# Ⅳ.　研究成果の刊行物・別刷

8. Amado RG, Wolf M, Peeters M, et al: Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26:1626-1634, 2008

9. Van Cutsem E, Lang I, D'haens G, et al: KRAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience. J Clin Oncol 26:5s, 2008 (suppl, abstr 2)

10. Bokemeyer C, Bondarenko I, Hartmann JT, et al: KRAS status and efficacy of first-line treatment of patients with metastatic colorectal cancer (mCRC) with FOLFOX with or without cetuximab: The OPUS experience. J Clin Oncol 26:178s, 2008 (suppl, abstr 4000)

11. Oudejans JJ, Slebos RJ, Zoetmulder FA, et al: Differential activation of ras genes by point mutation in human colon cancer with metastases to either lung or liver. Int J Cancer 49:875-879, 1991

12. Suchy B, Zietz C, Rabes HM: K-ras point mutations in human colorectal carcinomas: Relation to aneuploidy and metastasis. Int J Cancer 52:30-33, 1992

13. Al-Mulla F, Going JJ, Sowden ET, et al: Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 185:130-138, 1998

14. Tórtola S, Steinert R, Hantschick M, et al: Discordance between K-ras mutations in bone marrow micrometastases and the primary tumor in colorectal cancer. J Clin Oncol 19:2837-2843, 2001

15. Rajagopalan H, Bardelli A, Lengauer C, et al: Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934, 2002

DOI: 10.1200/JCO.2008.18.7286

# Detailed Analysis of Visitors to Cancer-Related Web Sites

**To the Editor:** Web sites are a valuable source of information for cancer patients.[1] Patients are seeking information necessary for their own treatment, as well as general cancer information. To satisfy such needs of cancer patients, it is necessary to build Web sites that are conducive to patients' individual needs, as well as to have organic linkage between a wide variety of sites. Although achieving this end requires sufficient study of the characteristics of cancer-related Web site users, there is little research on the topic, leaving an unclear picture of the actual state of cancer-related Web site users. Therefore, in this study, we conducted an access analysis of cancer-related Web sites to shed light on the characteristics of their visitors, which is information necessary for improving the user friendliness of such Web sites.

Using Keyword Advice Tool (Overture KK, Tokyo, Japan),[2] we first selected 96 keywords pertaining to cancer that have been used in more than 3,000 searches per month on Yahoo! as of September 2006. Next, we used the 96 selected keywords to conduct Yahoo! searches,[3] and then selected 2,000 Web sites that came up in these searches. We then used Keyword Advice Tool to obtain the number of searches performed with each keyword and ranked the Web sites proportionate to the number of searches. Then we computed a ranking score by giving the $n$th-ranking keyword of the converted ranking a $1/n$ value (eg, the first-ranking site gets 1,000 points, the second-ranking site half of that, and so on). We

**Table 1.** Web Sites Analyzed

| Classification | Name of Web Site | Aggregation Period | No. of Visitors (daily average) | No. of Page Views (daily average) |
|---|---|---|---|---|
| Cancer center | Cancer center Web site A | September 1, 2006 to November 30, 2006 | — | 42,663 |
| Cancer center | Cancer center Web site B | October 1, 2006 to November 30, 2006 | — | 62,181 |
| Hospital | Hospital Web site C | August 1, 2006 to November 30, 2006 | 8026 | — |
| Hospital | Cancer center Web site D | March 26, 2006 to November 18, 2006; October 15, 2006 to January 13, 2007 | — | — |
| Hospital | Hospital Web site D | October 1, 2006 to December 31, 2006 | — | 421 |
| Hospital | Hospital Web site E | November 1, 2006 to December 31, 2006 | — | — |
| Pharmaceutical company | Pharmaceutical company Web site A | November 1, 2006 to December 31, 2006 | — | — |
| Pharmaceutical company | Pharmaceutical company Web site B | November 1, 2006 to December 31, 2006 | — | — |
| Pharmaceutical company | Pharmaceutical company Web site C | November 1, 2006 to December 31, 2006 | — | — |
| Pharmaceutical company | Pharmaceutical company Web site D | November 1, 2006 to December 31, 2006 | — | — |
| Pharmaceutical company | Pharmaceutical company Web site E | November 1, 2006 to December 31, 2006 | — | — |
| Individual | Individual antiaging Web site A | December 1, 2006 to December 31, 2006 | — | — |
| Cancer patient | Cancer blog B | October 1, 2006 to December 31, 2006 | — | — |
| Cancer patient | Cancer blog C | October 1, 2006 to December 31, 2006 | — | — |
| Cancer patient | Cancer blog D | December 2, 2006 to January 12, 2007 | — | — |
| Cancer patient | Pediatric cancer blog E | December 10, 2006 to January 27, 2007 | — | — |
| Cancer patient | Pediatric cancer blog F | December 10, 2006 to January 27, 2007 | — | — |
| Cancer patient | Childhood leukemia blog G | December 10, 2006 to January 27, 2007 | — | — |
| Cancer patient | Leukemia blog H | October 8, 2006 to January 8, 2007 | — | — |
| Cancer patient | Leukemia blog I | October 6, 2006 to January 5, 2007 | — | — |
| Cancer patient | Breast cancer blog J | January 1, 2007 to February 28, 2007 | 198 | — |
| Cancer patient | Breast cancer blog K | January 1, 2007 to February 28, 2007 | 161 | — |
| Cancer patient | Leukemia blog L | January 1, 2007 to February 28, 2007 | 173 | — |
| Cancer patient | Ureteral cancer blog M | January 1, 2007 to February 28, 2007 | 51 | — |
| Cancer patient | Individual cancer link site N | January 1, 2007 to February 28, 2007 | — | — |

also assigned a hit frequency score for the frequency with which each Web site appeared in searches with each of the 96 keywords. We then computed the final score with the product of the ranking score and the hit frequency score and extracted the 100 highest scoring sites as the subject of this study. Blogs in the present study also included homepages on patients' personal experiences fighting cancer.

There are two main methods for conducting access analysis; these are analyzing the Web server logs and obtaining access logs through JavaScript tags embedded in each page of a Web site.[4] Both methods require the site author to collect the log data. It is possible to obtain the Uniform Resource Locator (URL) of the pages visited, the page viewed before visiting the site, the Internet Protocol (IP) address of the visitors, and the time of visit. In this study, we requested the following information from the selected 100 Web sites. We requested a summary of aggregated results from the Web sites already compiling access data on their own. For the Web sites that are not compiling access data on their own but that can obtain a server log, we obtained the access logs from the site authors and then compiled the data ourselves. For all of the other Web sites, we embedded tags to collect data for the purposes of this study and compiled the data.

Of the sites, 25 Web sites complied with our request and consented to participate in the study; characteristics of these sites are listed in Table 1. Each site operator agreed to participate in the study on the condition of anonymity. The number of visitors to cancer center sites was overwhelmingly higher than the number of visitors to cancer patients' blogs.

We were able to obtain data on page views by day of the week for three Web sites operated by cancer centers and general hospitals (Fig 1). Page views on nonworking days for all three sites were 64% to 70% of page views on weekdays.

We were able to analyze the number of visitors to one hospital Web site (hospital Web site C) and four cancer patients' blogs every 3 hours. The number of visitors to hospital Web site C peaked on weekdays around 12:00 to 3:00 PM. However, there were no evident day-to-day fluctuations on cancer patients' sites, whose accesses peaked around 9:00 PM to 12:00 AM. Although the average number of visitors per hour to hospital Web site C outside of business hours decreased to 38% of the number during business hours (from 9:00 AM to 6:00 PM on weekdays), the average number of visitors to the four cancer patients' blog sites decreased to only 61% (Fig 2).

The ratio of search engines used to access each Web site is shown in Table 2. The percentage of people who used MSN was lower for visitors to cancer patients' blogs than for visitors of hospital sites.

We were able to attain the repeat rate for six cancer patients' blog sites. Although the repeat rates for leukemia and ureteral cancer patients' blogs were extremely low, the repeat rate for breast cancer–related blog sites was high (Table 2). We compared four cancer patients' blog sites for which we were able to obtain this data (Fig 3). There was a group of visitors with a high degree of familiarity for each of the two breast cancer patients' blog sites. There was a smaller group of visitors familiar for leukemia blog L compared with the two breast cancer patients' blog sites. We found a group of visitors tending towards defection for ureteral cancer blog M.

We were able to obtain data on changes in visit frequency over the last year or more for cancer center Web site A, pharmaceutical company Web site A, pharmaceutical company Web site B, and pharmaceutical company Web site E. Although visit frequency for each site exhibited small fluctuations and overall increasing and decreasing trends, we did not observe any so-called seasonal variations. Furthermore, when we examined 3-month logs of search keywords that led to cancer blog C, we found no visible changes in search keywords during the 3-month period.

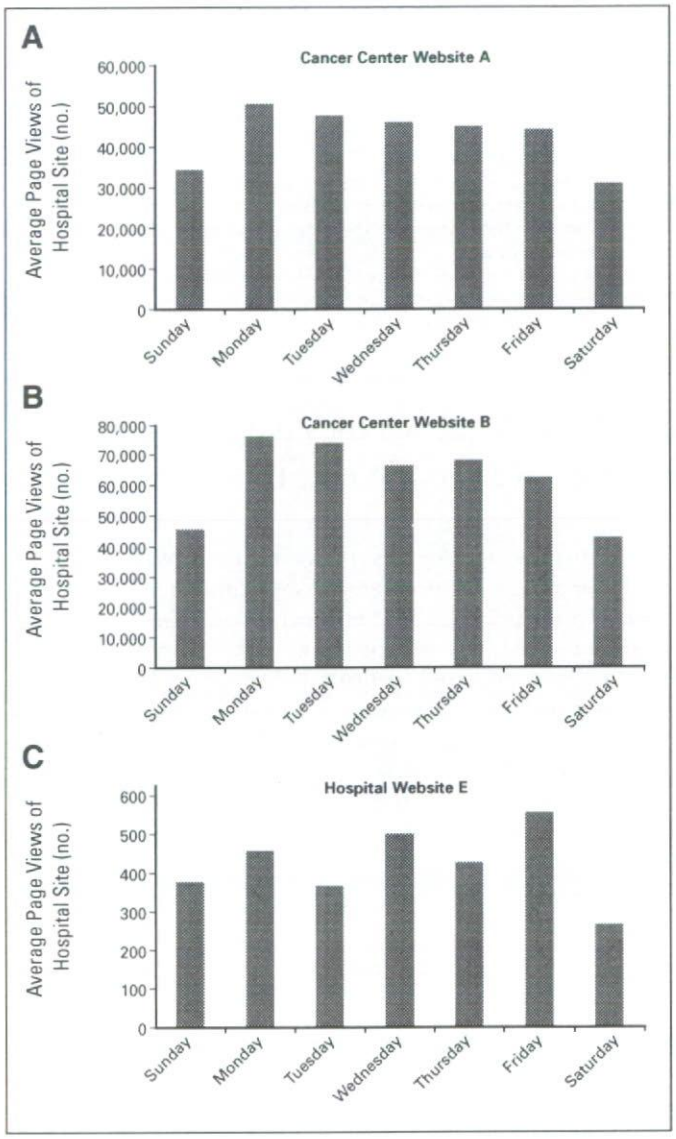


**Fig 1.** Average page views of hospital site by day of the week.

We selected Web sites in a wide range of categories for this study. In addition to hospital and pharmaceutical company Web sites, we also targeted a large number of homepage sites on patients' personal experiences fighting cancer. We first screened for sites that are influential among users. Cancer patients' homepage sites constituted 9% of the influential sites initially selected. Previous studies, however, have not focused on these cancer patients' homepage sites.[1,5-8] Blogging on one's experiences with cancer has enabled the flow of information among patients that goes beyond time and space. Homepages provide a means for communication among patients and their families that is more convenient and costs less than traditional face-to-face patient organizations. It is possible that these sites provide information that is

  

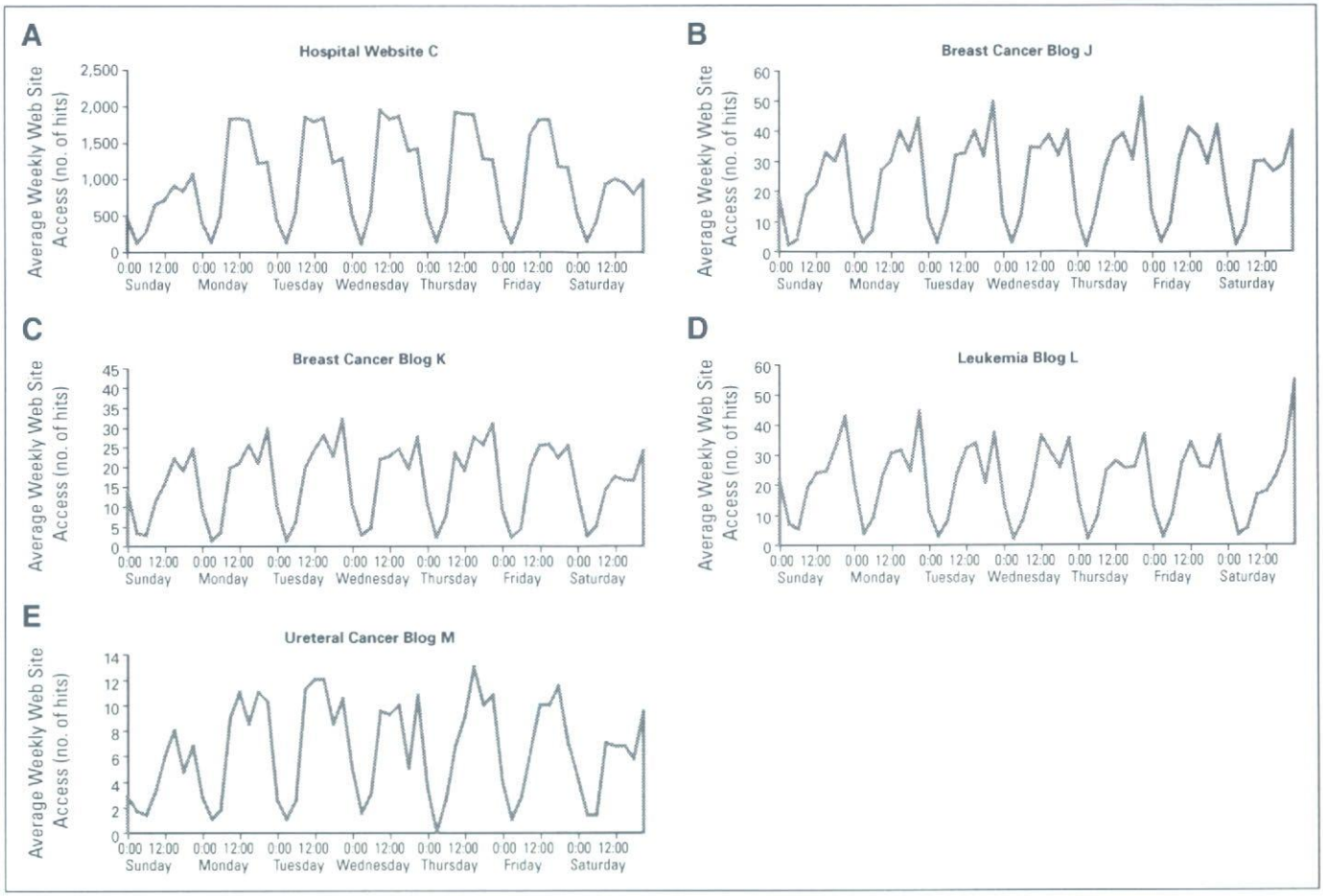


**Fig 2.** Average weekly access to homepages on fighting cancer. The value on the *y*-axis is number of accesses to the site every 3 hours.

not provided by medical care providers but that is useful to patients. It is likely that patients' blog sites will become an important category of Web sites in the future.

This study showed that visitors' access patterns vary among different types of Web sites. Many people visited hospital-type sites on weekday afternoons, whereas few visited these sites on nonworking days. In contrast, there was hardly any variation between days of the week in visits to cancer patients' blogs, which peaked at night (Fig 1). This fact demonstrates that the background or the status of use varies between users of hospital-type sites and homepage sites. Although we cannot draw any definitive conclusions as a result of insufficiently detailed data, we can infer from the fact that the peak in visits to hospital-type sites coincided with hospital consultation hours that many of these people use these sites as they prepare for hospital visits. At the same time, we can also deduce that there is a tendency for people to visit cancer patients' blogs during their spare time. This may reflect the fact that people use cancer patients' blogs not for one-way transmission of information but as a tool for communication among patients and their family members.

In this study, the rate of visitors who use MSN[9] to reach cancer-related Web sites was generally low compared with the Japanese national average. Moreover, cancer patients' homepage visitors tended to go through Yahoo! and Google[10] more often than MSN compared with visitors to other categories of Web sites. In general, people who frequently access the Internet use Google, whereas those who access it less frequently use Yahoo!, and those who access the Internet even less frequently use MSN, which comes bundled in many computers' initial setup configuration. Considering this, we can deduce that visitors to cancer-related Web sites and, in particular, to cancer patients' homepages are highly literate with information technology and frequently access the Internet.

This study demonstrated that the repeat rate of visitors varies depending on the attributes of particular Web sites. The repeat rate of visitors to breast cancer–related homepages was extremely high compared with the average repeat rate of information service–type sites, which is approximately 25% to 30% (T. Nobue, personal communication, 2006). We observed the same trend from the results of visitor familiarity as well. This indicates that there are many avid fans of breast cancer–related homepages, which represents a significant departure from information service–type sites such as those of cancer centers. Even among cancer-related homepages, the repeat rate for leukemia-related sites was extremely low. There are a number of possible reasons for this. First, compared with breast cancer, there are many different subtypes of leukemia, with varying symptoms and duration. Therefore, visitors may more often find that the leukemia-related Web site they visited was not describing the exact subtype of leukemia they intended to look up. Second, the

**Table 2.** Characteristics of Sites for Which Data Were Obtainable

| Name of Site | Search Engine Ratio (*v* Google.co.jp* + Google.com†) | | Repeat Ratio (%) |
|---|---|---|---|
| | Yahoo‡ | MSN§ | |
| Cancer center Web site A | 1.67 | 0.16 | — |
| Cancer center Web site B | 1.05 | 0.14 | — |
| Cancer center Web site D | 2.17 | 0.20 | — |
| Hospital Web site E | 7.13 | 0.89 | — |
| Hospital Web site F | 2.25 | 0.02 | — |
| Pharmaceutical company Web site A | 1.84 | 0.14 | — |
| Pharmaceutical company Web site B | 4.92 | 0.18 | — |
| Pharmaceutical company Web site C | 6.00 | 0.40 | — |
| Pharmaceutical company Web site D | 4.00 | 0.29 | — |
| Cancer blog B | 6.39 | 0.16 | — |
| Pediatric cancer blog E | — | — | 55.30 |
| Leukemia blog I | 1.50 | 0.08 | — |
| Breast cancer blog J | 2.87 | 0.08 | 44.60 |
| Breast cancer blog K | 1.66 | 0.08 | 29.30 |
| Leukemia blog L | 0.90 | 0.30 | 5.60 |
| Ureteral cancer blog M | 1.80 | 0.05 | 13.80 |
| Individual cancer link site N | 0.72 | 0.09 | 9.30 |
| Throughout Japan‖ | 1.86 | 0.51 | — |
| Kameda Medical Center | 0.35 | 0.10 | — |
| Hula dance class for homemakers | 8.14 | 0.63 | — |
| Optical device company | 0.92 | 0.15 | — |

*Web site: http://www.google.co.jp/.
†Web site: http://www.google.com/.
‡Web site: http://www.yahoo.co.jp/.
§Web site: http://jp.msn.com/.
‖Web site: http://internet.watch.impress.co.jp/cda/event/2006/04/21/11756.html.
¶Shows the percentage when OCN is set to 1.

survival rate is higher and the duration of illness is longer for breast cancer than for leukemia. Third, because information technology is less diffuse among elderly individuals, it is possible that there are more visitors at the Web sites related to the types of cancer that occur at an earlier age, such as breast cancer.

To our knowledge, this study was the first to shed light on the characteristics of cancer-related Web site visits. However, there are a number of issues that need to be considered. First, because the number of sites from which we obtained data is limited, we cannot generalize for all cancer-related Web sites. More large-scale studies with a wider scope of target sites will be needed in the future. Second, this study demonstrated that homepage sites on people's personal experiences fighting disease are forums for the communication of information among patients. It is possible for patients and their families to obtain information through these sites that they cannot get from medical care providers. With few previous studies on these sites, more research is needed on the role of these sites in improving patient literacy, as well as the limitations of these homepage sites. Finally, this study showed that the background and behavior of cancer Web site visitors differ among different types of Web sites. This suggests that visitors have diverse needs. Cancer-related Web sites need to be designed from this perspective to make them easy to use and beneficial to their users.

*Hiroto Narimatsu*
Division of the Strategic Outcome Research Program for Cancer Control, Ministry of Health, Labour and Welfare Commission, Japan Cancer Society; Division of Exploratory Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan

*Tomoko Matsumura, Tomohiro Morita, Yukiko Kishi, Koichiro Yuji, and Masahiro Kami*
Division of Exploratory Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan

*Tsunehiko Komatsu*
Third Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan

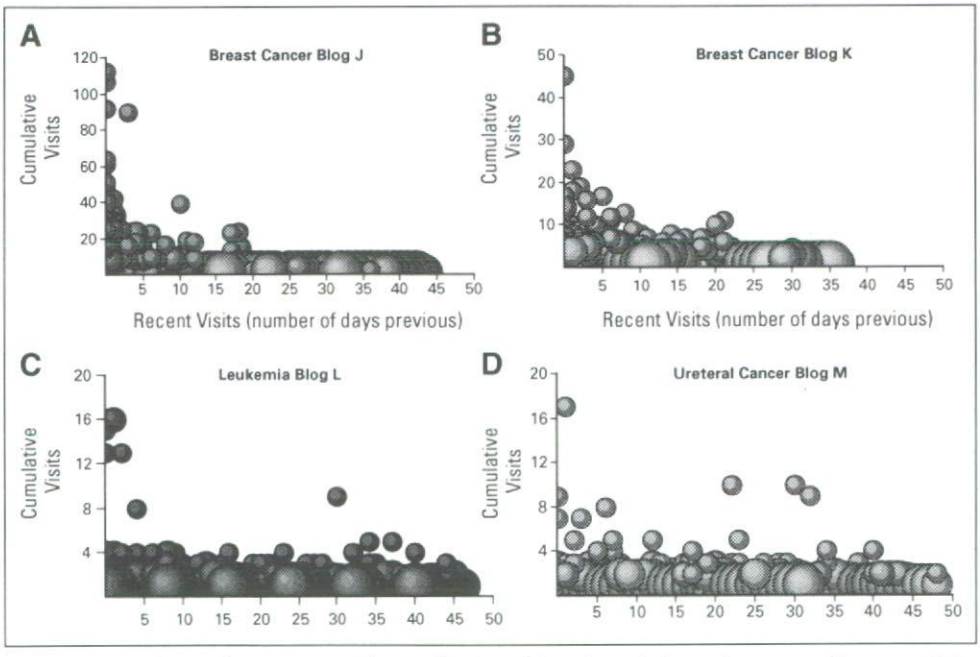


**Fig 3.** Distribution of visitor familiarity and defection. The size of the spheres indicates the amount of aggregated data.

  

*Yuji Tanaka*
Division of Exploratory Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan

*Tomohiro Sawa and Yoshinori Nakata*
Medical Information and System Research Center, Teikyo University School of Medicine, Tokyo, Japan

ACKNOWLEDGMENT

We are grateful to Toshiteru Nobue (Maikai Solutions, Tokyo, Japan) for his technical support. Supported by Grant from Health and Labor Sciences Research Grants, the third-term comprehensive control for cancer (H18-012 to Dr Nakata) from the Ministry of Health Welfare and Labor of the Japanese Government, Tokyo, Japan.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

**Conception and design:** Hiroto Narimatsu, Masahiro Kami, Tomohiro Sawa, Yoshinori Nakata
**Collection and assembly of data:** Hiroto Narimatsu, Tomoko Matsumura, Tomohiro Morita, Yukiko Kishi, Koichiro Yuji, Tsunehiko Komatsu, Yuji Tanaka
**Data analysis and interpretation:** Hiroto Narimatsu, Masahiro Kami, Tomohiro Sawa, Yoshinori Nakata
**Manuscript writing:** Hiroto Narimatsu, Masahiro Kami, Yoshinori Nakata
**Final approval of manuscript:** Hiroto Narimatsu, Tomoko Matsumura, Tomohiro Morita, Yukiko Kishi, Koichiro Yuji, Masahiro Kami, Tsunehiko Komatsu, Yuji Tanaka, Tomohiro Sawa, Yoshinori Nakata

REFERENCES

1. Newnham GM, Burns WI, Snyder RD, et al: Information from the Internet: Attitudes of Australian oncology patients. Intern Med J 36:718-723, 2006
2. Overture. http://inventory.jp.overture.com/d/searchinventory/suggestion/
3. Yahoo! Japan. Homepage. http://www.yahoo.co.jp/
4. Nikkei BP Consulting Inc: Access analysis. http://consult.nikkeibp.co.jp/consult/bizmark/feature.html
5. Alper BS: Usefulness of online medical information. Am Fam Physician 74:482:485, 2006
6. Berland GK, Elliott MN, Morales LS, et al: Health information on the internet: Accessibility, quality, and readability in English and Spanish. JAMA 285:2612-2621, 2001
7. D'Alessandro DM, Kingsley P, Johnson-West J: The readability of pediatric patient education materials on the World Wide Web. Arch Pediatr Adolesc Med 155:807-812, 2001
8. Morita T, Narimatsu H, Matsumura T, et al: A study of cancer information for cancer patients on the internet. Int J Clin Oncol 12:440-447, 2007
9. MSN Japan: Homepage. http://jp.msn.com/
10. Google Japan: Homepage. http://www.google.co.jp/

DOI: 10.1200/JCO.2008.18.7468

# Cetuximab Pharmacokinetics in End-Stage Kidney Disease Under Hemodialysis

TO THE EDITOR: Cetuximab, an anti–epidermal growth factor receptor chimeric mouse/human immunoglobulin 1 monoclonal antibody against the epidermal growth factor (Merck, Darmstadt, Germany), has been approved as a treatment for advanced head and neck cancer in combination with radiation therapy.[1,2] However, there is very little data on cetuximab in patients undergoing chronic dialysis.[3] The treatment of cancer in patients with impaired renal function is an emerging problem because the population is getting older and the rate of chronic dialysis increases by 5% yearly in Western countries. We report a pharmacokinetic study of cetuximab in a patient with renal insufficiency requiring hemodialysis. Cetuximab was instituted at a dose of 250 mg/kg weekly for a 55-year-old patient with head and neck cancer.

We characterized the pharmacokinetics and efficacy of cetuximab at conventional efficacious dose levels in combination with radiation therapy in a hemodialyzed patient with head and neck cancer. The aim of the study was to determine whether conventional doses of cetuximab in combination with radiotherapy were appropriate for hemodialyzed patients.

Cetuximab serum concentration was measured by a validated enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay method used a recombinant human epidermal growth factor receptor (extracellular domain) adsorbed onto microtiter plates to capture cetuximab in serum. The captured cetuximab was detected using a peroxidase-conjugated goat antihuman $F(ab')_2$ specific for Fc fragment (horseradish peroxidase anti–human immunoglobin G). Lower limit of quantitation and upper limit of quantitation were 0.75 and 15 $\mu$g/mL, respectively. The limit of detection was 0.012 $\mu$g/mL. Concentrations higher than the upper limit of quantification were diluted 1:10 or 1:100, deviation and variability of this procedure being lower than 4.5%. Serum samples were used to estimate cetuximab pharmacokinetics, assuming no time-dependence, with WINNonlin (Scientific Consultant, Apex, NC; Pharsight Corporation). One- and two-compartment models with first order distribution and elimination constants were tested. The best model was selected using the usual methods, which included the analysis of plots of observed versus predicted concentrations and the Akaike information criteria. The model that best fitted the observed data was a two-compartment model with first-order elimination from the central compartment (Fig 1). Clearance from central compartment was 0.025 L/h, central compartment volume was 3.8 L, and terminal elimination half-life was 11.9 days (Table 1).

Although analyses of cetuximab pharmacokinetics were previously reported, the results obtained in our patient cannot be readily compared with these publications. Tan et al[4] did not use a formal compartment model. In the studies of Baselga et al[5] and Delbaldo et al,[6] cetuximab pharmacokinetics were described by a one-compartment model. However, a two-compartment model has previously been shown to be the best to describe the pharmacokinetics of immunoglobulin 1 monoclonal antibodies, including trastuzumab,[7] inolimomab,[8] rituximab,[9] basiliximab,[10] clenoliximab,[11] alemtuzumab,[12] and adalimumab.[13] Dirks et al[14] used a two-compartment model but with a Michaelis-Menten type of elimination. This last approach necessitates a large number of patients and the study of different dose regimens, and could not be applied to our patient.

Breast Cancer Res Treat (2008) 110:485–491
DOI 10.1007/s10549-007-9747-9

PRECLINICAL STUDY

# Favourable long-term results after surgical removal of lung metastases of breast cancer

**Masataka Yoshimoto · Keiichiro Tada · Seiichiro Nishimura · Masujiro Makita · Takuji Iwase · Fujio Kasumi · Sakae Okumura · Yukitoshi Sato · Ken Nakagawa**

Received: 28 August 2007 / Accepted: 29 August 2007 / Published online: 27 September 2007


**Abstract** We retrospectively evaluated whether a surgical strategy benefits patients with operable lung metastasis of breast cancer. Between 1960 and 2000, 90 patients (mean age 55.1; range 32–77) with lung metastasis (79 solitary, 11 multiple) underwent surgery as follows: wedge resection ($n$ = 10), segmental resection ($n$ = 11), lobectomy ($n$ = 68) and pneumonectomy ($n$ = 1). The metastases were completely resected in 89% of them. One patient died due to surgical complications. The overall 5- and 10-year cumulative overall survival rates were 54% and 40%, respectively (median, 6.3 years). Fifteen patients survived without relapse for over 10 years. They were 24% of those who progressed for 10 years or more after lung surgery. The most significant prognostic factor was disease-free interval (DFI) and stage at breast surgery. The 10-year survival rates of those with ≧3 and <3 years of DFI were 47% and 26%, respectively ($P$ = 0.014). Survival times were significantly longer for patients with clinical stage I at breast surgery than those with stage II–IV ($P$ = 0.013). Our data, although limited and highly selective, suggest that surgical approach to lung metastasis from breast cancer may prolong survival in certain subgroups of patients to a greater extent than systemic chemotherapy alone. Surgical approach to lung metastasis of breast cancer, if possible, should be a treatment of choice to a great extent.

**Keywords** Breast cancer · Lung metastasis · Pulmonary metastasis · Surgery · Prognosis · Cure rate · Long survival

M. Yoshimoto · K. Tada · S. Nishimura · M. Makita · T. Iwase · F. Kasumi
Breast Oncology Group, Cancer Institute Ariake Hospital, Ariake 3-10-6, Koto-ku, Tokyo 135-8550, Japan

S. Okumura · Y. Sato · K. Nakagawa
Department of Chest Surgery, Cancer Institute Ariake Hospital, Ariake 3-10-6, Koto-ku, Tokyo 135-8550, Japan

M. Yoshimoto (✉)
Breast Center, Mita Hospital, International University of Health and Welfare, Mita 1-4-3, Minato-ku, Tokyo 108-8329, Japan
e-mail: myoshimoto@iuhw.ac.jp

## Introduction

Recent advancements in adjuvant chemotherapy and hormone therapy have significantly reduced the recurrence rate and increased the overall survival times of patients with operable breast cancer [1, 2]. However, once patients relapse, cure rates after systemic treatments remain hopelessly low [3, 4]. Thus, metastatic breast cancer is thought to be incurable [5] and systemic treatments are used for palliative under such circumstances [6, 7].

The lung is one of the most common sites of recurrent metastasis from breast cancer. Surgery has seldom been the treatment of choice since it is considered a manifestation of a systemic disease. Several studies of surgical approaches have indicated promising results [8–12], but most medical oncologists disapprove of surgical strategies.

Considering the poor results of systemic treatments, we postulated that surgical resection of operable lung metastases with postoperative systemic treatments might prolong survival times more than systemic treatments alone. Here, we describe a surgical approach to treating lung metastasis from breast cancer and discuss the controversial nature of this strategy.

## Patients and methods

Our principal indications for surgical intervention to treat lung metastasis consisted of a solitary and operable lung

metastasis, no clinical evidence of other recurrent lesions, no serious complications, written informed consent to undergo surgery after understanding the associated risks and remaining alive for at least 6 months.

Chest X-rays were taken every 6 months after treating the primary breast cancer to detect recurrence. Suspected lung metastasis was confirmed by CT scanning and bronchoscopy, which also evaluated the feasibility of surgery. Cytological assessment of specimens obtained by trans-bronchoscopic aspiration cytology (TBAC), trans-bronchial lung biopsy (TBLB) or CT-guided trans-cutaneous aspiration cytology was performed for almost all patients. Recurrent lesions outside the lung were investigated using X-rays, bone scintigraphy, ultrasonography, CT scanning and more recently FDG-PET scanning to avoid selecting inappropriate candidates for surgery. After confirming lung metastasis, patients were followed up from 2 until 388 weeks (mean, 14.8 weeks) to reconfirm the diagnosis and to determine surgical indications. Some patients who had undergone systemic front-line therapy but whose metastasis failed to respond were also candidates for surgical treatment. Lung and cardiac functions and other general conditions were also assessed to minimize surgical risk.

Between June 1960 and October 2000, 90 women with breast cancer underwent surgery to treat lung metastases. Among them, four had been treated for loco-regional skin and/or regional lymph node metastases surgically or radiologically and had been under complete remission before lung metastases, one had stage IV breast cancer with lung metastasis, and 85 had lung metastases as the first site of recurrence. Among these patients, 70 underwent breast surgery at our hospital and the remaining 20 were referred to our institution from other hospitals.

During the same period, 382 patients developed lung metastases as the first site of recurrence among 13,477 women with primary breast cancer who underwent breast surgery at our hospital. Among the 382 patients, 70 (18%) underwent lung surgery and 312 (82%) underwent systemic treatment alone. Although lung surgery was indicated, the selection of patients was inconsistent and mainly depended on the opinions of the physician in charge.

Clinical status, treatment methods and patient outcomes are listed in Table 1. The mean age of the patients was 55.1 years (range 32–77 years) at surgery for lung metastasis. The mean interval between surgery for the primary breast cancer and the discovery of recurrent disease (disease free interval: DFI) was 5.6 years (range 0–20.4 years).

Preoperative estimation of the largest metastatic lung tumour varied from 0.8 to 4.8 cm (mean, 2.3 cm). Preoperative estimations of the numbers of lung metastases were solitary in 79 patients, two in 9 patients, and three or more in 2 patients. None of the patients had bilateral lung metastasis, except one who had undergone lobectomy twice to treat bilateral lung metastasis, the second being 4 years after the first.

**Table 1** Patient characteristics

| Characteristics | Number | % |
|---|---|---|
| Age (years) | | |
| ≦40 | 6 | 7 |
| 41–50 | 26 | 29 |
| 51–60 | 30 | 33 |
| ≧61 | 28 | 31 |
| Mean (range) | 55.1 (32–77) | |
| Disease-free interval (years) | | |
| <3 | 33 | 37 |
| ≧3 | 57 | 63 |
| Mean (range) | 5.6 (0–20.4) | |
| Size of largest metastasis (cm) | | |
| ≦2 | 44 | 49 |
| >2 | 46 | 51 |
| Mean (range) | 2.2(0.8–4.8) | |
| Numbers of lung metastases, preoperatively | | |
| One | 78 | 88 |
| Two | 7 | 10 |
| Three or more | 5 | 2 |
| Method of surgery | | |
| Wedge resection | 10 | 11 |
| Segmental resection | 11 | 12 |
| Lobectomy | 68 | 76 |
| Pneumonectomy | 1 | 1 |
| Nodal dissection | | |
| Done | 84 | 93 |
| Not done | 6 | 7 |

The lung, pleural cavity and mediastinal node status was also intra-operatively assessed by the surgeon to reach a final decision about surgical resection of lung metastasis. In fact, the number of metastatic lesions in the lung in six patients was higher than that diagnosed preoperatively, and pleural dissemination was evident in 4 patients. Basically, hilar and unilateral mediastinal lymph nodes were simultaneously dissected. The postoperative systemic treatment was not uniform, but consisted essentially of standard systemic regimens at that time.

Survival was estimated by the Kaplan–Meier product-limit method from the date of surgical removal of lung metastasis to the date of death or of the last observation. Possible prognostic factors after surgery included DFI, clinical manifestations of nodal involvements at the hilus of the lung and/or the mediastinum, preoperative estimations of the number and the size of the largest lung metastasis, stage at breast surgery and others. The log-rank test (or Wilcoxon test) evaluated the significance of differences between survival curves.

## Results

The operative procedures consisted of wedge resection ($n$ = 10 patients), segmental resection ($n$ = 11), lobectomy ($n$ = 68) and pneumonectomy ($n$ = 1) were selected. Eighty and 10 patients underwent curative and palliative procedures, respectively. The reasons for the palliative operations were pleural dissemination ($n$ = 4), incomplete resection of lung metastasis ($n$ = 3), and incomplete resections of lymph node metastasis ($n$ = 3). After surgery, 78 patients had a single metastasis, seven had two, two had three, and two had four metastases. All 90 patients had histologically proven metastatic lung metastasis from breast cancer.

The lymph nodes were dissected in 81 patients and lymph nodes were sampled from 3. Neither lymph node sampling nor dissection was performed in the remaining 6 patients. Lymph node metastasis was present in 37 patients (44% of dissected or sampled patients), but not in 47. No information regarding nodal involvement was obtained from the remaining 6 patients. Among the 37 patients in whom lymph node metastasis was identified, metastasis was localized at the hilus of the lung and to the mediastinum in 11 and 26 patients, respectively.

Several minor complications developed such as postoperative infection, atelectasis and hepatitis due to blood transfusions. Serious complication developed in one patient who died within 1 month of surgery.

All patients were followed up until death or up to December 2003 or later with a median follow-up of 6.6 years. At the end of December 2003, 62 patients had relapsed again with recurrent disease, 28 had not relapsed. After lung surgery, first relapses were again found in the lungs, pleura, mediastinal lymph nodes, and others in 20, 5, 4, 33, respectively. Patients again relapsed from 3 weeks to 12.6 years (median 35.6 months) after lung surgery excluding palliative surgery. Two patients relapsed 10 years or more after lung surgery.

At the time of this writing, 56 patients are dead and 34 remain alive. Among the deceased patients, 54 died from recurrent disease, 1 from colon cancer and 1 from cerebrovascular disease. Among the survivors, 26 of 34 remain alive without recurrent disease. Twenty-two patients lived for over 10 years after lung surgery (Table 2). Fifteen of these remained free of recurrence for over 10-years, and 3 remained alive with recurrence, 4 died from recurrent diseases, one from contralateral breast cancer and 1 from colon cancer. Among 63 patients who progressed over 10-years from lung surgery, 15 (24%) survived for 10 years or more without further relapse. The longest survival time without relapse was 24.7 years after lung surgery.

The median overall cumulative survival time was 6.3 years, and the 5-, 10-, 20-year cumulative overall survival rates after lung surgery were 54, 40, and 25%, respectively (Fig. 1).

Table 3 shows the effects of possible prognostic factors that might affect survival after lung surgery. The most significant factors for survival after lung surgery were DFI and clinical stage at breast surgery. The 10-year overall survival rate for 33 patients with <3 year DFI was 26%, whereas that for 57 patients with ≥ 3-year DFI was 47%. The difference was statistically significant ($P$ = 0.014, log-rank test). Similarly, the 10-year overall survival rate for 20 patients with clinical stage I at breast surgery was 74%, which was significantly higher than that for patients with stage II–IV ($P$ = 0.013, log-rank test). The biggest size of metastasis and the presence of nodal metastases of the lung were marginally significant prognostic factors. The prognosis of patients with large tumours of ≤2 cm was significantly more favourable than that of patients with tumours that were >2 cm ($P$ = 0.043 in Wilcoxon test). The number of metastasis, type of lung surgery, age at lung surgery and others did not affect survival significantly. Lymph node dissection and curability did not also affect survival probably because of the small number.

The 10-year cumulative survival rate of 312 patients with lung metastases as the first site of recurrence and who underwent systemic chemo- and hormonal treatment alone was 6.5% and the median length of survival was 2.2 years. Among them, 11 patients survived for over 10 years, but eventually eight died from recurrent disease and only three remain alive without disease. Indeed, the extent or the status of metastatic diseases differed, but these survival data are obviously worse than those obtained from patients who underwent lung surgery.

## Discussion

Based on the assumption that metastatic breast cancer is a systemic disease, medical oncologists usually do not recommend surgical procedures for metastatic breast cancer or consider them only as palliative strategies [6]. Only systemic treatments are routinely considered for such patients. Yet recent systemic approaches even with anthracycline and/or taxanes have achieved small progress in terms of prolonging life expectancy [5, 6, 12]. High-dose chemotherapy with stem-cell transplantation has also failed to prolong survival times [13]. The median survival after chemotherapy remains at around 24 months, and cure rate is hopelessly low [3]. Thus, metastatic breast cancer is still regarded as incurable, and treatment is usually only palliative [4, 5]. Indeed, this may be true for most patients, but we found that some patients survived for long periods and some of them seemed to be cured of metastatic disease by surgery.

**Table 2** Patients survival over 10 years after surgery for lung metastasis of breast cancer

| Patient No. | Age at lung surgery | DFI (years) | Extra-pulmonary metastasis | No. metastasis | Max. metastasis size (cm) | Type of lung surgery | Node metastasis in mediastinum | Chemotherapy after surgery | Survival (years) | Recurrence after surgery | Prognosis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 45 | 3.7 | No | 1 | 2.8 | Lobect. | + | CPA + 5FU | 24.7 | No | Alive |
| 2 | 32 | 1.7 | No | 1 | 2.6 | Lobect. | – | CMFT | 22.2 | No | Alive |
| 3 | 65 | 6.1 | No | 1 | 3.1 | Lobect. | – | None | 19.3 | No | Alive |
| 4 | 50 | 1.9 | No | 1 | 2.5 | Lobect. | – | None | 16.4 | No | Alive |
| 5 | 50 | 8.9 | No | 1 | 1.8 | Lobect. | – | FACT | 16.0 | Pleura, brain | Alive with disease |
| 6 | 57 | 8.0 | No | 2 | 1 | Lobect. | – | CMF, FAC | 15.9 | Lung | Alive with disease |
| 7 | 74 | 9.0 | No | 1 | 2.5 | Lobect. | – | TAM, Ex | 15.4 | No | Alive |
| 8 | 62 | 1.0 | No | 3 | 3.2 | Lobect. | – | FAC | 15.2 | No | Alive |
| 9 | 65 | 11.5 | No | 1 | 3.3 | Lobect. | – | FAC | 15.1 | Liver | Died of BC |
| 10 | 37 | 2.3 | No | 1 | 1.3 | Lobect. | – | None | 14.8 | No | Died of other BC |
| 11 | 69 | 1.2 | No | 1 | 1.3 | Lobect. | – | FAC, CMF | 14.5 | No | Alive |
| 12 | 38 | 2.4 | No | 1 | 3.2 | Lobect. | + | FAC | 13.9 | No | Alive |
| 13 | 48 | 5.6 | No | 1 | 1.3 | Partial | + | FACT | 13.6 | No | Alive |
| 14 | 55 | 4.8 | No | 1 | 2 | Wedge | Not dissected | CMF | 13.3 | Lung | Alive with disease |
| 15 | 58 | 19.8 | No | 2 | 1.2 | Lobect. | + | FAC, CMF | 13.3 | No | Alive |
| 16 | 55 | 14.9 | Local skin | 1 | 0.7 | Lobect. | – | FACT | 13.2 | Lung | Died of BC |
| 17 | 49 | 1.6 | No | 1 | 1 | Partial | – | CMF | 11.9 | No | Alive |
| 18 | 50 | 7.4 | No | 1 | 1.3 | Lobect. | – | None | 11.4 | No | Died of colon cancer |
| 19 | 50 | 18.4 | No | 1 | 1.9 | Lobect. | + | TAM | 10.5 | No | Alive |
| 20[a] | 59 | 6.4 | No | 1 | 1.5 | Lobect. | + | FACT | 10.4 | Lung[a] | Died of BC |
| 21 | 50 | 4.9 | No | 2 | 1.7 | Lobect. | – | None | 10.2 | Brain | Died of BC |
| 22 | 44 | 10.4 | No | 1 | 3.2 | Pneumo. | + | None | 10.2 | No | Alive |

*Lobect* lobectomy, *Pneumo* pneumonectomy, *CPA* cyclophosphamide, *5FU* 5-fluorouracil, *CMF* CPA + methotrexate + 5FU, *T* tamoxifen, *FAC* 5FU + doxorubicin + CPA, *BC* breast cancer

[a] Patient undrewent second lung surgery

Under these general recognitions, our rationales for surgical treatment of lung metastasis of breast cancer are as follows: (1) To date, survival after lung metastasis has been discouragingly low despite intensive systemic treatment. (2) Several pioneers however, have found that surgical intervention can achieve promising results if curable resection can be performed. (3) Recent advances in lung surgery have rendered surgical resection a much safer operation with minimal physical trauma. (4) Recent advances in imaging techniques, such as CT and FDG-PET scanning, can detect early lung metastasis, allowing the selection of suitable surgical candidates. (5) Experimental tumour models indicate that the results of systemic approaches should be improved when the tumour burden is lowered [14]. (6) In addition to causing lung function to deteriorate, metastatic lung tumours might be a source of other systemic metastases if the tumour burden is large.

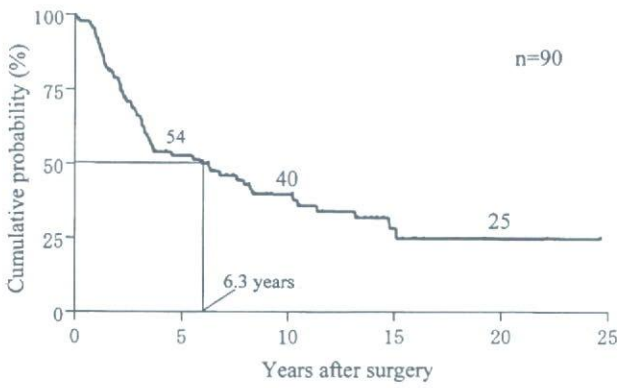


**Fig. 1** Cumulative survival curves after surgery for lung metastasis

Few reports have described the surgical treatment of lung metastasis, but some have indicated promising results (Table 4). Friedel et al. [7] studied 467 patients and

**Table 3** Survival after surgery for lung metastasis according to clinical status

| Clinical status | Number of patients | Median (50%) survival (year) | Cumulative 10-year survival rate (±SE) | Significance logrank test (Wilcoxon test) |
|---|---|---|---|---|
| Overall | 90 | 6.3 | 39.8 ± 5.6% | – |
| Age at lung surgery | | | | |
| ≦50 years | 32 | 6.3 | 46.4 ± 8.9 | NS |
| >50 years | 58 | 4.5 | 35.3 ± 7.2 | |
| Stage at breast surgery | | | | |
| I | 20 | >15 | 74.3 ± 10.0% | $P = 0.013$ |
| II | 53 | 3.4 | 29.6 ± 6.7% | |
| III/IV | 7 | 3.7 | 38.1 ± 19.9% | |
| Unknown | 10 | 3.1 | 34.3 ± 15.9 | |
| Disease-free interval | | | | |
| <3 years | 33 | 2.4 | 26.0 ± 8.1% | $P = 0.014$ |
| ≧3 years | 57 | 8.2 | 47.1 ± 7.3% | |
| Other recurrence site before lung surgery | | | | |
| None | 85 | 6.3 | 41.2 ± 5.8% | NS |
| Local recurrence (under control) | 5 | 1.5 | 20.0 ± 17.9% | |
| Number of lung metastasis | | | | |
| One | 78 | 5.9 | 39.3 ± 6.0% | NS |
| Two or more | 12 | 2.6 | 41.7 ± 7.3% | |
| Biggest size of metastasis | | | | |
| ≦2 cm | 44 | 8.2 | 49.4 ± 7.9% | $P = 0.168$ ($P = 0.043$) |
| >2 cm | 46 | 3.5 | 29.7 ± 7.6% | |
| Type of surgery | | | | |
| Wedge or segmental resection | 20 | 6.8 | 46.5 ± 13.1% | NS |
| Lobectomy or pneumonectomy | 70 | 3.5 | 37.6 ± 6.1% | |
| Radicality of surgery | | | | |
| Curative | 80 | 6.3 | 41.4 ± 5.8% | NS |
| Palliative | 10 | 2.7 | – | |
| Nodal dissection or sampling | | | | |
| Done | 84 | 5.5 | 40.2 ± 5.7% | NS |
| Not done | 6 | 4.6 | 26.7 ± 22.6% | |
| Nodal metastasis | | | | |
| Negative (including no information) | 53 | 7.1 | 44.1 ± 7.4% | $P = 0.186$ |
| Positive | 37 | 3.2 | 33.5 ± 8.4% | |

described them in the international registry of lung metastasis. The prognosis of these patients who underwent lung metastatectomy was 38% after 5 years, 22% after 10 years, and 20% after 15 years. Staren et al. [8] reported 5-year survival rates of 36% with a median survival of 55 months in a study of 33 patients. Lanza et al. [9] reported a 5-year survival rate of 50% with a median survival of 47 months in a study of 41 patients. Based on these results, Friedel expressed that lung metastatectomy is currently the best treatment option for selected patients with lung metastasis arising from breast cancer [7]. The present study adds more evidence that the surgical approach to treating lung metastases is beneficial.

The prognostic factors after surgery remain controversial. In the group of completely resected patients, a long DFI was one of the most significant favourable prognostic factors. The DFI was 36 months in this study and in that of Friedel et al. [7], but varies in other studies between 12 and 48 months [9, 15]. Other important factors were the clinical stage of the primary tumour in this study, the number of metastases [16], the biggest size of metastasis in this series [17]. Whether it is true or not, solitary lung metastasis without other remote metastases should be a best candidate, and waiting for several months to ensure a surgical candidate and routine lymph node dissections might have been a factor in our favourable results. Moreover, recent

**Table 4** Reports for surgical removal of lung metastases of breast cancer

| Author | Number of patients | Median age (range) | % of solitary metastasis | Curative surgery rate (%) | 5-year survival (%) | 10-year survival (%) | Median survival (months) |
|---|---|---|---|---|---|---|---|
| Lanza et al. [10] | 41 | 55 (32–79) | 73 | 90 | 50[a] | – | 47[a] |
| Staren et al. [9] | 33 | – | 82 | – | 36 | – | 55 |
| Friedel et al. [16][b] | 89 | 53 (23–78) | 59 | 76 | 27 | – | 31 |
| McDonald et al. [12] | 60 | 58 (21–81) | 52 | 67 | 38 | 8 | 42 |
| Friedel et al. [8][c] | 467 | 53 (21–87) | 69 | 84 | 38 | 22 | 35 |

[a] For cases with complete resection

[b] Single institute

[c] Multi-institutes

advancements in diagnostic instrumentation such as CT and FDG-PET scanning have surely conduct a favourable results of surgical strategy.

Opposition to surgical treatment for metastatic breast cancer is based on the oncological viewpoint that it is a systemic disease. Most medical oncologists assert that the surgical treatment of metastatic disease is pointless in the presence of systemic foci. The value of the systemic approach cannot be underestimated, but the hopelessly low cure rate achieved by systemic treatment is unjustifiable. We must consider that the success of systemic drug therapy in the adjuvant setting [1, 2] and its inability to cure patients in the metastatic setting [3, 4]. This evidence indicates that cytostatics can overcome microscopic, but not clinically large metastatic foci, which might be due to the heterogeneous nature of cancer cells that become resistant to cytostatics [18].

Since cancer cells can acquire heterogeneity during cell division, the nature of resistance to cytostatics is essentially proportional to tumour size [14]. Smaller metastatic foci will retain more clonal identity and less heterogeneity, and will thus be more sensitive to anticancer drugs than larger metastasis foci [14]. Moreover, a smaller disease burden presumably requires fewer "logs" to kill [18], or might confer more kinetic sensitivity to anticancer drugs [19]. If these notions are correct, then clinically evident metastatic tumours should be removed where possible, since the remaining microscopic metastatic foci will be more sensitive to anticancer drugs approximating their use as an adjuvant. This concept represents the foundation for our treating metastatic breast cancer by surgery. The results of the present study support the notion that this strategy is beneficial.

In conclusion, the data from our limited series of patients suggest that surgical treatment for operable lung metastasis of breast cancer will prolong survival in specific subgroups of patients to a greater extent than standard systemic drug therapy alone. To ascertain the significance of surgical treatment, a prospective randomized trial should compare conventional systemic drug therapies with and without surgery.

## References


1. Early Breast Cancer Trialist' Collaborative Group (1998) Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet 352:930–942
2. Early Breast Cancer Trialist' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351:1451–1467
3. Greenberg PA, Hortobagyi GN, Smith TL, Ziegler LD, Frye DK, Buzdar AU (1996) Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer. J Clin Oncol 14:2197–2205
4. Nabholtz JM, Lindsay MA, Hugh J, Mackey J, Smylie M, Au HJ, Tonkin K, Allen M (1999) The academic global virtual concept in clinical cancer research and its application to breast cancer: the breast cancer International Research Group. Semin Oncol 26:4–8
5. Sledge GW (1996) Should we dream the impossible dream? The meaning of long-term survival in metastatic breast cancer. J Clin Oncol 14:2191–2193
6. Hortobagyi GN (2002) Can we cure limited metastatic breast canacer? J Clin Oncol 20:620–623
7. Friedel G, Pastprino U, Ginsberg RJ, Goldstraw P, Johnston M, Pass H, Putnam JB, Yoomes H (2002) Results of lung metastasectomy from breast cancer: prognostic criteria on the basis of 467 cases of the International Registry of Lung Metastases. Eur J Cardiothorac Surg 22:335–344
8. Staren ED, Salerno C, Rongione A, Witt TR, Faber LP (1992) Pulmonary resection for metastatic breast cancer. Arch Surg 127:1282–1284
9. Lanza LA, Natarajan G, Roth JA, Putnam JB (1992) Long-term survival after resection of pulmonary metastasis from carcinoma of the breast. Ann Thorac Surg 54:244–248
10. Martini N, McCormack PM (1998) Evolution of the surgical management of pulmonary metastases. Chest Surg Clin N Am 8:13–27
11. McDonald ML, Deschamps C, Ilstrup DM, Allen MS, Trastek VF, Pairolero PC (1994) Pulmonary resection for metastatic breast cancer. Ann Thorac Surg 58:1599–1602
12. Giordano SH, Buzdar AU, Smith TL, Kau SW, Yang Y, Hortobagyi GN (2004) Is breast cancer survival improving? Cancer 100:44–52
13. Gerrero RM, Stein S, Stadtmauer EA (2002) High-dose chemotherapy and stem cell support for breast cancer: where are we now? Drugs Aging 19:475–485

14. Goldie JH, Coldman AJ (1983) Quantitative model for multiple levels of drug resistance in clinical tumors. Cancer Treat Rep 67:923–931
15. Friedel G, Linder A, Toomes H (1994) The significance of prognostic factors for the resection of pulmonary metastases of breast cancer. Thorac Cardiovasc Surg 42:71–75
16. Bodzin GA, Staren ED, Faber PL (1998) Breast carcinoma metastases. Chest Surg Clin N Am 8:145–156
17. Schlappack OK, Baur M, Steger G, Dittrich C, Moser K (1988) The clinical course of lung metastases from breast cancer. Klin Wochenschr 66:790–795
18. Skipper HE, Schabel FM (1984) Tumor stem cell heterogeneity: implications with respect to classification of cancers by chemotherapeutic effect. Cancer Treat Rep 68:43–61
19. Norton L, Simon R (1986) The Norton-Simon hypothesis revisited. Cancer Treat Rep 70:163–169

# Multiplex Reverse Transcription-PCR Screening for *EML4-ALK* Fusion Transcripts

Kengo Takeuchi,[1] Young Lim Choi,[3] Manabu Soda,[3] Kentaro Inamura,[1] Yuki Togashi,[1] Satoko Hatano,[1] Munehiro Enomoto,[3] Shuji Takada,[3] Yoshihiro Yamashita,[3] Yukitoshi Satoh,[2] Sakae Okumura,[2] Ken Nakagawa,[2] Yuichi Ishikawa,[1] and Hiroyuki Mano[3,4]

**Abstract**

**Purpose:** EML4-ALK is a fusion-type protein tyrosine kinase that is generated by inv(2)(p21p23) in the genome of non–small cell lung cancer (NSCLC). To allow sensitive detection of *EML4-ALK* fusion transcripts, we have now developed a multiplex reverse transcription-PCR (RT-PCR) system that captures all in-frame fusions between the two genes.

**Experimental Design:** Primers were designed to detect all possible in-frame fusions of *EML4* to exon 20 of *ALK*, and a single-tube multiplex RT-PCR assay was done with total RNA from 656 solid tumors of the lung ($n = 364$) and 10 other organs.

**Results:** From consecutive lung adenocarcinoma cases ($n = 253$), we identified 11 specimens (4.35%) positive for fusion transcripts, 9 of which were positive for the previously identified variants 1, 2, and 3. The remaining two specimens harbored novel transcript isoforms in which exon 14 (variant 4) or exon 2 (variant 5) of *EML4* was connected to exon 20 of *ALK*. No fusion transcripts were detected for other types of lung cancer ($n = 111$) or for tumors from 10 other organs ($n = 292$). Genomic rearrangements responsible for the fusion events in NSCLC cells were confirmed by genomic PCR analysis and fluorescence *in situ* hybridization. The novel isoforms of EML4-ALK manifested marked oncogenic activity, and they yielded a pattern of cytoplasmic staining with fine granular foci in immunohistochemical analysis of NSCLC specimens.

**Conclusions:** These data reinforce the importance of accurate diagnosis of EML4-ALK–positive tumors for the optimization of treatment strategies.

**Authors' Affiliations:** [1]Division of Pathology, The Cancer Institute, [2]Department of Thoracic Surgical Oncology, Thoracic Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan; [3]Division of Functional Genomics, Jichi Medical University, Tochigi, Japan; and [4]CREST, Japan Science and Technology Agency, Saitama, Japan

Received 4/19/08; revised 6/23/08; accepted 7/3/08.

**Grant support:** Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and grants from the Japan Society for the Promotion of Science; from the Ministry of Health, Labor, and Welfare of Japan; from the National Institute of Biomedical Innovation; from the Smoking Research Foundation; and from the Vehicle Racing Commemorative Foundation.

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

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

K. Takeuchi and Y.L. Choi contributed equally to this work.

Current address for Y. Satoh: Department of Thoracic Surgery, Kitasato University School of Medicine, Kanagawa 228-8520, Japan.

The nucleotide sequences of the *EML4-ALK* variant 4, 5a, and 5b cDNAs have been deposited in DDBJ/EMBL/Genbank under the accession numbers AB374363, AB374364, and AB374365, respectively.

**Requests for reprints:** Kengo Takeuchi, Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan. Phone: 81-3-3520-0111; Fax: 81-3-3570-0558; E-mail: kentakeuchi-tky@umin.net.



doi:10.1158/1078-0432.CCR-08-1018

Chromosome rearrangement is a major mechanism giving rise to transforming potential in human cancers, especially in hematologic malignancies (1). A balanced translocation between chromosomes 9 and 22, for instance, generates an activated protein tyrosine kinase, BCR-ABL, that plays an essential role in the pathogenesis of chronic myeloid leukemia (2). The gene for another protein tyrosine kinase, ALK, is fused to those for NPM1 or other partner proteins in anaplastic lymphoma and soft tissue tumors, resulting in an increase in the kinase activity of ALK (3).

Mitelman et al. have suggested that chromosome translocations, in addition to being common in hematologic malignancies, are not rare in epithelial tumors (4, 5). These researchers also proposed that the genetic mechanisms underlying oncogenesis might not differ fundamentally between hematologic and epithelial malignancies, and that the current apparent difference in the frequency of chromosomal translocations between these two types of cancer is likely to disappear with the advent of new and more powerful investigative tools.

Consistent with this notion, recurrent chromosome rearrangements involving genes for ETS transcriptional factors have been identified in many cases of prostate cancer and may contribute to the hypersensitivity of prostate cancer cells to androgens (6, 7). In addition, we recently discovered another

### Translational Relevance

EML4-ALK is a fusion-type protein-tyrosine kinase generated through a recurrent chromosome rearrangement, inv(2)(p21p23), observed in non–small cell lung cancer (NSCLC). Because both *EML4* and *ALK* genes are mapped to the short arm of chromosome 2 in opposite orientations, PCR with primer sets flanking the fusion points of the two genes would not produce any specific products from cells without inv(2)(p21p23). Reverse transcription (RT)-PCR for the fusion point would, therefore, become a highly sensitive and accurate means to detect tumors positive for *EML4-ALK*. Such analyses may detect small amounts of cancer cells in sputa from individuals with NSCLC at early clinical stages. Because several isoforms have been already reported for *EML4-ALK*, it is mandatory to detect all isoforms of the fusion kinase in a sensitive and reliable way. Toward this goal, we here developed a single-tube multiplex RT-PCR screening system to capture all possible isoforms of EML4-ALK. Examination of various tumor samples ($n$ = 656) with our multiplex RT-PCR has indeed identified 11 specimens positive for the variants of EML4-ALK only among lung adenocarcinoma ($n$ = 253). Our system, thus, paves a way for a sensitive molecular detection of this intractable disorder at early curable stages.

recurrent chromosome translocation in non–small cell lung cancer (NSCLC; ref. 8), a major cause of cancer deaths in humans. A small inversion within the short arm of chromosome 2, inv(2)(p21p23), was found to be present in <10% of NSCLC cases and to give rise to a novel fusion-type tyrosine kinase, EML4-ALK, that exhibited marked transforming activity *in vitro* (8). Transgenic mice that specifically express EML4-ALK in lung epithelial cells were also found to develop hundreds of adenocarcinoma nodules in both lungs at only a few weeks after birth, and such nodules disappeared rapidly in response to oral administration of a specific inhibitor of the catalytic activity of ALK.[5] These data thus indicate that EML4-ALK plays a pivotal role in malignant transformation in lung cancer, and they suggest that chemical compounds that inhibit the tyrosine kinase activity of EML4-ALK may provide an effective treatment for EML4-ALK–positive lung cancer. The selection of suitable drugs for individuals with lung cancer will thus require accurate determination of the absence or presence of the *EML4-ALK* fusion gene in biopsy specimens.

Given that *EML4* and *ALK* map in opposite orientations within the short arm of chromosome 2, reverse transcription-PCR (RT-PCR) analysis with primers designed to amplify the fusion points of *EML4-ALK* transcripts would not be expected to yield specific products from normal cells or cancer cells without inv(2)(p21p23). Such analysis should thus provide a highly reliable and sensitive means to detect *EML4-ALK* in clinical specimens. Given that sputum has been shown to be a suitable specimen for such molecular diagnosis of *EML4-ALK* positivity (8), detection of *EML4-ALK*–positive cells by RT-PCR analysis of sputa may be effective for the identification of lung cancer at early clinical stages. The accurate diagnosis of *EML4-ALK*–positive tumors, however, will require that all isoforms of *EML4-ALK* are detected.

The fusion of intron 13 or 20 of *EML4* to intron 19 of *ALK* gives rise to variant 1 or 2 of *EML4-ALK*, respectively (8). We have recently discovered another isoform (variant 3) of *EML4-ALK* in which intron 6 of *EML4* is ligated to intron 19 of *ALK* (9). Theoretically, in addition to such fusion of exons 6, 13, and 20 of *EML4*, an in-frame fusion to exon 20 of *ALK* can occur with exons 2, 18, or 21 of *EML4*. Given that the amino-terminal coiled-coil domain of EML4 is responsible for the dimerization and constitutive activation of EML4-ALK (8) and that exon 2 of *EML4* encodes the entire coiled-coil domain, all of these possible fusion genes would encode EML4-ALK proteins containing the coiled-coil domain and therefore likely produce oncogenic EML4-ALK kinases.

To establish a highly sensitive and accurate PCR-based screening system for *EML4-ALK*–positive cancer, we have now developed a high-throughput multiplex RT-PCR assay for the detection of all potential *EML4-ALK* in-frame fusion transcripts. Among a consecutive series of lung adenocarcinoma specimens ($n$ = 253) as well as other solid tumor samples ($n$ = 403), we have now identified a total of 11 lung adenocarcinoma specimens positive for *EML4-ALK*, two of which harbor previously unidentified fusion mRNAs.

## Materials and Methods

***Clinical samples and RNA extraction.*** This study was done with clinical samples from 253 lung adenocarcinomas, 90 other NSCLCs (71 squamous cell carcinomas, 7 adenosquamous carcinomas, 7 large cell carcinomas, 2 pleomorphic carcinomas, and 3 large cell endocrine carcinomas), 21 small cell lung carcinomas, 50 breast carcinomas, 46 renal cell carcinomas, 48 colon carcinomas, 13 prostate carcinomas, 29 urothelial carcinomas, 33 gastric carcinomas, 10 uterine carcinomas, 9 hepatocellular carcinomas, 8 pancreatic carcinomas, and 46 malignant fibrous histiocytomas. All specimens were collected with the approval of the ethical committee at the Cancer Institute Hospital (Tokyo, Japan) and with the informed consent of individuals undergoing surgery from May 1995 to July 2003. The NSCLC cases were consecutive and spanned a period of 19 mo. Histologic diagnosis of NSCLC was made according to the WHO classification (10). All lesions were grossly dissected, rapidly frozen in liquid nitrogen, and stored at -80°C until RNA extraction with an RNeasy Mini Kit (Qiagen). RNA quality and the absence of contamination with genomic DNA were verified by formaldehyde-agarose gel electrophoresis.

***Multiplex RT-PCR analysis and nucleotide sequencing.*** Total RNA was subjected to RT with random primers and SuperScript III reverse transcriptase (Invitrogen). For detection of *EML4-ALK* fusion cDNAs, multiplex PCR analysis was done with AmpliTaq Gold DNA polymerase (Applied Biosystems), the forward primers EML4 72F (5′-GTCAGCTCTTGAGTCACGAGTT-3′) and Fusion-RT-S (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′), and the reverse primer ALK 3078RR (5′-ATCCAGTTCGTCCTGTTCAGAGC-3′). The *GAPDH* cDNA was amplified by PCR with the primers 5′-GTCAGTGGTGGACCTGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. For amplification of *EML4-ALK* fusion cDNAs, the samples were incubated at 94°C for 10 min and then subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and polymerization at 72°C for 1 min. For amplification of *GAPDH* cDNA, the samples were subjected to 35 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 30 s. Virtual gel electrophoresis of multiplex RT-PCR products was done with a 2100 Bioanalyzer (Agilent Technologies).

[5] M. Soda et al., submitted for publication.

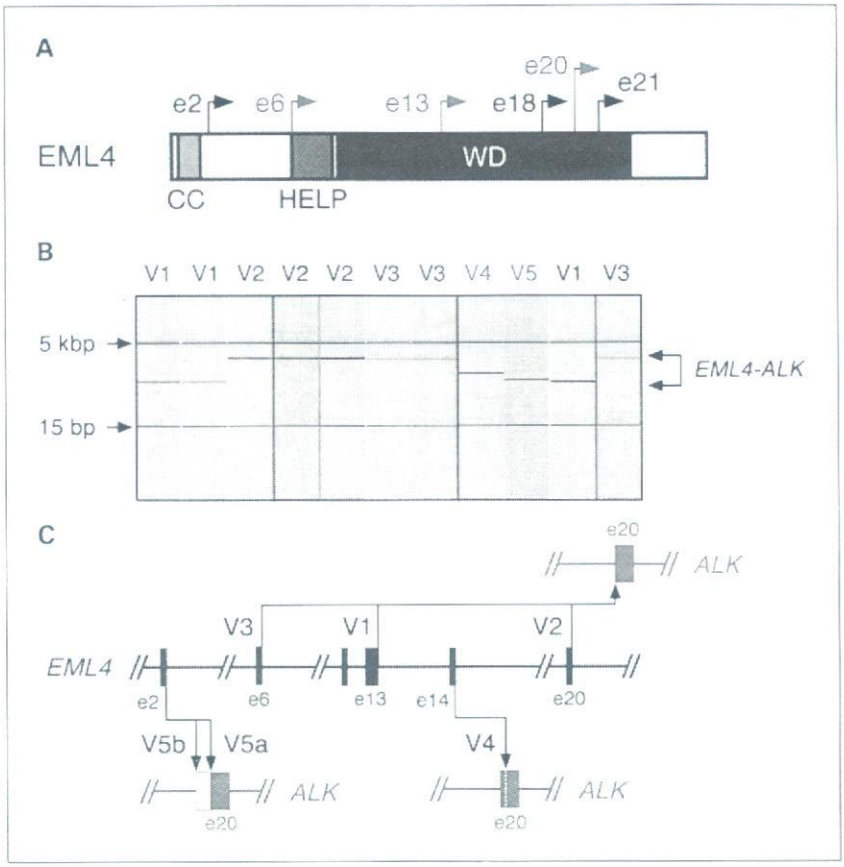


Fig. 1. Identification of *EML4-ALK* variants 4 and 5. *A*, schematic representation of the structure of EML4. The corresponding positions of exons (*e*) that can theoretically be fused in-frame to exon 20 of *ALK* are indicated by arrows, with known fusion points being denoted in red. CC, coiled-coil domain; HELP, hydrophobic EMAP (echinoderm microtubule-associated protein) – like protein domain; WD, WD repeats. *B*, virtual gel electrophoresis of multiplex RT-PCR products derived from lung adenocarcinoma specimens. Seven samples (*blue*) were known to harbor *EML-ALK* variants (*V*) 1, 2, or 3, whereas four samples were newly detected by multiplex RT-PCR. Two of the latter four specimens yielded PCR products corresponding to the newly identified variants 4 and 5. The positions of the fusion products of *EML4-ALK* are indicated on the right, and those of DNA size standards (5 kbp and 15 bp) are shown on the left. *C*, fusions between exons of *EML4* and *ALK*. Fusion of exons 6, 13, or 20 of *EML4* to exon 20 of *ALK* gives rise to variants 3, 1, and 2 of *EML4-ALK*, respectively. In addition, nucleotide sequencing of the PCR products shown in *B* revealed that exon 14 or 2 of *EML4* was fused to exon 20 of *ALK* in the cDNAs for *EML4-ALK* variants 4 and 5, respectively.

The primers used for direct amplification of the fusion points of individual cDNAs were 5′-AGGAGAGAACTCAGCGACACTACC-3′ and 5′-TCCACGCTCAAAAGTGCCAAGTCC-3′ for variant 4 and 5′-GCTTTCCCCGCAAGATGGACGG-3′ and 5′-AGCTTGCTCAGCTTGTACTCAGGG-3′ for variant 5. Full-length cDNAs for *EML4-ALK* variants were amplified with PrimeSTAR DNA polymerase (Takara Bio) and the primers 5′-ACTCTGTCGGTCCGCTGAATGAAG-3′ and 5′-CCACGGTCTTAGGGATCCCAAGG-3′.

***Fluorescence in situ hybridization analysis.*** Surgically resected lung cancer tissue was fixed in 20% formalin, embedded in paraffin, sectioned at a thickness of 4 μm, and placed on glass slides. The unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescently labeled bacterial artificial chromosome clone probes for *EML4* and *ALK* (GSP Laboratory) or for genomic regions upstream and downstream of the *ALK* break point (Dako), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus).

***Immunohistochemical analysis.*** Unstained paraffin-embedded sections were depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and then subjected to heat-induced antigen retrieval with Target Retrieval Solution pH 9.0 (Dako) before immunohistochemical staining with a mouse monoclonal antibody to ALK (ALK1, Dako) at a dilution of 1:20. Immune complexes were detected with the use of an EnVision+DAB system (Dako) with minor modifications.[6]

***Transforming potential of EML4-ALK proteins.*** Protein analysis of EML4-ALK variants was done as described previously (8). In brief, the *EML4-ALK* variant 4, 5a, or 5b cDNAs were fused with an oligonucleotide encoding the FLAG epitope tag and inserted into the retroviral expression plasmid pMXS (11). The resulting plasmids and similar pMXS-based expression plasmids for EML4-ALK variant 1, variant 1(K589M), variant 2, variant 3a, and variant 3b were individually introduced into HEK293 cells. Lysates of the transfected cells were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were subjected either to immunoblot analysis with the same antibodies or to an *in vitro* kinase assay with the YFF peptide (12). Mouse 3T3 fibroblasts were also infected with recombinant retroviruses for each of the EML4-ALK variants or wild-type ALK and were then cultured for 12 d for a focus formation assay. The same set of 3T3 cells was injected s.c. into nu/nu mice, and tumor formation was examined after 20 d.

## Results

***Multiplex RT-PCR screening for EML4-ALK fusion transcripts in lung adenocarcinoma.*** As described above, exons 2, 6, 13, 18, 20, and 21 of *EML4* may participate in an in-frame fusion to exon 20 of *ALK* (Fig. 1A). To identify all possible *EML4-ALK* fusion cDNAs in a single-tube experiment, we designed a mixture of two sense primers (one targeted to exon 2 and the other to exon 13 of *EML4*) and a single antisense primer (targeted to exon 20 of *ALK*) and did multiplex RT-PCR with these primers and total cDNA preparations from tumor specimens. The exon 2 primer for *EML4* would be expected to generate a PCR product of 458 bp with the exon 2 (*EML4*)-exon 20 (*ALK*) fusion cDNA or of 917 bp with the exon 6-exon 20 fusion cDNA (variant 3). In addition, the exon 13 primer for *EML4* would be expected to generate PCR products of 432, 999, 1,185, or 1,284 bp with the exon 13-exon 20 (variant 1), exon 18-exon 20, exon 20-exon 20 (variant 2), and exon 21-exon 20 fusion cDNAs, respectively.

[6] K. Takeuchi et al., manuscript in preparation.

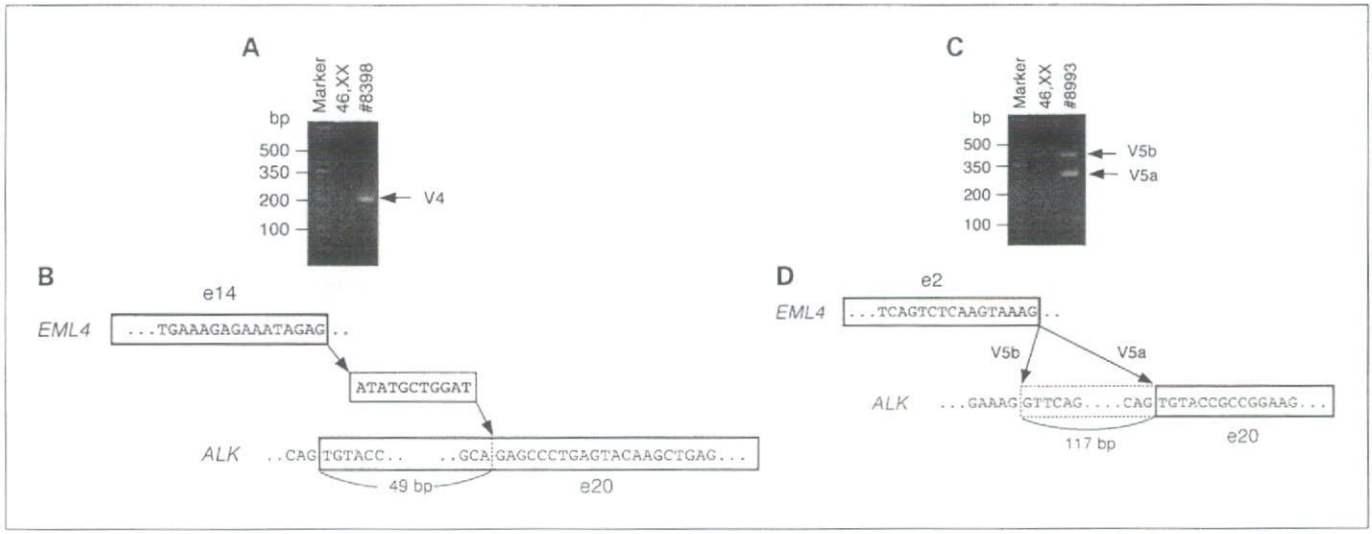


Fig. 2. Structure of *EML4-ALK* variant 4 and 5 cDNAs. *A*, RT-PCR amplification of the fusion point of *EML4-ALK* variant 4 mRNA in NSCLC specimen ID no. 8398 as well as in peripheral blood mononuclear cells of a female volunteer (46,XX). A PCR product of 203 bp corresponding to *EML4-ALK* variant 4 was specifically amplified from the tumor cells. The left lane contains DNA size standards (50-bp ladder). *B*, nucleotide sequencing of the PCR product in *A* revealed that exon 14 of *EML4* (*blue*) was connected to an 11-bp cDNA fragment of unknown identity (*black*), which was ligated in turn to the nucleotide at position 50 of exon 20 of *ALK* (*red*). *C*, RT-PCR amplification of the fusion point of *EML4-ALK* variant 5 mRNA in NSCLC specimen ID no. 8993 as well as in peripheral blood mononuclear cells of a female volunteer (46,XX). Two specific products of 415 and 298 bp were obtained, corresponding to variants 5b and 5a, respectively. The left lane contains DNA size standards (50-bp ladder). *D*, nucleotide sequencing of the PCR products in *C* revealed that exon 2 of *EML4* was fused either to exon 20 of *ALK*, generating the variant 5a cDNA, or to a position 117 bp upstream of exon 20 of *ALK*, generating the variant 5b cDNA.

Virtual gel electrophoresis of the multiplex RT-PCR products (Fig. 1B) revealed that 11 samples (4.35%) were positive for *EML4-ALK* cDNA among a consecutive series of 253 lung adenocarcinoma specimens, including those examined in our previous studies (8, 9, 13). All of the specimens previously shown to harbor *EML4-ALK* (two cases with variant 1, three with variant 2, and two with variant 3) were faithfully detected with our multiplex RT-PCR system. No specific PCR products were obtained for other types of lung cancer ($n = 111$) or other solid tumors ($n = 292$). Nucleotide sequencing of the PCR products for the newly identified positive cases revealed that one specimen was positive for variant 1 and another for variant 3 of *EML4-ALK*, but that the remaining two specimens harbored previously unidentified variants (Fig. 1B and C). Exon 14 of *EML4* was ligated to a position within exon 20 of *ALK* in the product from tumor ID no. 8398 (designated variant 4), whereas exon 2 of *EML4* was ligated to exon 20 of *ALK* in the product from tumor ID no. 8993 (designated variant 5).

***Structure of EML4-ALK variant 4 cDNA.*** To verify the presence of novel *EML4-ALK* variants in the cancer cells, we first did direct RT-PCR analysis for the cDNA of tumor ID no. 8398 with a new set of primers encompassing the putative fusion point of variant 4. This analysis showed the presence of the fusion cDNA (Fig. 2A). Nucleotide sequencing of the PCR product revealed that exon 14 of *EML4* was fused to an unknown sequence of 11 bp, which in turn was connected to the nucleotide at position 50 of exon 20 of *ALK* (Fig. 2B). (We failed to detect a region of the human genome (build 36) homologous to the 11-bp connecting sequence in a BLAST search.[7]) Although exon 14 of *EML4* is not expected to produce an in-frame fusion to exon 20 of *ALK*, insertion of the unknown 11-bp sequence and its ligation to a position within the *ALK* exon allows an in-frame connection between the two genes. Fusion cDNAs in which the point of connection is located within, rather that at the 5′ terminus of, exon 20 of *ALK* have also been described for *MSN-ALK* (14) and *MYH9-ALK* (15).

We further examined whether a full-length cDNA encoding such an unexpected EML4-ALK variant could be isolated from the cancer cells. For this purpose, we designed a sense primer targeted to the 5′ untranslated region of *EML4* cDNA as well as an antisense primer targeted to the 3′ untranslated region of *ALK* cDNA. Direct RT-PCR analysis with this primer set yielded a single PCR product of ~3.4 kbp with total cDNA of tumor ID no. 8398 (Supplementary Fig. S1A). Complete nucleotide sequencing of the PCR product revealed that the cDNA contained an open reading frame for 1,097 amino acids comprising residues 1 to 547 of human EML4, residues 1,075 to 1,620 of human ALK, and 4 amino acids of unknown origin between these two sequences (Supplementary Fig. S1B). The isolation of a full-length cDNA containing the 11-bp insert indicated that the variant 4 protein was likely expressed in the cancer cells.

***Structure of EML4-ALK variant 5 cDNAs.*** We similarly investigated the presence of variant 5 mRNA in the cells of tumor ID no. 8993. Direct RT-PCR analysis to amplify the fusion point of this variant cDNA yielded two independent products of 298 and 415 bp (Fig. 2C). Nucleotide sequencing of each product revealed that the former contained exon 2 of *EML4* and exon 20 of *ALK*, as expected, whereas in the latter, exon 2 of *EML4* was connected to a position within intron 19 of *ALK* located 117 bp upstream of exon 20 (Fig. 2D). These fusion constructs were designated variants 5a and 5b, respectively.

Although no mRNAs or expressed sequence tags in the nucleotide sequence database were found to contain the

[7] http://www.ncbi.nlm.nih.gov/genome/seq/blastgen/blastgen.cgi?taxid=9606

117-bp sequence of intron 19 of *ALK*, the human genome sequence surrounding the 5′ terminus of this 117-bp sequence is AG-GT (Fig. 2D), which conforms to the consensus sequence for a splicing acceptor site. To show that such a cryptic exon is indeed involved in the production of an oncogenic kinase, we attempted to detect full-length cDNAs for variants 5a and 5b from total cDNA of tumor ID no. 8993. A doublet of PCR products of ~2.0 kbp was obtained (Supplementary Fig. S1A), and nucleotide sequencing of these products revealed that they indeed encode EML4-ALK variant 5a and 5b proteins (Supplementary Fig. S1C). Genomic PCR and fluorescence *in situ* hybridization (FISH) analyses further revealed that the cells of tumor ID no. 8993 harbor a single *EML4-ALK* fusion gene, suggesting that variant 5a and 5b mRNAs are generated by alternative splicing of the primary transcript of this single fusion gene (see below).

***Detection of the EML4-ALK fusion genes by FISH.*** To confirm the rearrangements involving the *ALK* locus in the specimens harboring variants 4 and 5 of *EML4-ALK* cDNA, we did FISH analysis with tissue sections. We first designed a FISH-based "fusion assay" for *EML4* and *ALK* genes. Bacterial artificial chromosome fragments encompassing the entire genes were fluorescently labeled green and red, respectively. An overlapping signal for both probes was readily identified in a merged image for the tumor cells harboring variants 4 or 5 of *EML4-ALK* (Fig. 3A). To confirm further the breakage of the *ALK* locus, we did an "*ALK* split assay" with bacterial artificial chromosome fragments encompassing the 5′ or 3′ regions of the locus and labeled green and red, respectively. In this assay, the normal *ALK* locus would be expected to yield an overlapping signal, whereas a pair of separate green and red signals would indicate genomic breakage within *ALK*. As expected, a proportion of cells of tumor ID no. 8398 or no. 8993 in the histologic sections generated one overlapping signal and one pair of split signals (Fig. 3B), suggesting that these tumor cells each have at least one normal and at least one rearranged *ALK* locus.

These data, together with genomic PCR analysis (data not shown), thus indicated that the cells of each of these tumors harbor one normal chromosome 2 and a chromosome 2 with an inv(2)(p21p23) rearrangement. The other *EML4-ALK* cDNA-positive specimens (variants 1 to 3) in this cohort showed a similar FISH labeling profile, consistent with the presence of the corresponding *EML4-ALK* rearrangements (data not shown).

***Detection of EML4-ALK proteins* in situ.** To detect EML4-ALK proteins in the cancer cells, we did immunohistochemical analysis with the ALK1 monoclonal antibody to ALK (16). The cytoplasm of tumor cells harboring *EML4-ALK* variant 1 (ID no. 9034), variant 4 (ID no. 8398), or variant 5 (ID no. 8993) manifested a diffuse pattern of immunoreactivity with fine granular concentrations (Fig. 3C). No normal pulmonary epithelial cells or lymphocytes in the sections of these specimens reacted with the antibody.

***Transforming activity of EML4-ALK variants.*** We prepared expression plasmids for FLAG epitope-tagged EML4-ALK variants 1, 2, 3a, 3b, 4, 5a, and 5b, the predicted molecular sizes of which are 118,356; 146,913; 87,613; 88,874; 122,541; 71,046; and 74,867 Da, respectively. Each of these proteins, as well as a kinase-inactive mutant of EML4-ALK variant 1 (8), was expressed independently in HEK293 cells, immunoprecipitated, and subjected to immunoblot analysis with antibodies to FLAG. Each cDNA generated an EML4-ALK protein of the expected molecular size (Fig. 4A). The same immunoprecipitates were subjected to an *in vitro* kinase assay with the synthetic peptide YFF (12). Each variant protein (with the exception of the kinase-inactive mutant of variant 1) was shown to possess protein tyrosine kinase activity, with that of variants 3a, 3b, and 5b being most prominent (Fig. 4A).

To examine the transforming potential of the EML4-ALK variants, we transfected mouse 3T3 fibroblasts with the corresponding expression plasmids and then cultured the cells for 12 days. Transformed foci were readily detected for the cells expressing the variants of EML4-ALK but not for cells overexpressing wild-type ALK (Fig. 4B). Furthermore, s.c. injection of the transfected 3T3 cells into the shoulder of nude mice revealed that those expressing the various EML4-ALK isoforms, but not those overexpressing wild-type ALK, formed large tumors *in vivo* (Fig. 4B).

## Discussion

We have done multiplex RT-PCR analysis to detect all possible isoforms of *EML4-ALK* transcripts in NSCLC cells, and unexpectedly identified two novel subtypes of the fusion event. This finding was supported by detection of the corresponding fusion genes by genomic PCR and FISH

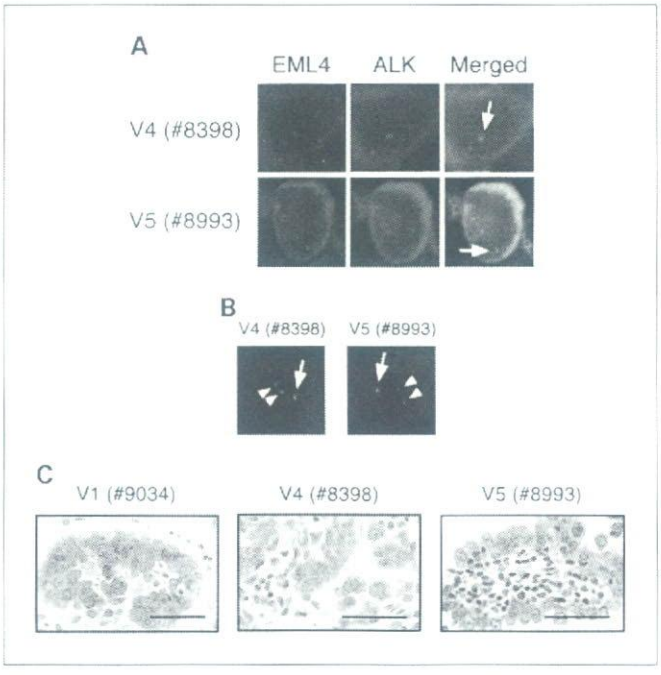


**Fig. 3.** FISH and immunohistochemical analyses of NSCLC specimens. *A*, FISH analysis of representative cancer cells in sections of lung adenocarcinoma harboring *EML4-ALK* variant 4 (ID no. 8398) or variant 5 (ID no. 8993). Each section was subjected to hybridization with differentially labeled probes for *EML4* (*left*) or for *ALK* (*center*). A fusion signal (*arrow*) and a pair of green (*EML4*) and red (*ALK*) signals are present in each merged image (*right*). *B*, the same clinical specimens as in *A* were subjected to FISH analysis with differentially labeled probes for the 5′ (*green*) or 3′ (*red*) regions of the *ALK* locus. A pair of split signals (*arrowheads*) and an overlapping signal (*arrow*) indicate the rearranged and normal *ALK* loci, respectively. *C*, immunohistochemical analysis of NSCLC specimens positive for *EML4-ALK* variants 1 (ID no. 9034), 4 (ID no. 8398), or 5 (ID no. 8993) with a monoclonal antibody to ALK. A pattern of diffuse staining with fine granular foci was apparent in the cytoplasm of all three tumors. Scale bars, 50 μm.

analyses and by that of the encoded proteins by immunohistochemical analysis in the NSCLC cells. Together with the previously isolated variants (8, 9), we have to date identified a total of seven distinct isoforms of EML4-ALK (variants 1, 2, 3a, 3b, 4, 5a, and 5b). Given that each of these isoforms possesses marked transforming activity, they all likely play an important role in the development of NSCLC. Our failure to detect *EML4-ALK* cDNA in the other solid tumors ($n$ = 313) examined suggests that *EML4-ALK* may be an oncogene specific to NSCLC, especially to lung adenocarcinoma.

In our multiplex RT-PCR analysis, a sense primer targeted to exon 2 of *EML4* was designed to detect fusion events involving exon 2 or 6 of *EML4*, and PCR products of the expected sizes were indeed obtained with NSCLC specimens positive for such fusion events (variants 5 and 3, respectively). The other sense primer was targeted to exon 13 of *EML4* and was designed to detect fusion events involving exon 13, 18, 20, or 21 of *EML4*. Given that we were able to readily amplify a specific product of 1185 bp corresponding to the fusion event involving exon 20 of *EML4* (variant 2), it is likely that all possible fusions giving rise to PCR products up to this size would have been detected in our cohort. It should be noted, however, that a possible fusion between exon 21 of *EML4* and exon 20 of *ALK* would be expected to generate a PCR product of 1,284 bp. Although the size difference between the 1,185- and 1,284-bp products is small (99 bp), it is still possible that our multiplex RT-PCR analysis failed to efficiently amplify the longer product and that there may be as-yet-undetected fusion events for *EML4-ALK* in our cohort.

All EML4-ALK isoforms manifested a similar subcellular distribution profile despite marked differences in the size and domain structure of the EML4 portions of these chimeric proteins. In addition, the intracellular signaling systems activated by EML4-ALK may be shared among variants 1 to 5 (Supplementary Fig. S2). The EML4 portion of variant 5 comprises only the coiled-coil domain. This domain of EML4 may therefore play an essential role not only in the dimerization and activation of EML4-ALK isoforms (8) but also in tethering EML4-ALK to specific subcellular components. The pattern of subcellular immunostaining for EML4-ALK (cytoplasmic staining with fine granular foci) was distinct from that for other ALK fusion proteins associated with other malignancies (17, 18), suggesting that the subcellular localization of ALK fusion kinases varies substantially. The first such fusion kinase to be identified, NPM-ALK, preferentially phosphorylates STAT3, which is thought to participate in mitogenic signaling by NPM-ALK (19–21). Five ALK fusion kinases (NPM-ALK, TFG-ALK, ATIC-ALK, TPM3-ALK, and CLTC-ALK) were shown to differ markedly in their abilities to transform 3T3 fibroblasts, to phosphorylate STAT3 and AKT, and to activate phosphoinositide 3-kinase (17). Furthermore, a proteomics approach to identify tyrosine-phosphorylated proteins failed to detect marked phosphorylation of STAT3 in NSCLC specimens positive for EML4-ALK (22). It is therefore likely that each ALK fusion kinase exerts its effects through fusion-specific (although possibly partially overlapping) downstream pathways. In addition, we detected slight differences in catalytic and transforming activities among the variants of EML4-ALK (Fig. 4). These differences are likely due to the different portions of EML4 present in the different variants, which may affect dimerization affinity or the recruitment of substrates.

In addition to *EML4-ALK*, NSCLC cells harbor other potent oncogenes such as mutant versions of *EGFR* or *KRAS*. These three oncogenes, however, were found to be mutually exclusive

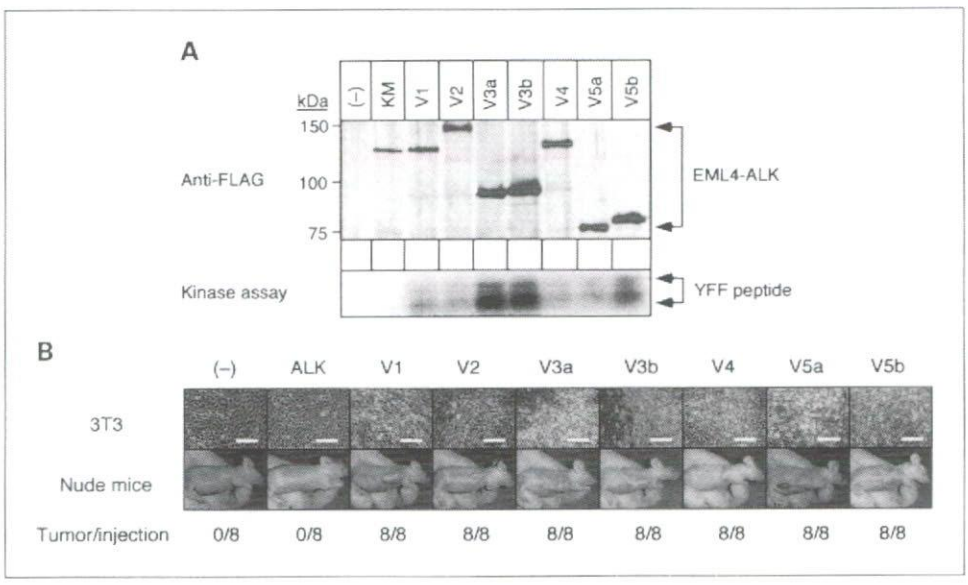


**Fig. 4.** Transforming potential of EML4-ALK variants. *A*, HEK293 cells expressing FLAG-tagged variant 1, 2, 3a, 3b, 4, 5a, or 5b of EML4-ALK were lysed and subjected to immunoprecipitation with antibodies to FLAG. The resulting precipitates were then either subjected to immunoblot analysis with antibodies to FLAG (*top*) or assayed for kinase activity with the synthetic YFF peptide (*bottom*). Cells transfected with the empty vector (-) or with a vector for a kinase-inactive mutant (*KM*) of EML4-ALK variant 1 were also analyzed. The positions of molecular size standards (kDa) and of EML4-ALK proteins are indicated on the left and right of the top panel, respectively. *B*, mouse 3T3 fibroblasts were transfected with expression plasmids for wild-type ALK or FLAG-tagged EML4-ALK variants, or with the empty plasmid (-), and were photographed after culture for 12 d (*top*). Scale bars, 200 μm. Alternatively, the transfected cells were injected s.c. into the shoulder of nu/nu mice and tumor formation was examined after 20 d (*bottom*). The number of tumors formed per eight injections is indicated at the bottom.

in our previous NSCLC cohort (8, 13), suggesting that EML4-ALK–positive NSCLC is a distinct subclass of lung cancer. Given that a selective inhibitor of the kinase activity of ALK rapidly induces cell death in EML4-ALK–positive cancer cells both *in vitro* (8, 9) and *in vivo*,[8] determination of the presence or absence of *EML4-ALK* in a given tumor may in the future inform the choice of treatment strategy for NSCLC. The demonstration of the existence of multiple isoforms of *EML4-ALK* transcripts will necessitate optimization of the detection systems so that all isoforms are detected with a high accuracy and sensitivity.

[8] M. Soda et al., submitted for publication.

## Note Added in Proof

During our revision process, a novel EML4-ALK fusion variant was reported by Koivunen et al. (Clin Cancer Res 2008;14:4275–83). They have designated it as variant 4, which is different from our variant 4 in the present study.

## Disclosure of Potential Conflicts of Interest

K. Takeuchi is a consultant providing advisory services to Dako for their antibodies.

## Acknowledgments

We thank Kazuko Yokokawa, Motoyoshi Iwakoshi, Miyuki Kogure, and Tomoyo Kakita for technical assistance as well as Yuki Takano for help in preparation of the manuscript.

## References

1. Aplan PD. Causes of oncogenic chromosomal translocation. Trends Genet 2006;22:46–55.
2. Sherbenou DW, Druker BJ. Applying the discovery of the Philadelphia chromosome. J Clin Invest 2007;117:2067–74.
3. Pulford K, Morris SW, Turturro F. Anaplastic lymphoma kinase proteins in growth control and cancer. J Cell Physiol 2004;199:330–58.
4. Mitelman F, Johansson B, Mertens F. Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat Genet 2004;36:331–4.
5. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 2007;7:233–45.
6. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644–8.
7. Tomlins SA, Laxman B, Dhanasekaran SM, et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 2007;448:595–9.
8. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–6.
9. Choi YL, Takeuchi K, Soda M, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res. In press 2008;68:4971–6.
10. Travis WD, Elisabeth B, Muller-Hermelink HK, Harris CC, editors. Pathology and genetics of tumours of the lung, pleura, thymus and heart. Lyon: IARC Press; 2004.
11. Onishi M, Kinoshita S, Morikawa Y, et al. Applications of retrovirus-mediated expression cloning. Exp Hematol 1996;24:324–9.
12. Donella-Deana A, Marin O, Cesaro L, et al. Unique substrate specificity of anaplastic lymphoma kinase (ALK): development of phosphoacceptor peptides for the assay of ALK activity. Biochemistry 2005;44:8533–42.
13. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 2008;3:13–7.
14. Tort F, Pinyol M, Pulford K, et al. Molecular characterization of a new ALK translocation involving moesin (MSN-ALK) in anaplastic large cell lymphoma. Lab Invest 2001;81:419–26.
15. Lamant L, Gascoyne RD, Duplantier MM, et al. Non-muscle myosin heavy chain (MYH9): a new partner fused to ALK in anaplastic large cell lymphoma. Genes Chromosomes Cancer 2003;37:427–32.
16. Pulford K, Lamant L, Morris SW, et al. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood 1997;89:1394–404.
17. Armstrong F, Duplantier MM, Trempat P, et al. Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. Oncogene 2004;23:6071–82.
18. Duyster J, Bai RY, Morris SW. Translocations involving anaplastic lymphoma kinase (ALK). Oncogene 2001;20:5623–37.
19. Wan W, Albom MS, Lu L, et al. Anaplastic lymphoma kinase activity is essential for the proliferation and survival of anaplastic large-cell lymphoma cells. Blood 2006;107:1617–23.
20. Marzec M, Kasprzycka M, Ptasznik A, et al. Inhibition of ALK enzymatic activity in T-cell lymphoma cells induces apoptosis and suppresses proliferation and STAT3 phosphorylation independently of Jak3. Lab Invest 2005;85:1544–54.
21. Galkin AV, Melnick JS, Kim S, et al. Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. Proc Natl Acad Sci U S A 2007;104:270–5.
22. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007;131:1190–203.