputum specimens (data not shown), indicating that it was an artifact of the initial PCR or the deep-sequencing step. We did, however, readily confirm both 4374G→A and 4493C→A changes. Among 73 *EML4-ALK* cDNA clones from the patient's pleural-effusion specimens, 34 (46.6%) were positive for 4374G→A and 11 (15.1%) were positive for 4493C→A (Fig. 1). (The remaining 28 [38.4%] were negative for both point mutations.) These rates of detection are similar to those from the deep sequencing of *ALK*, indicating that wild-type *ALK* mRNA was present at a low level in lung tissue, as reported previously.[1]

The PCR analyses covered both nucleotide positions, yet none of the patient's specimens contained both mutations, indicating that each mutation occurred independently. Genomic fragments encompassing the 4374G and 4493C positions were also amplified by means of a PCR assay and were then subjected to nucleotide sequencing, which confirmed the presence of each of the two mutations in the tumor genome (Fig. 4 in the Supplementary Appendix).

The 4374G→A and 4493C→A substitutions result in cysteine→tyrosine (C→Y) and leucine→methionine (L→M) changes at the positions corresponding to amino acids 1156 and 1196, respectively, of wild-type human ALK (Fig. 2 in the Supplementary Appendix). We examined whether such amino acid changes affect the sensitivity of EML4-ALK to ALK inhibitors.

Cells of the mouse interleukin-3–dependent cell line BA/F3 that were made to individually express primary EML4-ALK and secondary mutant EML4-ALK (with the C1156Y or L1196M mutation) were exposed to ALK inhibitors. Crizotinib inhibited the growth of BA/F3 cells expressing primary EML4-ALK, in a concentration-dependent manner (Fig. 2A). In contrast, cells expressing either the C1156Y or L1196M mutant form manifested a markedly reduced sensitivity to the drug. Cells expressing the L1196M mutant form of EML4-ALK were more resistant to crizotinib than were those expressing the C1156Y mutant form (Fig. 2A, and Fig. 5 in the Supplementary Appendix).

We also examined whether cells expressing these EML4-ALK mutants are also refractory to other ALK inhibitors. A 2,4-pyrimidinediamine derivative (PDD) has a median inhibitory concentration for ALK of less than 10 nM,[11] and oral administration of PDD has been shown to eradicate lung-cancer nodules in transgenic mice with EML4-ALK expression.[4] BA/F3 cells expressing EML4-ALK with either the C1156Y or L1196M mutation were markedly less sensitive to PDD than were those expressing the primary EML4-ALK (Fig. 2A). Thus, although these mutations appear to develop during clinical treatment with crizotinib, their generation probably renders EML4-ALK resistant not only to crizotinib but also to other ALK inhibitors. In contrast to the resistance profile for crizotinib, BA/F3 cells expressing the EML4-ALK C1156Y mutant form were slightly more resistant to PDD than were those expressing the L1196M mutant form (Fig. 2A, and Fig. 6 in the Supplementary Appendix), indicating that the resistance profiles for the two mutations may be, in part, inhibitor-dependent, as was previously shown for BCR-ABL mutants.[12]

We examined tyrosine phosphorylation of

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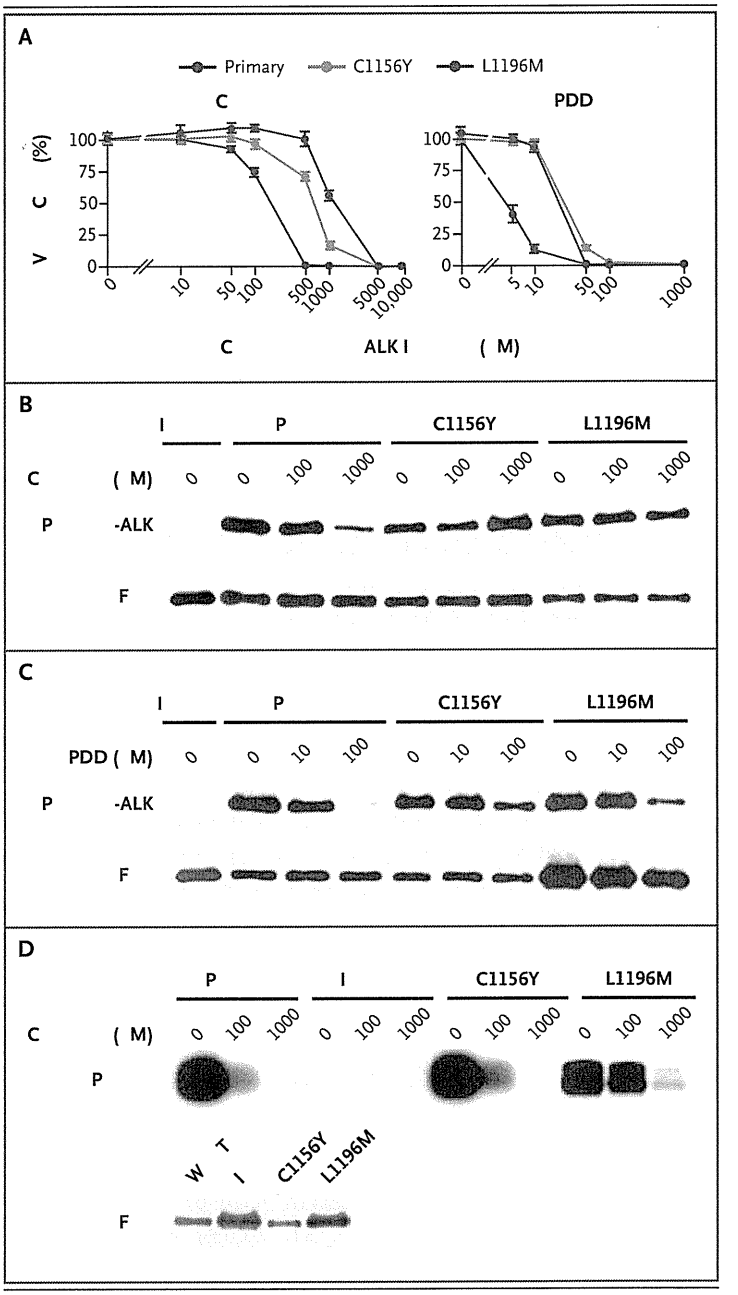
Panel A shows the percentage of viable BA/F3 cells expressing primary EML4-ALK, EML4-ALK with the C1156Y mutation, or EML4-ALK with the L1196M mutation, after 5×10^5 cells were incubated for 48 hours with the indicated concentration of crizotinib (left) or 2,4-pyrimidinediamine derivative (PDD) (right). Data are expressed as the mean value, from three separate experiments, for the percentage of cells expressing primary EML4-ALK after incubation in the vehicle (dimethyl sulfoxide) only. The I bars indicate standard deviations. Because primary EML4-ALK, EML4-ALK with the C1156Y mutation, and EML4-ALK with the L1196M mutation each abrogate the interleukin-3 dependence of BA/F3 cells, the assays were performed in the absence of the interleukin. Panels B and C show the effect of ALK inhibitors on EML4-ALK and its secondary mutant forms, tagged with the Flag epitope, in BA/F3 cells. Panel B shows the results of exposure to various concentrations of crizotinib for 15 hours, after which EML4-ALK was immunoprecipitated from cell lysates with antibodies against the Flag epitope and the immunoprecipitate was subjected to immunoblot analysis with the use of antibodies specific for ALK phosphorylated at the tyrosine at position 1604 (Phospho-ALK) or for the Flag epitope. Cells expressing an inactive mutant form of EML4-ALK were examined as a negative control. Panel C shows the results of a similar experiment, involving PDD instead of crizotinib. Panel D shows the results of an in vitro kinase assay for Flag-tagged EML4-ALK or its secondary mutants immunoprecipitated from BA/F3 cells with antibodies against the Flag epitope. The immunoprecipitates were incubated with [γ-^{32}P]ATP, a synthetic peptide, and various concentrations of crizotinib (top). Separate immunoprecipitate samples were subjected to immunoblot analysis with antibodies against the Flag epitope (bottom).



EML4-ALK by means of immunoblot analysis, using antibodies specific for ALK phosphorylated at the tyrosine at position 1604. The exposure of BA/F3 cells to crizotinib markedly inhibited the tyrosine phosphorylation of EML4-ALK but did not substantially affect that of the C1156Y and L1196M mutants (Fig. 2B). Exposure to PDD also inhibited the tyrosine phosphorylation of EML4-ALK, in a concentration-dependent manner, with a lesser effect on the mutants (Fig. 2C). The results of an in vitro kinase assay were consistent with these findings, showing pronounced inhibition of the enzymatic activity of primary EML4-ALK with crizotinib, whereas the effect on the C1156Y mutant was less pronounced and the effect on the L1196M mutant was much less pronounced (Fig. 2D).

Figure 3 shows the cysteine at position 1156 (C1156) and the leucine at position 1196 (L1196) of the kinase domain of ALK.[13] C1156 is positioned adjacent to the N-terminal of the predicted helix αC as well as close to the upper edge of the ATP-binding pocket. No activating mutations have been reported at this position in other tyrosine kinases in cancer specimens. L1196 of ALK corresponds to the threonine at position 315 in ABL and at position 790 in EGFR, each of which is the site of the most fre-

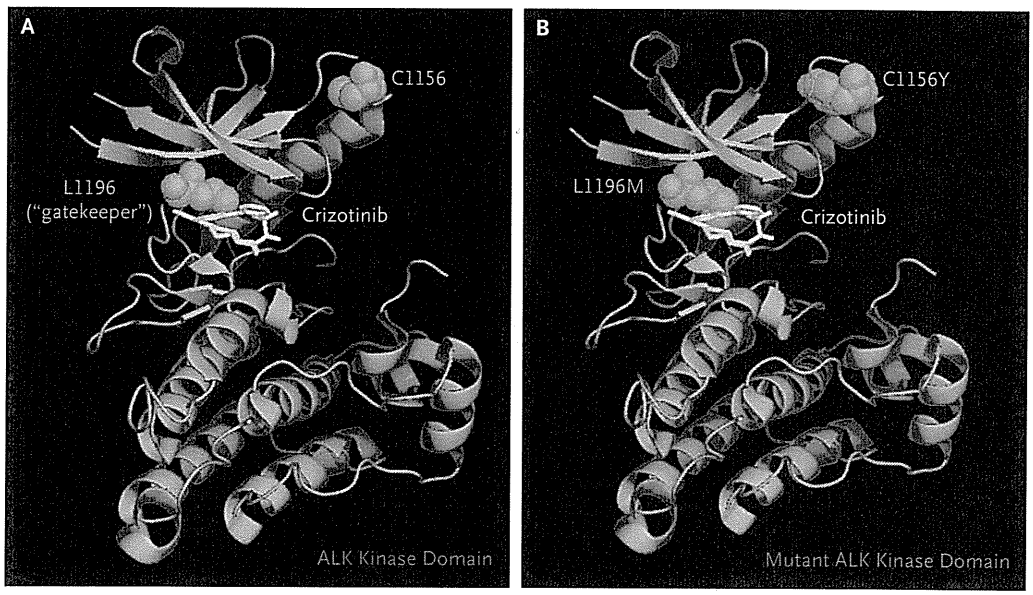


Figure 3. Predicted Crystal Structure of the Kinase Domain of ALK.

Panel A shows the nonmutant kinase domain and the site of binding of crizotinib. Panel B shows the EML4-ALK secondary mutations at positions 1156 and 1196. Adapted from Bossi and colleagues.[13]

quently acquired mutations that confer resistance to tyrosine kinase inhibitors in these kinases (Fig. 7 in the Supplementary Appendix).[14,15] This site is located at the bottom of the ATP-binding pocket (Fig. 3), and the presence of an amino acid with a bulky side chain at this "gatekeeper" position may interfere with the binding of many tyrosine kinase inhibitors.[7,16]

DISCUSSION

We identified two de novo mutations within the kinase domain of EML4-ALK from the tumor of a single patient that confer resistance to multiple ALK inhibitors. Given that we did not detect any *EML4-ALK* cDNA harboring both mutations, we propose that each mutation developed independently in distinct subclones of the tumor. Because we were not able to examine pleural-effusion specimens from the patient before he received crizotinib treatment, we do not know whether the resistant clones were present initially or developed secondarily, during the treatment.

Amino acid substitutions at the gatekeeper position of several tyrosine kinases have been detected in tumors treated with tyrosine kinase inhibitors (Fig. 7 in the Supplementary Appendix).[7-9,17,18] Whereas no mutations at this site have previously been reported for EML4-ALK or ALK, the effects of various artificial amino acid substitutions at the gatekeeper position of nucleophosmin (NPM)–ALK, another fusion-type "oncokinase" form of ALK, were recently examined.[19] The findings were consistent with the results of our analysis of tumor cells in vivo: the introduction of methionine at this position rendered NPM-ALK resistant to ALK inhibitors. It is therefore likely that gatekeeper alterations constitute a universal mechanism for the acquisition of tyrosine kinase–inhibitor resistance in oncogenic tyrosine kinases.

In contrast to gatekeeper substitutions, activating mutations at the position adjacent, on the N-terminal side, to the αC helix (e.g., C1156 in ALK) have not been confirmed for other tyrosine kinases in cancer specimens. Though a T→I change at the corresponding position of EGFR was described in one case of non–small-cell lung cancer, its relevance to drug sensitivity was not examined.[16] The importance of helix αC for allosteric regulation of enzymatic activity has been shown, however, for serine–threonine kinases.[20] A change at C1156 of ALK might therefore interfere allosterically with the binding of tyrosine

kinase inhibitors. Determination of the crystal structure of the ALK kinase domain with the C1156Y or L1196M mutation should shed light on these matters, as well as provide a basis for the development of next-generation ALK inhibitors that may effectively eradicate tumors harboring EML4-ALK with the acquired mutations.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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original article

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Bone metastasis and poor performance status are prognostic factors for survival of carcinoma of unknown primary site in patients treated with systematic chemotherapy

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Background: Cancer of unknown primary site (CUP) generally has a poor prognosis, and there is no established standard therapy. There have been no reports of a prognostic model for CUP patients treated with a single regimen of systemic chemotherapy.

Methods: Univariate and multivariate prognostic factor analysis for overall survival (OS) were conducted retrospectively in 58 consecutive CUP patients treated with carboplatin plus paclitaxel (Taxol) therapy as a first-line treatment.

Results: Univariate prognostic factor analysis revealed baseline performance status (PS) of two or more, low serum albumin level, pleural effusion, bone metastasis, and liver metastasis as adverse prognostic factors. Cox proportional hazards analysis showed that poor PS and bone metastasis had the most powerful adverse impact on survival. We developed a prognostic model using those two variables—a good-risk group (PS 0–1 without bone metastasis) and a poor-risk group (PS ≥2 or bone metastasis). The poor-risk group showed significantly poorer OS than the good-risk group (1 year OS 36.8% versus 67.1%, $P = 0.0003$).

Conclusions: Poor PS and bone metastasis were identified as independent adverse prognostic factors in CUP. A simple prognostic model was developed and seems useful for decision making as to whether chemotherapy is indicated for CUP patients.

Key words: cancer of unknown primary site, carboplatin plus paclitaxel, bone metastasis

introduction

Cancer of unknown primary site (CUP) is pathologically diagnosed metastatic carcinoma in which no obvious primary site is identified with a conventional work-up. It is not a rare clinical entity, accounting for 3%–5% of all solid malignancies [1, 2]. The prognosis of CUP is generally considered poor, with median survival ~6–12 months [3]. Briasoulis et al. [4] reported encouraging results from phase II data of carboplatin and paclitaxel combination therapy for patients with CUP. In this study, the overall response rate by an intention-to-treat analysis was 38.7%, and median overall survival (OS) was 13 months at median follow-up time of 28 months. Platinum and taxane combination therapy is now widely used in clinical practice [4–8], but recent multiple-treatment meta-analysis showed that no type of chemotherapy has been proven to prolong survival in patients with CUP [9]. CUP consists of heterogeneous neoplasms with variable biological features, making it difficult to identify clinically useful prognostic survival factors. But several subsets have been identified requiring a specific treatment and having a better prognosis. Women with peritoneal carcinomatosis of serous adenocarcinoma [10], women with adenocarcinoma of axillary lymph nodes [11] or cervical lymph node metastasis of squamous cell carcinoma [12], young adults with poorly differentiated carcinoma of midline distribution [13], and undifferentiated carcinoma with neuroendocrine features [14] are CUP subgroups known to have a better prognosis. But the majority of CUPs have a poor prognosis, as mentioned above. In this article, we report the results of a prognostic factor analysis conducted in a population of 58 patients of CUP treated with carboplatin and paclitaxel as a first-line systemic chemotherapy. We retrospectively investigated baseline characteristics as prognostic factors for survival to identify a subset of patients who would benefit from chemotherapy.

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methods

patient characteristics

The medical and pathological records of 58 consecutive newly diagnosed patients with CUP who received carboplatin and paclitaxel (Taxol, Bristol-Myers Squibb, Tokyo, Japan) combination therapy as first-line therapy at the Cancer Institute Hospital, Japanese Foundation for Cancer Research, from March 2004 to January 2008 were retrospectively reviewed. Patients had pathologically confirmed metastatic cancer and were surveyed for detailed medical history, complete physical examination, blood counts, chemistry profile, chest radiograph, computed tomography (CT) scan of chest and abdomen, and further radiological survey or endoscopy of suspected areas. Serum prostate-specific antigen (PSA) was measured in male patients, and CA 125 was measured in female patients. Women with adenocarcinoma of axillary lymph nodes also received mammography and breast ultrasound. Young adults with poorly differentiated adenocarcinoma involving the mediastinal region were surveyed with α-fetoprotein and β-human chorionic gonadotropin. The gastrointestinal tracts of male and female patients with adenocarcinoma involving abdominal and pelvic lesion were surveyed by upper gastrointestinal endoscopy and colonoscopy. Gynecologic examination was carried out in female patients with abdominal and pelvic disease. Patients with squamous cell carcinoma of cervical lymph nodes also underwent laryngeal endoscopy and upper gastrointestinal endoscopy. Bone metastases were assessed by the combination of bone scintigraphy or positron emission tomography with chest X-ray, CT, or magnetic resonance imaging. Histopathological review including immunohistochemistry (IHC) was carried out to detect primary sites and to exclude other malignancies. Low-molecular cytokeratins (CKs) 7 and 20 were routinely stained for all patients with CUP, and thyroid transcription factor 1, caudal type homeobox transcription factor 2, and PSA were stained for patients with adenocarcinoma of CUP. When a specific origin was suspected by morphological examination and clinical history, distinctive IHC was carried out (chromogranin, synaptophysin, and CD56 for neuroendocrine cell carcinoma; D2-40, placental alkaline phosphatase, human chorionic gonadotropin, and CD30 for germ-cell tumor; and D2-40 and calretinin for mesothelioma). In the case of difficulty in diagnosing epithelial carcinoma, several IHC of S100, vimentin, leukocyte common antigen, and CKs are used for distinguishing melanoma, sarcoma, and lymphoma from the anaplastic cell type of carcinoma.

We excluded patients in favorable subsets that have specific treatments other than carboplatin and paclitaxel—such as women with adenocarcinoma of axillary lymph nodes or cervical lymph node metastasis of squamous cell carcinoma, young adults with poorly differentiated carcinomas of midline distribution, and patients with undifferentiated carcinomas of neuroendocrine features. However, women with peritoneal carcinomatosis of adenocarcinoma who were treated with carboplatin and paclitaxel as first-line treatment were included in this study.

treatment

Carboplatin was administered by a 2-h i.v. infusion, dosed with 6 mg/ml/min target area under the free carboplatin plasma concentration versus time curve and was followed by paclitaxel 200 mg/m^2 in 500 ml of normal saline administered over 3 h. The Calvert formula was used for carboplatin dosing, on the basis of a glomerular filtration rate calculated by the Cockcroft–Gault equation using serum creatinine, body surface area, and age. Chemotherapy cycles were repeated every 3 weeks and responding patients continued the chemotherapy until disease progression or intolerable toxicity. Response to chemotherapy was assessed by Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0). Progression-free survival (PFS) and OS were calculated from day 1 of the first cycle of chemotherapy.

statistical analysis

Survival curves were estimated using the Kaplan and Meier method, compared using the log-rank test, and prognostic factors were identified by univariate analysis. Then the forward stepwise Cox proportional hazards analysis was carried out to identify independent prognostic factors. Statistical analyses were carried out using SPSS software (version 17.0; SPSS Inc., Chicago, IL).

results

patient characteristics

Patient characteristics are shown in Table 1. Fifty-eight CUP patients treated with at least one cycle of carboplatin and paclitaxel combination therapy were retrospectively analyzed. Twenty-eight (48.3%) patients were male, and the median age was 64 years (range 28–79 years). Forty-nine patients (84.5%) had a good performance status (PS) of zero to one. Twenty-six (44.8%) patients had well-differentiated adenocarcinoma, 21 (36.2%) patients had anaplastic or poorly differentiated carcinoma, and 5 patients (8.6%) had squamous cell carcinoma. Another six (10.3%) patients had clear-cell carcinoma, transitional cell carcinoma, or adenosquamous cell carcinoma. Metastatic sites are listed in Table 1. Lymph nodes, lung, bone, and liver were frequently involved sites and cervical, mediastinum, and retroperitonium were common sites for lymph node metastasis.

PSA was measured in 20 male patients (median PSA level 2.04 ng/ml, range 0.34–4.04 ng/ml), and CA 125 was obtained in 26 female patients (median CA 125 level 462 U/ml, range 4.8–50000 U/ml). Five of six male patients with bone metastasis showed a PSA level <4.0 ng/ml, and the PSA value before treatment of one young male patient was not available.

outcome of chemotherapy

A total of 315 cycles were administered, and patients received a median of five cycles of treatment (range 1–21 cycles).

Table 1. Patient characteristics

Number of patients	58
Age, median (range)	64 (28–79)
Sex	
Male	28
Female	30
Performance status	
0–1	49
2–4	9
Pathology	
Adenocarcinoma	26
Squamous cell carcinoma	5
Poorly differentiated/anaplastic carcinoma	21
Other	6
Sites of metastasis	
Lung	15
Bone	13
Liver	11
Pleural effusion	15
Ascites	11
Lymph node	44

The response rates by main histopathological types of adenocarcinoma, squamous cell carcinoma, poorly differentiated carcinoma, or poorly differentiated adenocarcinoma were 42.3%, 60.0%, and 23.8%, respectively (Table 2). For other histology types, one patient with transitional cell carcinoma had partial response. Sixteen patients were treated with second-line chemotherapy. At a median follow-up time of 12 months (range 6–1659 days), median OS and PFS were 16.7 months and 5.9 months, respectively. Six patients had PFS >2 years and one of these patients survived >4 years.

prognostic model of clinical and biological variables

The outcome of univariate analysis of clinical and biological factors is listed in Table 3. Five parameters have prognostic relevance: poor PS (≥2) ($P = 0.01$), low serum albumin level (<3.7 g/dl) ($P = 0.003$), pleural effusion ($P = 0.04$), bone metastasis ($P = 0.02$), and liver metastasis ($P = 0.02$). Multivariate analysis for these five variables was conducted and showed that bone metastasis ($P = 0.002$) and PS of two or more ($P = 0.016$) had significant adverse impact for survival (Table 3). Poor PS was not correlated with presence of bone metastasis.

Table 2. Treatment results

	N	CR (*n*)	PR (*n*)	ORR (%)
Total	58	5	15	34.5
Pathology				
Adenocarcinoma	26	5	6	42.3
Squamous cell carcinoma	6	0	3	50.0
Poorly differentiated anaplastic carcinoma	21	0	5	23.8

CR, complete response; PR, partial response; ORR; overall response rate.

Table 3. Univariate and multivariate analysis of prognostic factors for survival

	Univariate	Multivariate	
	P value	HR (95% CI)	*P* value
PS ≥2	0.01	2.93 (1.22–7.04)	0.016
Age (>65 years)	0.29		
Sex (male)	0.41		
ALP (>UNL)	0.13		
LDH (>UNL)	0.45		
ALB (<3.7 g/dl)	0.003		
Hb (<11.0 g/dl)	0.77		
Pleural effusion	0.04		
Ascites	0.69		
Lung metastasis	0.58		
Bone metastasis	0.02	3.48 (1.56-7.78)	0.002
Liver metastasis	0.02		
Adenocarcinoma	0.81		
Poorly/anaplastic carcinoma	0.32		

HR, hazard ratio; CI, confidence interval; PS, performance status; ALP, alkaline phosphatase; UNL, upper normal limit; LDH, lactate dehydrogenase; ALB, albumin; Hb, hemoglobin.

The incidence of bone metastasis was not significantly different between males and females (6 of 28 males, 5 of 30 females). A prognostic model was developed with those two variables. Nineteen (32.8%) patients were assigned to the good-risk group (defined as PS 0–1 without bone metastasis), and 38 (67.2%) patients were assigned to the poor-risk group (defined as PS ≥2 or bone metastasis). The poor-risk group ($n = 19$) showed significantly poorer OS than good-risk group ($n = 39$) (1 year OS 36.8% versus 67.1%, $P = 0.0003$) (Figure 1).

discussion

To identify a favorable or poor prognostic group of patients with CUP is of great concern when physicians consider whether systemic chemotherapy is indicated. No randomized trial showed better survival with chemotherapy than best supportive care. To our knowledge, the current study is the first that assesses prognostic factors for survival of patients with CUP treated with a single first-line regimen and should give us information as we choose an optimal therapy.

We demonstrated an overall response rate of 34.5% and a median OS of 16.7 months in CUP patients by an intention-to-treat analysis. This result seems similar to the results previously reported by Briasoulis et al. [4] and slightly better than other reports. One reason might be that both studies included female patients with peritoneal carcinomatosis (11 of 58 in ours and 19 of 75 in Briasoulis et al.). In our study, seven women (63.6%) responded to chemotherapy. A second reason might be that our group included a marginally larger number of patients with good PS. Golfinopoulos et al. [9] reported in recent multiple-treatment meta-analysis for CUP that 10 randomized trials assessed in that study included variable rates for patients with poor PS (median 24.5%, interquartile range 12.8%–38.9%). Third, our study included a slightly smaller number of patients

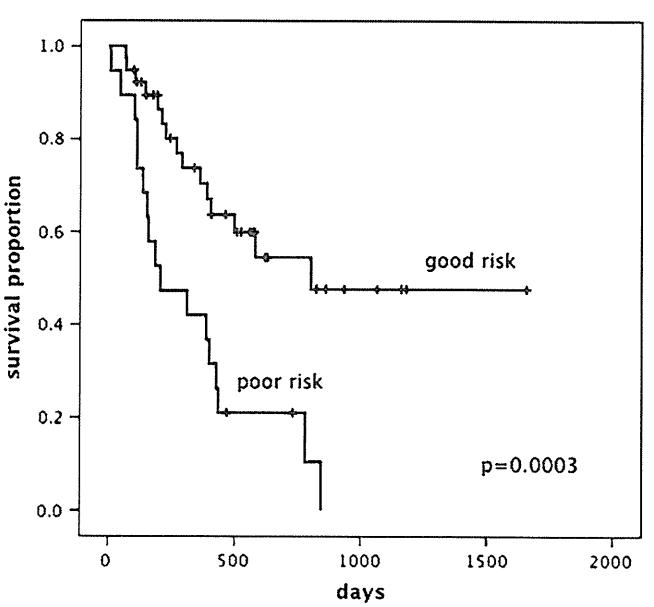

Figure 1. The prognostic model incorporating two variables. The good-risk group ($n = 39$) was defined as performance status (PS) of zero to one without bone metastasis and the poor-risk ($n = 19$) group as PS of two or more, or bone metastasis.

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with liver metastasis, which was reported as an independent poor prognostic factor by Seve et al. [15]. But the rates of liver metastases in previous studies are variable from 16% to 76% [4–8, 16, 17]. The patients in the present study were treatable with combination therapy, so most of them maintained good PS and end organ function. No prospective studies or meta-analysis of prognostic factors for CUP have been published. But several retrospective studies have shown a number of independent adverse factors such as age, male gender, poor PS, adenocarcinoma histology, number of metastatic sites, liver metastasis, bone metastasis, lung metastasis, pleural metastasis, brain metastasis, comorbidity scoring of adult comorbidity evaluation-27 (ACE-27), low serum albumin, high serum lactate dehydrogenase (LDH), high serum alkaline phosphatase, lymphopenia, anemia, thrombocytopenia, high serum carcinoembryonic antigen, and high serum CA 125 [15, 18–20]. Abbruzzese et al. [18] reported adverse prognostic variables from a study of 657 cases of CUP at M. D. Anderson Cancer Center, and multivariate analysis identified male gender, a large number of metastatic sites, adenocarcinomatous histological type, and the presence of liver metastasis as unfavorable indicators. Culine et al. [19] proposed a simple prognostic model using PS and serum LDH levels in a population of 150 CUP patients, excluding favorable subsets, at a French cancer center. More recently, Seve et al. conducted a retrospective study assessing the influence of comorbidities, age, PS, and chemotherapy on survival in a population of 389 patients with CUP in Canada. Multivariate analysis showed that patients who had a PS of two or more and a high overall ACE-27 score had a poor prognosis. They concluded that the impact of comorbidity on survival was limited to patients with low PS [20]. The same author showed in another study that low serum albumin level and liver metastasis were the two most powerful adverse prognostic factors. The prognostic significance of those two factors was validated in another set of 124 patients with CUP [15]. In our study, bone metastases and poor PS (≥2) had a powerful adverse impact on survival. In clinical practice, bone metastases could be the cause of declining PS, but in this study, bone metastases and poor PS were not significantly correlated. Poor PS was also an adverse prognostic factor in studies by Culine et al. and by Seve et al. Bone metastases have been identified as an independent poor prognostic factor for the first time in uniformly treated patients with CUP. Prognostic significance of bone metastases in advanced cancer depends on the primary sites. In breast cancer or prostate cancer, the presence of bone metastases or bone-only metastases indicates a better prognosis [21]. On the other hand, the presence of bone metastases indicates a worse prognosis in lung cancer [22], thyroid cancer [23], or renal cell carcinoma [24]. The worse prognosis of patients with bone metastases in our series might be due to the apparent absence of occult breast cancer or prostate cancer in this set of patients.

Although our study might be small for finding independent prognostic factors retrospectively, it is important to identify clinically useful prognostic factors for CUP patients treated with platinum and taxane combination therapy, which are used frequently in daily practice. It has not been proven that systemic chemotherapy would prolong the survival of unfavorable CUP patients, and the best supportive care is a reasonable choice for patients who have little benefit from systemic chemotherapy.

We designed a new prognostic model that incorporated those two factors, poor PS and bone metastasis. The OS of patients with at least one or more prognostic factor was significantly shorter than those with no adverse prognostic factor. This model might be useful for decision making regarding the use of chemotherapy for CUP patients in daily clinical practice. A validation study of our prognostic model is warranted in the near future.

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disclosure

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Tumor Size Is a Potential Predictor of Response to Tyrosine Kinase Inhibitors in Renal Cell Cancer

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OBJECTIVES To investigate the correlations between the initial tumor size and size reduction rate in patients treated with targeted agents. To select the patients who can benefit the most from treatment with targeted agents, it will be necessary to find a tumor characteristic that predicts their effectiveness.

METHODS The data from 139 metastatic and 16 primary lesions treated with the targeted agents were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions in 54 patients with metastatic renal cell carcinoma who had undergone treatment from April 2008 to July 2010. The relationship between the longest tumor diameter at baseline and the rate of reduction in tumor size was assessed using the Spearmancorrelation test.

RESULTS A linear, moderate to strong association between the initial tumor size and tumor size reduction rate was shown (correlation coefficient -0.441, $P < .001$). When these tumors were divided into 2 groups at the threshold value (23.95 mm), which was decided by the receiver operating characteristic curve analysis, the smaller tumors demonstrated a significantly greater size reduction than the larger tumors according to the Mann-Whitney U test ($P < .001$). Both univariate and multivariate linear regression analyses revealed that only the initial tumor size was associated with the rate of reduction in individual tumors ($P < .001$).

CONCLUSIONS The initial tumor size was a good predictor of the tumor size reduction. This simple observation could be useful for physicians who treat patients with metastatic renal cell carcinoma. In addition, in assessing clinical trials of targeted agents for metastatic renal cell carcinoma using the ResponseEvaluation Criteria in Solid Tumors, perhaps this association should be considered. UROLOGY xx: xxx, xxxx. © 2011 Elsevier Inc.

Surgical excision remains the standard and, indeed, the only curative therapy for patients with localized renal cell carcinoma (RCC). However, at the initial diagnosis, one third of patients with RCC will have visceral metastasis, and one half of the remainder will eventually develop distant metastases.[1] Previously, despite its limited clinical activity and significant toxicity, cytokine-based therapy was the mainstay treatment of metastatic RCC (mRCC).[1,2] A better understanding of the molecular biology of RCC has identified signaling pathways related to a hypoxia-inducible factor as rational targets, including the receptors of vascular endothelial growth factor and the mammalian target of rapamycin kinase for anticancer therapy for patients with mRCC.[3]

Because the agents aimed at these molecular targets have demonstrated significant objective responses with moderate and easily manageable toxic effects, a major breakthrough in the treatment paradigm for mRCC has occurred. Among them, sorafenib (Nexavar, Bayer Pharmaceuticals Corporation, West Haven, CT) and sunitinib (Sutent, Pfizer Inc., New York, NY) are tyrosine kinase inhibitors (TKIs), and target vascular endothelial growth factor receptors and platelet-derived growth factor receptors.[4,5]

As other new agents with alternative molecular targets emerge in RCC therapy, to select the patients who can benefit the most from these vascular endothelial growth factor receptor-targeted agents, it is necessary to find a biomarker or tumor characteristic that can predict their effectiveness. Because these agents function as angiogenesis inhibitors,[4,5] the initial tumor size and volume might be important in whether tumors can be expected to shrink using these treatments. Initially, we hypothesized

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that the initial tumor size would be inversely associated with the tumor size reduction rate. In the present study, we investigated the relationship between the initial tumor size and the tumor size reduction rate of patients treated with TKIs.

MATERIAL AND METHODS

Patient and Treatment

The data from 139 metastatic and 16 primary lesions treated with targeted therapeutics were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions from 54 patients with mRCC who had undergone treatment at our hospital from April 2008 to July 2010. Each patient signed an institutional review board-approved protocol-specific informed consent form in accordance withnational and institutional guidelines. Sunitinib was administered orally at a dose of 50 mg/d, consistingof 4 weeks of treatment followed by a 2-week rest period. Sorafenib was administered orally at a continuous dose of 800 mg/d. Dose reductions of sunitinib (to 37.5 mg and then to 25 mg) and sorafenib (to 400 mg/d and then to 400 mg every other day) were performed, depending on the type and severity of the adverse events. All the target lesions were evaluated using multidetector computed tomography (CT) (Lightspeed Pro16, GE Healthcare Japan, Tokyo, Japan), which scans every 5 mm. The tumor measurements were performed by the physicians in charge of the respective patients in clinical practice and calculated separately for the response in the individual primary or metastatic sites. The response was assessed by multidetector CT at least every 2 cycles of treatment, according to the ResponseEvaluation Criteria in Solid Tumors, version 1.0 (RECIST).[6]

Statistical Analysis

To identify an optimal threshold for the prediction of >30% tumor reduction (partial response), receiver operating characteristics analysis was performed by incrementally increasing the cutoff values and recalculating the corresponding true-positive and false-negative rates. The relationship between the longest tumor diameter at baseline the tumor size reduction rate was assessed using the Spearmancorrelation test and the Mann-Whitney *U* test. Independent Student's *t* tests and analyses of variance were used in the univariate analysis for binomial variables, and correlation coefficient analyses were used for continuous variables. Multivariate linear regression analysis was used for the multivariate analysis. Statistical analyses were performed using the Statistical Package for Social Sciences, version 17.0, for Windows (SPSS, Chicago, IL). Two-tailed $P < .05$ was considered significant.

RESULTS

Patient Characteristics

The clinical and pathologic characteristics of the patients treated with TKIs are listed in Table 1. The median follow-up was 12.2 months (range 3.8-29.7). Overall, 16 patients (30%) demonstrated a partial response and 26 (48%) had stable disease according to the RECIST, indicating that 78% of the patients experienced a clinical benefit from these targeted agents. Progression was observed in 9 patients (17%) and early treatment failure before the initial assessment occurred in 3 patients (5%) owing to sorafenib-induced erythema multiforme.[7]

Table 1. Patient characteristics

Characteristic	Patients (n)
Total	54 (100)
Sex	
Male	43 (80)
Female	11 (20)
Age (y)	
Median	62
Range	25-80
ECOG performance status	
0	32 (59)
1	16 (30)
2	6 (11)
Tumor histologic type	
Clear cell	43 (80)
Clear cell plus sarcomatoid components	6 (11)
Papillary	2 (6)
Chromophobe	1 (2)
Xp translocation	1 (2)
Nephrectomy	
Yes	39 (72)
No	15 (28)
Cytokine therapy	
IL-2 and IFN	11 (20)
IFN	20 (37)
None	23 (43)
Tyrosine-kinase inhibitor	
Sunitinib	33 (61)
Sorafenib	21 (39)
Baseline serum laboratory findings	
Hemoglobin (g/dL)	
Median	11.8
Range	6.2-17.7
Corrected calcium (mg/dL)	
Median	9.3
Range	8.5-10.5
Lactate dehydrogenase (U/L)	
Median	173
Range	101-550
C-reactive protein	
Median	0.77
Range	0.03-19.4

ECOG, Eastern Cooperative Oncology Group; IL-2, interleukin-2; IFN, interferon.
Data in parentheses are percentages.

Response to Individual Targeted Lesions

We investigated the objective response of the individual primary or metastatic sites. A total of 155 tumors were examined, including 16 primary kidney lesions and 92 pulmonary, 26 lymph node, 10 liver metastatic, 6 adrenal gland, and 5 soft tissue sites. The mean ± standard deviation tumor size reduction rate was 23.8% ± 56.6%, and the tumor size was reduced by >30% in 103 tumors (66.5%) and >50% in 75 tumors (48.3%).

Correlation Between Initial Tumor Size and Tumor Size Reduction of Individual Targeted Lesions

We investigated the correlation between the initial tumor size and the tumor size reduction rate of the indi-

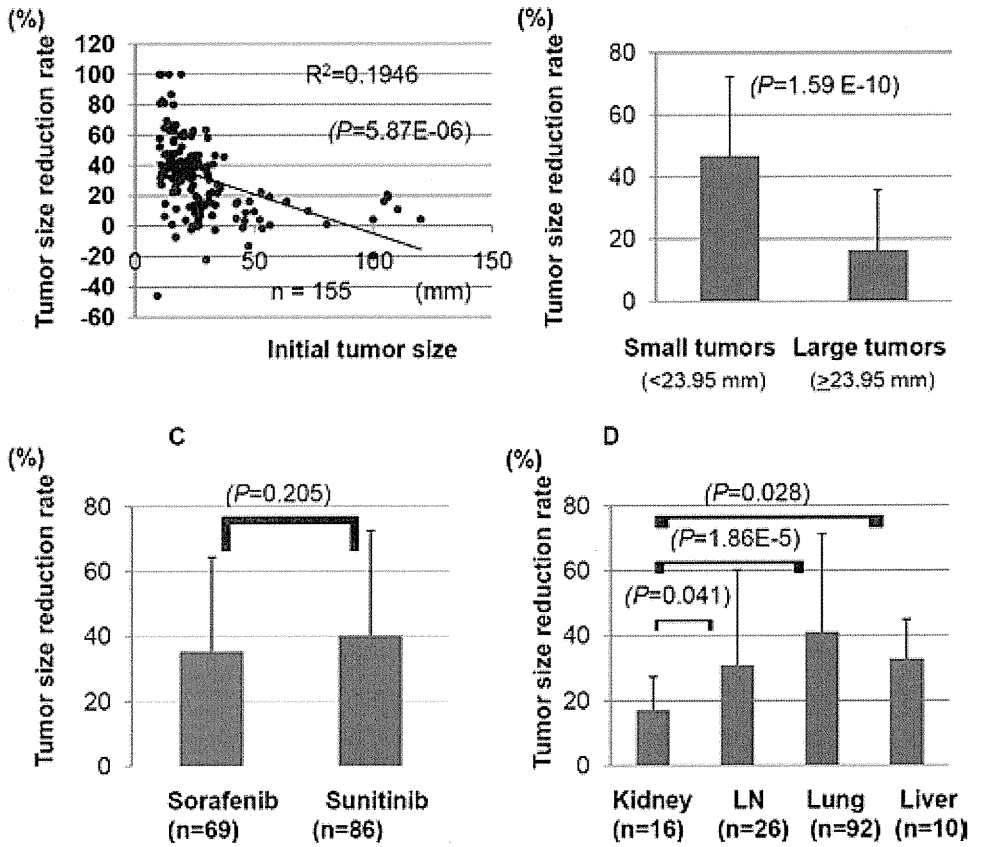

Figure 1. Association between primary tumor size and tumor size reduction. **(A)** Correlations between primary tumor size and tumor size reduction for individual tumor lesions. Smaller tumors demonstrated significant tumor size reduction compared with larger tumors. **(B)** Optimal threshold for prediction of >30% reduction (partial response) was 23.95 mm, as identified by receiver operating characteristics analysis. **(C)** No difference in tumor reduction rate demonstrated between sorafenib and sunitinib. **(D)** Primary kidney tumors demonstrated significantly small tumor size reduction compared with lymph node, metastatic lung, or liver lesions.

vidual targeted lesions. The linear association between the initial tumor size and the tumor size reduction is shown in Figure 1A. The correlation coefficient (r) was −0.441, indicating that a moderate to strong reverse association was confirmed between them ($P < .001$, Fig. 1A). Receiver operating characteristic curve analysis was performed using the clinical criteria of a partial response (>30% reduction) to separate those with and without a response. The area under the receiver operating characteristic curve was 0.814 ± 0.040, and the optimal detection threshold was 23.95 mm, with a sensitivity of 80.0% and specificity of 74.1%. When these tumors were divided into 2 groups at the threshold value (23.95 mm), the smaller tumors demonstrated a significantly greater size reduction than did the larger group ($P < .001$, Fig. 1B). In addition, among these patients, 16 had evaluable primary tumor and metastatic sites, when they were started with TKIs as induction therapy. The initial size of the 16 primary kidney tumor (77.8 ± 27.8 mm) was significantly larger than the metastatic lesions of the same patients (24.0 ± 12.7 mm, $P < .001$). Similarly, the tumor reduction rate of the primary tumor (16.1% ± 17.1%) was also significantly smaller than the metastatic lesions (43.2% ± 26.5%, $P < .001$).

Variables for Tumor Size Reduction

The relationship between the tumor reduction rate and the studied factors was investigated. The studied factors included initial tumor size, disease site, performance status, history of nephrectomy, history of cytokine therapy, TKI used (sunitinib or sorafenib), blood hemoglobin concentration, blood neutrophil count, blood thrombocyte count, serum calcium concentration, and serum lactate dehydrogenase concentration before the administration of TKIs. In the present study, no difference was found between the sorafenib-treated and sunitinib-treated lesions (Fig. 1C). In addition, the tumor reduction rate of the primary kidney was significantly smaller than that of the metastatic lymph node, pulmonary lesion, or liver lesion (Fig. 1D). However, no difference in the reduction rate was seen among the lymph node, lung metastatic, and liver metastatic lesions. On univariate analysis, the initial tumor size and the target organ were associated with the individual size reduction rate (Fig. 1A,B,D). Multivariate linear regression analysis revealed that only

with the individual size reduction rate ($P < .001$).

COMMENT

It has long been proposed that bulky disease is an adverse prognostic factor during chemotherapy for lymphoma or solid cancer.[8,9] In contrast, the significance of bulky disease when using target therapies has not yet been made clear. For instance, many had assumed that antibody therapy would be ineffective against bulky disease; however, a Phase II study of rituximab (anti-CD20 antibody) for bulky (>10 cm) low-grade lymphoma showed that standard rituximab therapy resulted in a good response rate (43%). In addition, however, they showed that the serum antibody concentration correlated negatively with the baseline tumor bulk.[10] Antiangiogenesis therapy had also been assumed to be ineffective against bulky disease, but this has not been clinically proved. We have demonstrated for the first time that the initial tumor size correlated negatively with the tumor reduction rate in targeted therapy for mRCC.

Up-front cytoreductive nephrectomy, followed by systemic therapy, been established as the standard of care for mRCC in the cytokine era.[1,2] Even for the patients with high-risk and locally advanced RCC, neither preoperative nor postoperative medical treatment has been recommended because of the real lack of effective systemic therapiespreviously available. Therefore, the treatment strategy for RCC must be reconsidered in this targeted therapy era.

According to our results, large tumors will seldom become smaller when TKIs are administered. Therefore, it might be infrequent that an unresectable tumor would become resectable, although no objective criteria exist to define surgical resectability. We believe that the greatest benefit of preoperative approaches in the setting of mRCC is that they can as a litmus test to reserve cytoreductive nephrectomy for only those whowill benefit from the procedure.

Possibly, the resolution of CT scans could affect the tumor size reduction rate. Small lesions might appear to shrink more owing to slice variation and not true size changes. However, we used multidetector CT, which scans every 5 mm. The minimal initial tumor size was 10 mm in the present study, because we excluded tumors that were <10 mm in diameter. Therefore, we believe that the potential issues regarding the resolution of the CT scans did not have a major effect on our conclusions. In addition, when micrometastasis is considered to be of a very small size, adjuvant therapy after radical nephrectomy could meet our expectation of reduced recurrence. Sorafenib and sunitinib have been the focus of adjuvant therapy for patients with resected primary tumors with a high risk of recurrence. Three randomized trials are comparing these agents to placebo in the adjuvant setting: Sunitinib Treatment in Renal Adjuvant Cancer (S-TRAC), Adjuvant Sorafenib or Sunitinib for Unfavorable Renal Carcinoma (ASSURE), Primary Renal Cell (SORCE).[11]

Very recently, a study similar to ours was published by Han et al.[12] In their study, the initial tumor enhancement on contrast-enhanced CT could be useful as a clinical predictor during targeted therapy, because it was associated with tumor size reduction of the individual metastases in patients with mRCC who had received targeted therapy.[12] Because of the antiangiogenic therapy, their rationale was quite reasonable. Tumor enhancement was associated not only with tumor size reduction, but also with progression-free survival of the treated patients. However, compared with their study, our study was simpler and could be performed without contrast medium, which can be detrimental to patients with a solitary kidney. In addition, it can be adapted for patients with renal dysfunction, as well as patients who are allergic to contrast medium.

In addition to the treatment paradigm of RCC, our results suggest a weakness in the RECIST, currently the most commonly used system to determine the response in clinical trials and clinical practice. In our study, the longest diameter of large tumor demonstrated a relatively smaller reduction rate than thatof the metastatic small tumor. Therefore, large primary tumors will have an important effect on the overall objective when these are included in the RECIST measurements.Therefore, whether the target lesion includes the large primary and/or metastatic lesions should be considered in calculating the overall response according to the RECIST.

CONCLUSIONS

The initial tumor size was inversely associated with the tumor reduction rate of the individual metastatic sites and primary tumors in patients with mRCC who underwent targeted therapy. Although our small study was preliminary and additional investigations are necessary, we believe that this simple observation might be useful for physicians who treat patients with mRCC, as exemplified by the consideration of the pre- and postoperative approaches. In addition, in assessing clinical trials of targeted agents for metastatic RCC using the RECIST, we might need to consider this association.

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Original Article

Circulating Endothelial Progenitors and CXCR4-Positive Circulating Endothelial Cells Are Predictive Markers for Bevacizumab

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BACKGROUND: Bevacizumab plus chemotherapy is a standard option in the treatment of metastatic colorectal cancer (mCRC). The aim of this study was to investigate the potential of circulating endothelial cell progenitors (CEPs) and phenotypical circulating endothelial cells (CECs) as surrogate markers of clinical outcome in mCRC patients to identify responders to bevacizumab in combination with chemotherapy. **METHODS:** A total of 69 patients with measurable mCRC were enrolled in this prospective study. Whole blood samples were analyzed before initiation of treatment and on days 4 and 14. Phenotypical CECs and CEPs were then isolated and enumerated by using flow cytometry. **RESULTS:** CEP levels of less than 0.04% on day 4 were significantly associated with longer progression-free survival (PFS) and overall survival (OS) ($P < .001$, $P = .002$, respectively) as compared with levels of 0.04% or more. In addition, CXCR4-positive CEC levels of less than 20% at baseline were significantly associated with longer PFS and OS as compared other indicators investigated ($P < .001$, $P = .002$, respectively). **CONCLUSIONS:** Levels of CEPs on day 4 and proportion of CXCR4-positive CECs at baseline were correlated with the prognosis of bevacizumab combination chemotherapy, suggesting that these surrogate markers may play a core role in the selection of candidates for bevacizumab treatment. *Cancer* **2011;117:4026–32.** © *2011 American Cancer Society.*

KEYWORDS: circulating endothelial progenitors, CXCR4-positive circulating endothelial cells, bevacizumab, metastatic colorectal cancer, chemotherapy.

Antiangiogenic agents such as bevacizumab that target the vascular endothelial growth factor (VEGF) pathway have shown promise in the treatment of a variety of malignancies.[1] However, clinical biomarkers are needed for quantitative evaluation of the effect of bevacizumab.

VEGF is known to promote the mobilization of bone-marrow–derived circulating endothelial progenitors (CEPs) and survival by activating antiapoptotic pathways in circulating endothelial cells (CECs),[2–4] which may subsequently differentiate into mature endothelial cells.[5,6] Recently, CEPs were reported to be involved in tumor angiogenesis in tumor implantation models[7–10] and in clinical studies.[11,12] According to several clinical reports, baseline CEC levels in cancer patients have shown higher values compared with those in healthy controls and were correlated with response and outcome.[13–15]

The aim of this study was to investigate the potential of CEPs and phenotypical CECs as surrogate markers of clinical outcome in metastatic colorectal cancer (mCRC) patients to identify responders to chemotherapy with bevacizumab.

MATERIALS AND METHODS

Patients

Principal inclusion criteria were measurable mCRC and commencement of a new systemic therapy. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, adequate organ function, and

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radiographic evidence of disease progression as defined by the Response Evaluation Criteria in Solid Tumors (RECIST). All patients were enrolled on protocols approved by the institutional review board at the Cancer Institute Hospital in The Japanese Foundation for Cancer Research. Written informed consent was obtained from all patients.

Assessment of Biomarkers

Whole blood samples were collected and analyzed at the following times: before initiation of treatment (baseline), immediately after completion of 1 course (day 4), and before commencement of a second cycle (day 14). Blood samples were drawn into 8.5-mL evacuated tubes (BD Biosciences, Franklin Lakes, NJ).

Mononuclear cells isolated by density gradient centrifugation were analyzed using the method established by Duda DG et al.[16] Briefly, Ficoll gradient was used to isolate peripheral blood mononuclear cells (PBMC) and remove red cells and platelets before incubation with antibodies. The following directly conjugated monoclonal antibodies were used for detection of CECs and CEPs by 4-color flow cytometry in peripheral blood: anti-CD31-FITC (BD Pharmingen, San Diego, Calif), anti-CD133-PE (Miltenyi Biotec, Auburn, Calif), anti-CD34-APC (BD Pharmingen), and anti-CD45-PerCP/Cy5.5 (BD Pharmingen). The proportions of CECs (CD31-positive and CD45 negative fractions) and CEPs (CD31-positive, CD34 highly positive, CD133-positive, and CD45 dimly positive fractions) were calculated as percentages of the total number of mononuclear cells after evaluation of at least 50,000 cellular events. Phenotypical CECs expressing VEGFR1, VEGFR2, Tie-2, or CXCR4 were also analyzed. The proportions of these CEC phenotypes were calculated as percentages of the total number of CECs.

Observation of CECs and CEPs

For morphological and immunohistological observation of CECs and CEPs, a small portion of mononuclear cells was fractionated into CXCR4-positive CECs or CEPs by using FACSVantage (Becton Dickinson, Franklin Lakes, NJ). The nuclei of the isolated live CECs and CEPs were stained with DRAQ5 (Alexis, now part of Enzo Life Sciences, Farmingdale, NY) and then observed by confocal laser scanning microscopy (FV1000; Olympus, Center Valley, Penn).

Table 1. Characteristics of Patients Treated With FOLFOX Plus Bevacizumab

Characteristics	Regimen
N=69	FOLFOX+bevacizumab
Median age (range)	61 (27-73)
Sex men/women	38/31
Primary site rectum/colon	24/45
Prior colorectomy +/-	6/63
Metastatic site	
Liver	37
Lung	36
LN	28
Local recurrence	5
Peritoneum	17
Bone	3
Chemotherapy +/-	14/55
5-FU	7
Other	7
CR/PR/SD/PD	2/46/15/6

CR indicates complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Statistical Analysis

Kaplan-Meier survival plots were generated based on CEC levels at each time point of blood sampling, and the curves were compared by using the log-rank test. The Cox proportional hazards regression model was used to determine univariate and multivariate hazard ratios for progression-free survival (PFS) and overall survival (OS).

RESULTS

Patient Characteristics

A total of 69 patients were enrolled. Patient characteristics at baseline are summarized in Table 1. Among 69 patients treated with FOLFOX4 plus bevacizumab assessable for response, we observed complete response in 2 (3%), partial response in 46 (67%), stable disease in 15 (22%), and progressive disease (PD) in 6 (8%) during treatment. Overall response rate was 70%.

Relation Between CEP Levels and Outcome

Univariate Cox regression analysis revealed that CEP levels on day 4 were significantly associated with PFS in 30 of the 69 patients in the training set. To identify the level of CEPs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 0.01%-0.20% of the total number of PBMCs on day 4 were systematically correlated with PFS. Median PFS in patients with levels above or below each threshold differed at 0.04% CEPs of the total number of PBMCs, reaching a plateau at approximately that level. At this level, the Cox

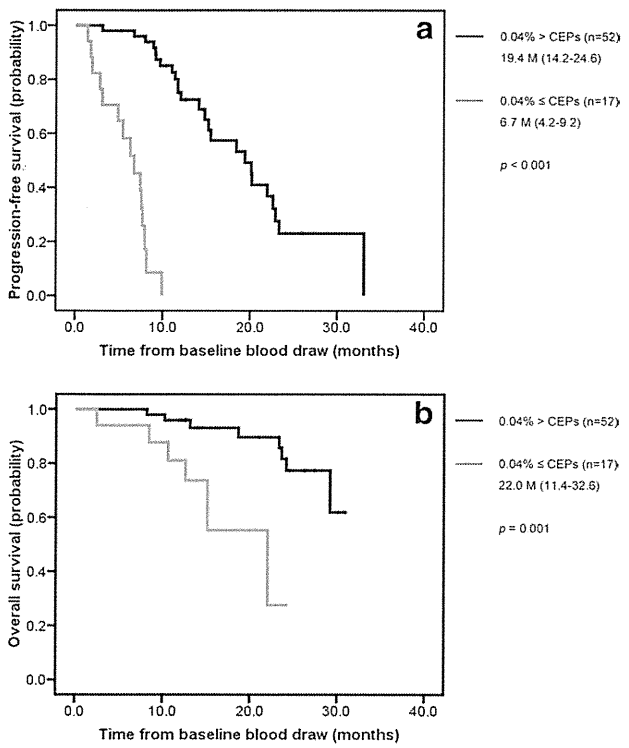

Figure 1. Depicted are (a) Kaplan-Meier plots of progression-free survival (PFS) and (b) Kaplan-Meier plots of overall survival (OS).

proportional-hazard ratio signifying the difference between slow and rapid progression of disease also reached a peak. Therefore, a cutoff of 0.04% CEPs was chosen to distinguish patients. The Kaplan-Meier 0.04% CEP counts were available on day 4 for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 0.04% or more CEPs on day 4 had a shorter median PFS (6.7 months; 95% CI, 4.2-9.2 months) than those with less than 0.04% CEPs on day 4 (19.4 months; 95% CI, 14.2-24.6 months) ($P <$ .001) (Fig. 1a). Patients with 0.04% or more CEPs on day 4 had a shorter median OS (22 months; 95% CI, 11.4-32.6 months) than those with less than 0.04% CEPs on day 4 ($P = .001$) (Fig. 1b).

Relation Between CEC Phenotype and Efficacy

Levels of CXCR4 in patients with PD were significantly higher than in those with no PD. Other phenotypes showed no differences between patients with PD and those without (Fig. 2).

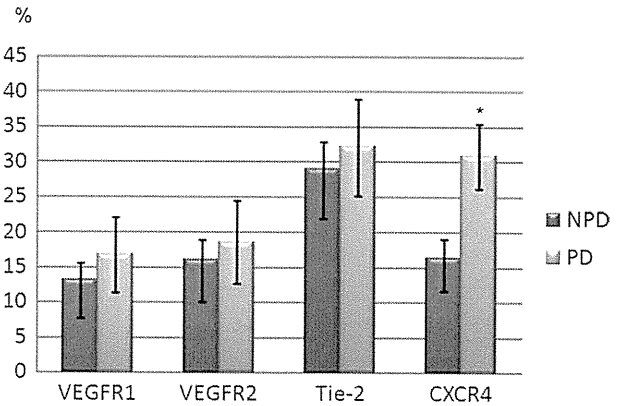

Figure 2. The relation is shown between levels of CEC phenotypes at baseline and bevacizumab efficacy in bevacizumab combination chemotherapy. PD indicates progressive disease; NPD, nonprogression disease. Results are expressed as mean ± standard error of the mean (SE). *$P < .05$

Relation Between CEC Phenotype and Outcome

According to univariate Cox regression analysis, CEC levels at baseline were significantly associated with PFS. To explore the predictive potential of CEC phenotypes at baseline, we analyzed the relation between baseline levels of CEC phenotypes and PFS. Univariate Cox regression analysis revealed that CXCR4-positive CEC levels at baseline were significantly associated with PFS. On the other hand, no correlation was observed between baseline VEGFR1-positive, VEGFR2-positive, or Tie-2–positive CEC levels and PFS. To identify the level of CXCR-positive CECs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 1% to 45% of the total number of CECs at baseline were systematically correlated with PFS. Median PFS in patients with levels of above or below each threshold differed at 20% CXCR4-positive CECs. At this level, the Cox proportional-hazards ratio signifying the difference between slow and rapid progression of disease also reached a plateau. Therefore, a distinguishing cutoff of 20% CXCR4-positive CECs was chosen. The Kaplan-Meier CXCR4-positive CEC count was available at baseline for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. No significant difference was observed in either PFS or OS in either set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 20% or more CXCR4-positive CECs at baseline had a shorter median PFS (6.7 months; 95% CI, 4.1-9.3 months) than those with less than 20% CXCR4-positive