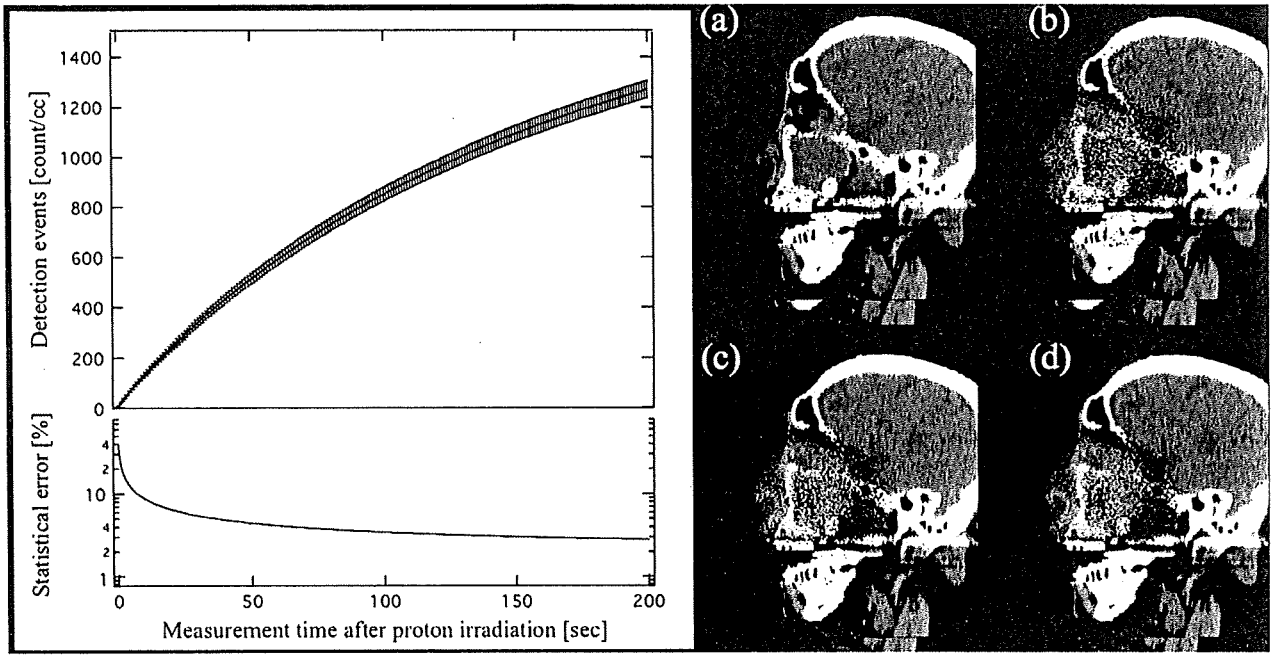

Fig. 3. The number of detection events per volume and PET images obtained during the detection period after proton beam irradiation. The PET images for detection period of (a) 0, (b) 50, (c) 100, and (d) 200 s are shown.

the detection event of the activity measured in the gating window will became about one third of the total detection events, and the statistical error will increase. Therefore, the measurement was performed with no synchronization with organ motion by respiration.

Changes in the activity distribution during the treatment period

In each treatment site, the activity distribution changed probably by reduction of the tumor size and changing of the body shape was conspicuously observed in some cases of the head and neck.

The verification was performed for a case involving tumors of the head and neck. Proton beam irradiation was performed in three fields of view: Port 1: 123 MeV, 90-mm SOBP, 350° gantry angle, 0° bed angle; Port 2: 121 MeV, 90-mm SOBP, 10° gantry angle, 20° bed angle; and Port 3: 117 MeV, 80-mm SOBP, 340° gantry angle, 350° bed angle. The irradiation dose was 2.5 GyE. Figure 5 shows a calculated proton dose distribution, an activity distribution, and a depth profile of a 2.5-GyE dose irradiation after a delivery dose of 2.5 (reference image), 10.0, 17.5, or 32.5 GyE from Port 1, a delivery dose of 5.0 (reference image), 12.5, 20.0, or 35.0 GyE, from Port 2, and a delivery dose of 7.5 (reference image), 15.0, 22.5, or 30.0 GyE from Port 3. Changes of the activity distribution were observed according to changes of the proton beam range and the dose delivered by previous irradiations resulted in a reduction of the tumor (see the arrow and the area surrounded by the dotted line in Fig. 5). The changing values of the activity range for each irradiation field (Port 1, Port 2, and Port 3) are shown in upper left of Fig. 6. The activity range was defined by the depth point of 50% distal falloff in the activity distribution normalized at the iso-center. The changing value of the activity range fully exceeded a 10-mm length. Moreover, to observe the changes in the activity distribution in the depth direction in a similar manner, the ratio of the integration of the detected numbers between 20 mm and 70 mm from the iso-center was expressed as follows:

$$R(D) = \frac{\int_{20}^{70} \left(dA(D)/dZ\right) dz}{\int_{20}^{70} \left(dA(0)/dZ\right) dz}. \quad (1)$$

Here, z is the depth, D is the delivery dose, A(D) is the depth activity distribution, and A(0) is the reference depth activity distribution. The ratio of the delivery dose is shown in the middle left of Fig. 6. The bottom left of Fig. 6 is the proton beam irradiation time per fraction dose at each irradiation. The average of the irradiation time was 30 s, and the difference of the irradiation time at random was within 3 s.

In this case, a new CT image was scanned and a retreatment planning was produced after the delivery of 35 GyE of the prescribed dose of 65 GyE. The volume of the tumor was decreased from 184 mL to 125 mL (the arrow in right of Fig. 6 shows the visible tumor reduction), and the maximum beam range was shortened by 20-mm water equivalent length. In the other 2 cases of 18 clinical cases of the head and neck, the changing activity range of more than 10 mm was observed. Similarly, the new CT image acquisition and the retreatment planning were immediately performed after the observation of the changing activity range. The reduction

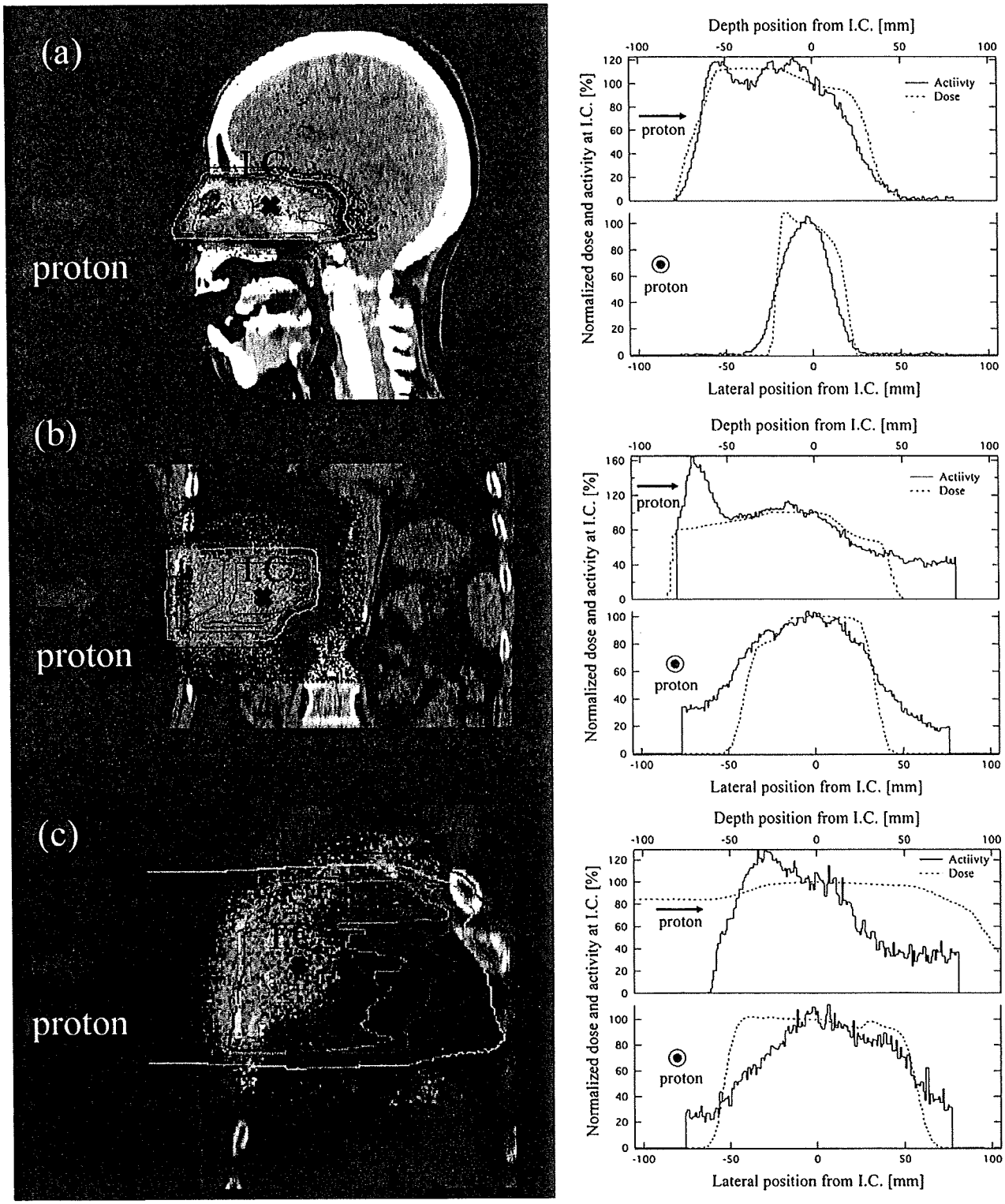

Fig. 4. The calculated dose distribution and the measured activity distribution (left figure), and corresponding lateral and depth profiles (right figure) of the irradiation fields (see Table 1) in each case involving tumors of the head and neck (a), the liver (b), the lungs (c), the prostate (d), and the brain (e), respectively. The iso-dose line of 100% is red, 80% yellowish green, 50% light blue, and 20% purple. The iso-activity wash between 30% and 100% changed from light blue to red.

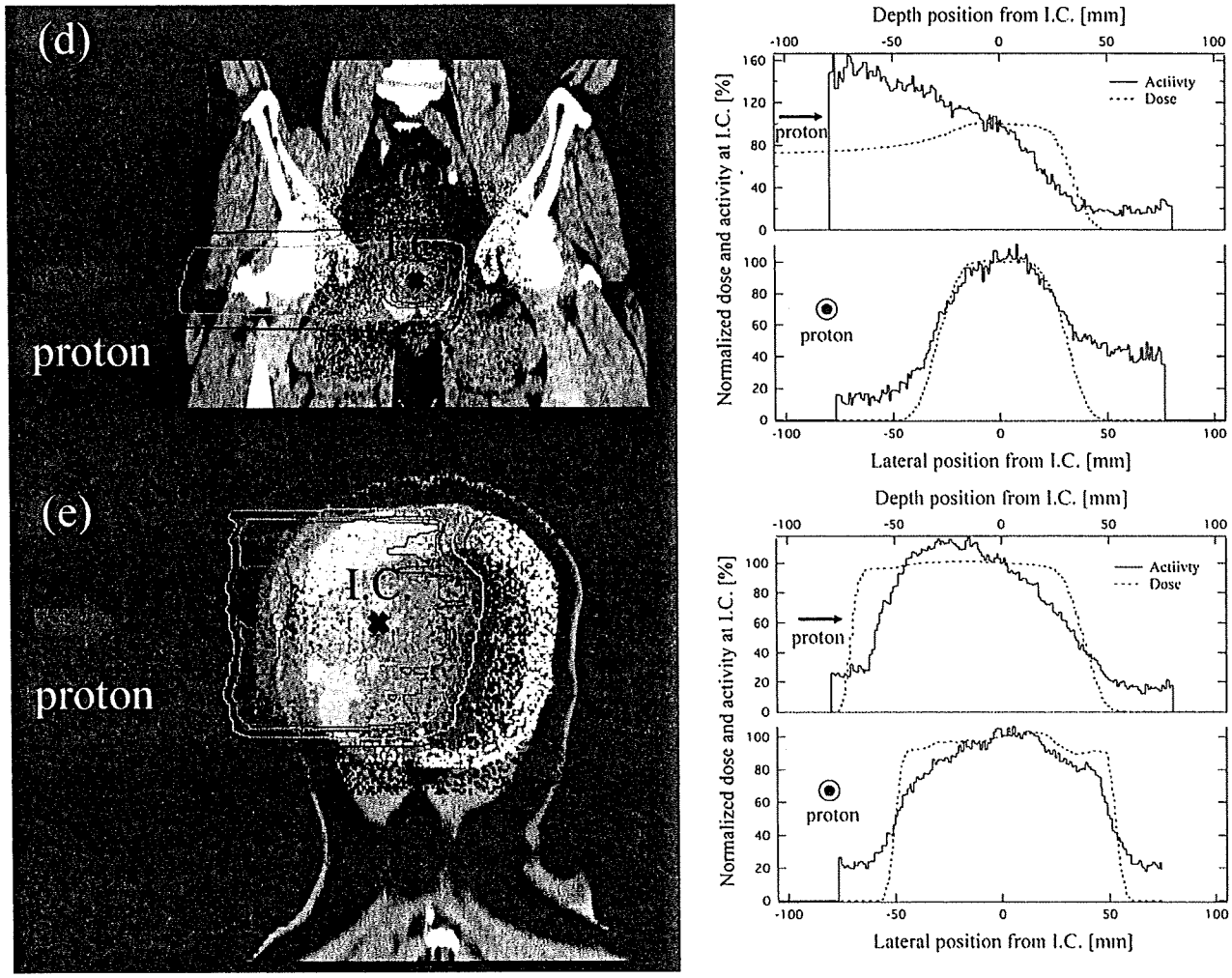

Fig. 4. (*continued*).

of the tumor's volume was more than 100 mL. Also, in carbon therapy, similar observation of the tumor shrinkage has been reported in (7). The BOLPs-RGp indicated that the proton irradiation dose was delivered to the brain stem of organs at risk.

Washout effect of the activity in the treatment period

A histopathologic examination demonstrated that higher activity was observed in regions containing necrotic liver cells than in any other region. The upper panel of Fig. 7 shows the calculated dose distribution and the measured activity distribution on a CT image taken at the first treatment of a 3.8 GyE delivery dose. The bottom left panel of Fig. 7 shows the number of detection counts per 20 s of activity in the regions of interest of areas A and B in the liver. Hence, the region of interest of area A is the necrotic region of the tumor, and area B is the normal tumor region. Therefore, area B-A is equivalent to the area of the tumor minus the necrotic region. The observed decay curves in the region of interest of area A and B-A were fitted well enough using a double exponential equation. The two half-lives of the double exponential fitting were 31 ± 8 s and 146 ± 20 s in the area A, and 21 ± 4 s and 164 ± 11 s in the area B-A, respectively. The half-life was longest in the necrotic region of the tumor. The activity images for the 200 s measurement by the BOLPs-RGp are shown in the left of Fig. 8. The high activity

Table 1. Summary of proton beam irradiation parameters

	Treatment site	Proton energy [MeV]	SOBP [mm]	Gantry angle [deg.]	Bed angle [deg.]	Fractional dose [GyE]	Irradiation time [sec.]
(a)	Head and Neck	123	90	0	0	2.5	39
(b)	Liver	137	70	270	0	3.8	229
(c)	Lungs	145	70	160	0	2.0	38
(d)	Prostate	187	50	270	0	2.0	15
(e)	Brain	122	90	330	90	2.5	40

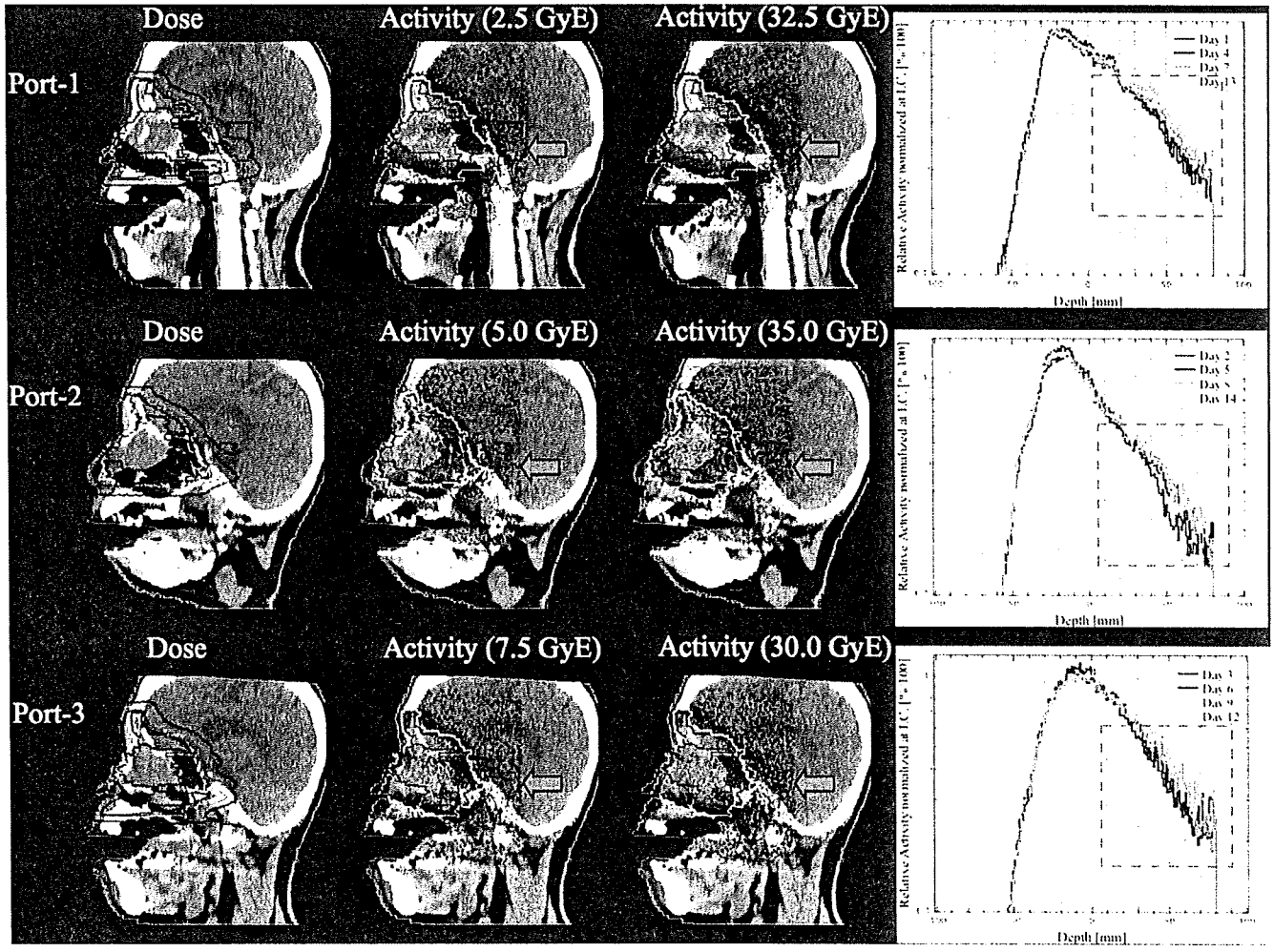

Fig. 5. The calculated proton dose distribution, measured activity distribution of a 2.5-GyE dose irradiation, and the depth profile of the measured activity normalized to the iso-center (0-mm depth) of the reference activity after a delivery dose of 2.5–35.0 GyE.

of the necrotic region decreased to same level as the normal parts of the liver in the last treatment. The ratio F of the detection activity normalized to the activity data from the first treatment for the delivery doses in the area A and the area B-A is expressed as follows:

$$F(D) = \frac{\int_0^{S_A} (dN(D)/dS)\, dS / \int_0^{S_A} dS}{\int_{S_A}^{S_B} (dN(D)/dS)\, dS / \int_{S_A}^{S_B} dS}. \quad (2)$$

Here, N is the detection number, S_A is the square of area A, and S_B is the square of area B. Ratio of the F values normalized at the value in first treatment calculated by using Eq. 2 and proton beam irradiation time per fraction dose are shown in the right of Fig. 8. The average of the irradiation time at random was 159 ± 77 s. There was no correction in the irradiation time and the decrease of the activity shown in Fig. 8. A decrease in the activity of the necrotic region was observed after the delivery dose was increased without depending on the beam irradiation time per fraction dose.

DISCUSSION

This study focused on the development of the BOLPs-RGp and its clinical use against tumors of the head and neck, liver, lungs, prostate, and brain in the proton therapy. Quick measurement of the activity generated in a patient's body after proton irradiation is feasible by using the BOLPs-RGp. The elements tracked by the activity imaging are ^{11}C (20.39 min), ^{10}C (19.26 s), ^{13}N (9.965 min), ^{15}O (122.2 s), ^{14}O (70.61 s), ^{30}P (2.498 min), and ^{38}K (7.636 min), and according to the results of a simulation by Parodi *et al.*, the "key" positron emitter nuclei are ^{11}C and ^{15}O (14). The measurement of this activity must be immediately performed after proton irradiation as the half-life of ^{15}O is about 2 min. As a result, the information for activity imaging is obtained in a short period. On the other hand, in the case of a beam OFF-LINE PET system used with a commercial based PET or PET/CT apparatus, it is very difficult to measure the activity of ^{15}O for several minutes even at the start of the activity measurement after proton irradiation. The main elements used for activity imaging are ^{15}O for measurements with

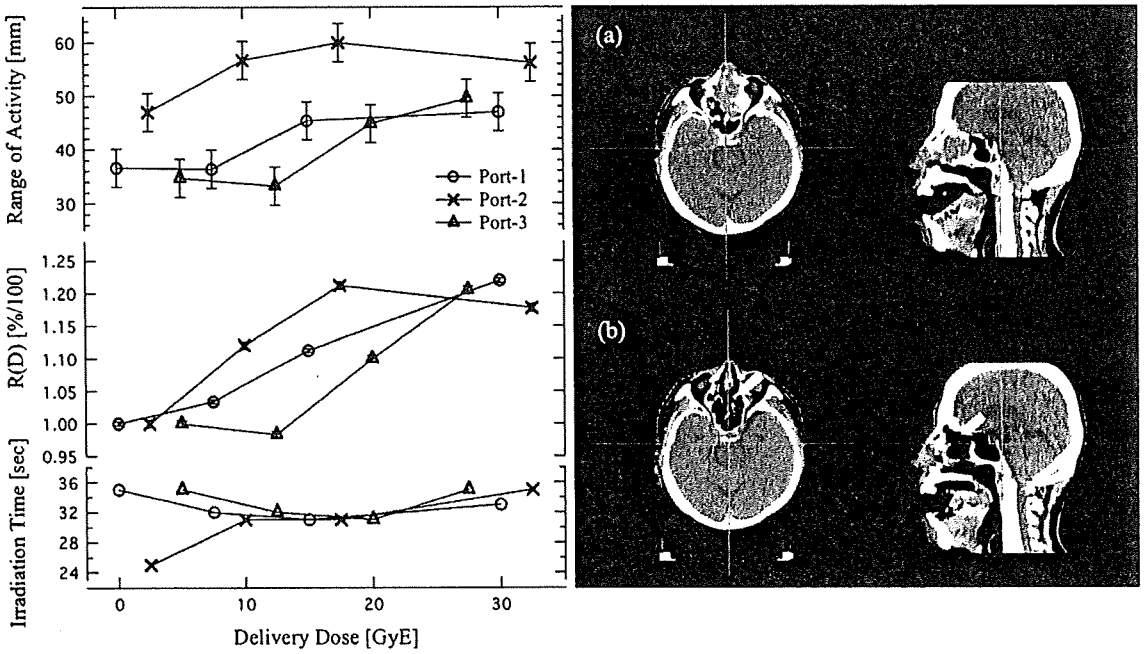

Fig. 6. Changes in the values of the activity range and proton beam irradiation time per fraction dose at each irradiation field of Port-1, Port-2, and Port-3. Axial and sagittal CT images of the head and neck before treatment (a) and after delivery doses of 35 GyE (b).

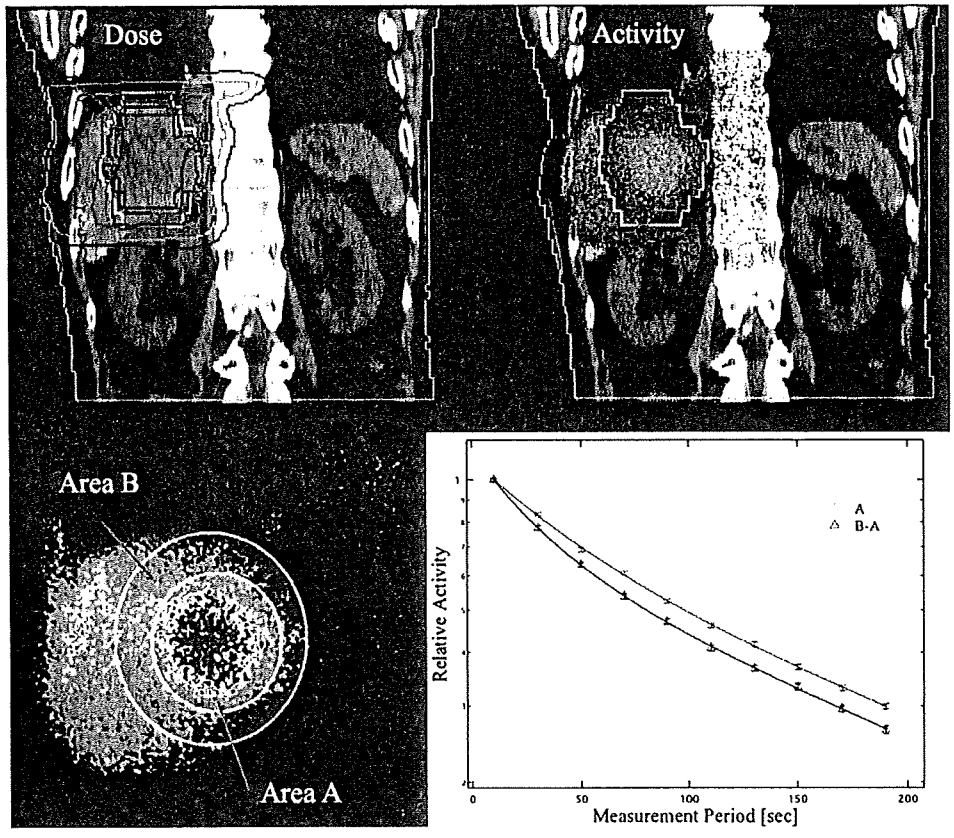

Fig. 7. The calculated dose distribution and the measured activity distribution on a CT image after the first treatment with a 3.8-GyE delivery dose, and the number of detection counts per 20 seconds of the activity in the region of interest (ROI) of areas A and B in the liver.

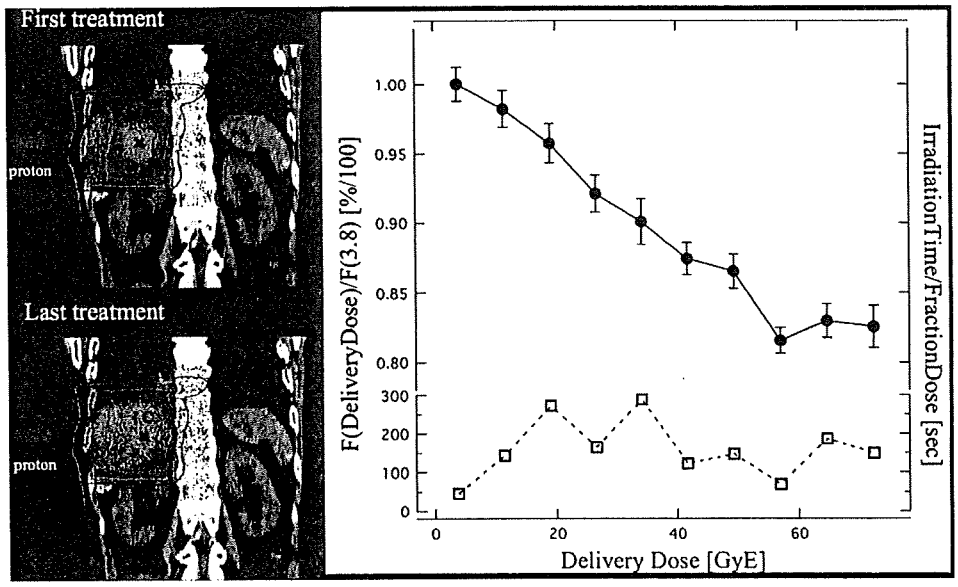

Fig. 8. The activity image and the ratio of the detection number to the measured activity calculated according to Equation (2) in the necrotic region of the liver tumor, and proton beam irradiation time per fractional dose.

the BOLPs-RGp and ^{11}C for measurements with the beam OFF-LINE PET system. As a tumor is equivalent to soft tissue, the measurement of the many ^{15}O nuclei generated in a tumor by proton irradiation is very important for the observation and evaluation of the changing form and the delivery dose response of the tumor. ^{12}C is present and ^{11}C is generated in the adipose tissue. Therefore, high activity is indicated in the region under the skin when using the beam OFF-LINE PET system. Furthermore, the BOLPs-RGp has the advantage of taking measurements with the patient in same position during proton irradiation. However, the CT image for the patient positioning can not be acquired at the same time as the activity measurement when using the BOLPs-RGp. This problem can be solved by the technological introduction of a CBCT.

At present, the length of activity measurement with the BOLPs-RGp after proton beam irradiation is 200 s; but, it may be possible that the measurement time can be shortened to less than 200 s as a result of this research. However, the measurement time must be determined with consideration to the detection efficiency by the delivery dose of each treatment site, the distance between the detector heads, and the activity measurement synchronized with the organ motion caused by respiration in the case of the liver and lungs.

The BOLPs-RGp has been used in the daily proton treatment of 48 patients. The monitoring of the accuracy of the proton beam irradiation was performed by comparing and verifying the daily activity images with reference activity images obtained at the start of the proton treatment. Specially, optimized proton treatment was performed by quickly replanning treatment in three clinical cases involving head-and-neck tumors, because different activity distribution were observed in the two images during the treatment period. The decrease of the activity in the region of necrotic cells in the liver tumor found during the histopathological examination was linked to an increase in the delivery dose. It is suggested that the increase in the washout effect in the necrotic region is caused by a decrease in the number of necrotic cells in the liver because of increased blood flow caused by the higher proton delivery dose. This effect may indicate a need to adapt the treatment to the dose response of the tumors in individual patients as well as the observation of the functional metabolism of organs.

The quality of the activity image is reduced by the large organ motion of the liver and the lungs. In cases of the prostate, the verification of changes in the activity distribution against the condition of the bladder and the position of the head of the femur will be reported in future. Moreover, a study concerning the experimental determination of cross sections of the target nuclear fragment reaction has been completed, and a simulation system that includes our cross-section data for calculating activity distribution in a patient's body with a high accuracy has been constructed using a cluster computer system. Many results of the research of the simulation have been already reported by the study group of Parodi *et al.* (12, 14). Finally, the ideal DGPT will be achieved through these developments and the research.

CONCLUSIONS

A BOLPs-RGp was constructed in our proton treatment room. The BOLPs-RGp has been used in many clinical cases. Report of the clinical use with beam ON-LINE PET or in-beam PET in the proton therapy has been never done before. The daily activity images obtained indicated the proton irradiation volume of the treatment administered to patients. Information about the positron-emitting nuclei provided by the BOLPs-RGp will be important for improving proton treatment accuracy in the future. DGPT (10) will thereby be achieved via daily proton treatment using the BOLPs-RGp.

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Reasons for response differences seen in the V15-32, INTEREST and IPASS trials

Nagahiro Saijo, Masahiro Takeuchi and Hideo Kunitoh

Abstract | The first phase III study to assess the effect of gefitinib and docetaxel on the survival of Japanese patients with non-small-cell lung cancer who received previous treatment with platinum doublets, the V15-32 trial, did not establish noninferiority of gefitinib over docetaxel in terms of the effect on overall survival, despite the results showing a twofold higher response rate to gefitinib. The overall survival favored docetaxel for the first 18 months and gefitinib thereafter. The INTEREST trial, which compared docetaxel and gefitinib, demonstrated noninferiority of gefitinib, and the survival curves were completely superimposed. In this trial, patients had been recruited from 24 countries from Europe, Asia, and North and South America. Results of the IPASS trial showed superior progression-free survival for gefitinib compared with the combination of carboplatin and paclitaxel as first-line treatment in Asian patients who were nonsmokers and had adenocarcinoma histology. In this Review, we discuss the reasons for the differences in the effects of molecular-targeted drugs and cytotoxic antineoplastic agents observed in these trials. We also highlight the magnitude of the antitumor activity of these two different categories of drugs, and discuss how this could affect future clinical trial design and analysis.

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Introduction

At present, the consensus opinion is that the efficacy of lung cancer chemotherapy with cytotoxic agents has reached a plateau, and it is difficult to expect superior efficacy with any novel cytotoxic anticancer agents that will become available in the near future. It is generally believed that the results seen with different platinum doublet regimens are of a similar magnitude, no matter which combination is used. However, slight differences were seen in results reported by the Eastern Cooperative Oncology Group (ECOG) study,[1] Four Arm Clinical Study (FACS),[2] South Western Oncology Group (SWOG) trial,[3] and Tax 326 study.[4] In the ECOG trial, progression-free survival seen with gemcitabine plus cisplatin was better than in the other treatment arms that included paclitaxel plus cisplatin, docetaxel plus cisplatin and paclitaxel plus carboplatin.[1] In the FACS trial, the overall survival rates observed for carboplatin plus paclitaxel and cisplatin plus vinorelbine were inferior compared with the gemcitabine plus cisplatin and irinotecan plus cisplatin,[2] and overall survival of cisplatin plus docetaxel was significantly better than that of cisplatin and vinorelbine.[3] In everyday clinical practice, treatment arms are selected taking into consideration factors such as the toxicity profile and ease of use on an outpatient basis. The choices of treatment used in combination with radiation therapy and surgery are based on consideration of patient adherence to the drugs administered.

Competing interests
N. Saijo has declared associations with the following companies: AstraZeneca, Bristol-Myers Squibb, Chugai-Roche, and Eli Lilly. H. Kunitoh declared associations with the following companies: AstraZeneca, Bristol-Myers Squibb and Sanofi-Aventis. See the article online for full details of the relationships. M. Takeuchi declared no competing interests.

Clinical outcomes with EGFR inhibitors

EGFR is a member of the HER family, which consists of four members: EGFR/HER1/erbB1, HER2/neu/erbB2, HER3/erbB3, and HER4/erbB4.[5] Once the ligands bind to the extracellular domain of EGFR proteins, the receptors dimerize with other EGFR family members to form homodimers or heterodimers, which induce phosphorylation of the tyrosine kinase EGFR and activation of downstream signal pathways.[6] EFGR–tyrosine kinase inhibitors (EGFR-TKIs) are molecular-targeted drugs that, in general, target the ATP binding site of protein kinases and show competitive inhibition, thereby preventing correct functioning of the receptor in tumor cells. Great advances are expected in the treatment of non-small-cell lung cancer (NSCLC) when these agents become available because they have demonstrated impressive tumor shrinkage in patients with disease refractory to platinum and taxane therapy even in phase I clinical trials.[7,8] It has been difficult to demonstrate any survival benefit of these agents in the clinical setting.[9–13] In phase III studies that compared erlotinib with placebo as second-line and third-line chemotherapy, a survival benefit in favor of erlotinib was demonstrated. In the ISEL (Iressa Survival Evaluation in Lung Cancer) trial that compared gefitinib with placebo in similar populations of patients, no survival advantage was seen with gefitinib; however, significant prolongation of survival

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Key points

- Many unexpected results were observed in the randomized, controlled trials of EGFR-targeted tyrosine kinase inhibitors (TKIs)
- The nature and quantity of antitumor effects are different between cytotoxic chemotherapy and molecular-targeted drugs
- Selection of patients is extremely important for future clinical trials that test EGFR-TKIs
- Results from the IPASS trial demonstrate that EGFR-TKIs provide superior progression-free survival compared with platinum-based doublet chemotherapy in selected patients with non-small-cell lung cancer, especially those with mutated *EGFR*

Table 1 | Data from randomized, controlled trials of EGFR-TKIs for NSCLC treatment

Study	EGFR-TKI agent	Selection of patients	Difference in end points between treatment and control
ISEL[a]	Gefitinib vs placebo	None	Negative
BR.21[a]	Erlotinib vs placebo	None	Positive
INTACT 1&2	Gefitinib vs combination	None	Both negative
TALENT & TRIBUTE[a]	Erlotinib vs combination	None	Both negative
V15-32	Gefitinib vs docetaxel	Japanese	Negative
INTEREST[a]	Gefitinib vs docetaxel	None	Positive
IPASS	Gefitinib vs carboplatin plus PTL	Adenocarcinoma, Asian, nonsmoking	Positive
WJTOG0203	Gefitinib vs platimum doublet (consolidation)	Japanese	Not available

[a]Discrepancies: BR.21 versus TALENT & TRIBUTE; ISEL versus INTEREST. Abbreviations: NSCLC, non-small-cell lung cancer; PTL, paclitaxel, trastuzumab and lapatinib; TKI, tyrosine kinase inhibitor.

time was observed in Asian patients.[14,15] Four large, randomized, controlled trials of standard platinum-based chemotherapy (carboplatin plus paclitaxel or cisplatin plus gemcitabine) with or without EGFR-TKIs yielded negative results in patients with advanced NSCLC who had not received previous chemotherapy.[9–12] In addition, the SWOG trial showed that the intensification with gefitinib after chemoradiotherapy in stage III NSCLC provided significantly poorer survival than in the control group.[13] As the reported response rates to EGFR-TKIs in Western populations are ≤10%, this low percentage does not reflect the prolongation of survival.

By contrast, gefitinib has been found to have outstanding therapeutic effect in a phase II clinical trial of Japanese patients, with reported response rates of 27.5%, median duration of response of 114 days, and median survival time of 13.8 months.[16] Subsequent clinical trials that included Asian populations showed higher response rates and better survival rates associated with this drug compared with placebo;[14,17] however, no such benefit was seen in Western patients.[14] A phase II trial of gefitinib in nontreated, nonselected, Japanese patients with NSCLC produced a similar response rate compared to patients with previous therapy.[18] Analysis of clinical factors has demonstrated that Asian ethnicity, female gender, adenocarcinoma histology and nonsmoking status are favorable factors in relation to the efficacy of EGFR-TKIs.[15,19,20]

In 2004, the presence of activating mutations of *EGFR* in tumor cells was reported to be extremely important for achieving the antitumor effect of EGFR-TKIs.[21,22] In patients with these *EGFR* mutations the response rate to EGFR-TKIs is approximately 80%.[23–31] The response duration ranged from 7.0 months to 10.7 months. The frequency of *EGFR* mutations is higher in Asian populations (30–40%) compared with Western populations (5–10%).[30] A higher frequency of these mutations in Japanese populations was also shown to correlate with the presence of favorable clinical factors such as adenocarcinoma, female gender and nonsmoking status.[26,29,31] Some have suggested that other biomarkers, such as *EGFR* amplification status detected by fluorescent *in situ* hybridization, could also be useful indicators of the response to EGFR-TKIs; however, these biomarkers are not reliable.[29,32] The problem with results obtained using fluorescent *in situ* hybridization is that this technique might detect two genetic abnormalities, namely, *EGFR* amplification and high polysomy. High polysomy is usually not well-correlated with the presence of *EGFR* mutations.[32–34] In Japan, gefitinib has been approved by the Ministry of Health, Welfare and Labour on the basis of data from the IDEAL (phase II) study and data from trials showing the survival benefit of gefitinib in Japanese populations.

Data from the V15-32 study of gefitinib

Two randomized, controlled trials conducted in Western patients have reported the effects of docetaxel in patients with previously treated NSCLC.[35,36] Prolongation of survival was demonstrated in the docetaxel-treated groups compared with groups given best supportive care or treated with ifosfamide and/or vinorelbine. Docetaxel was, therefore, established as the gold standard for second-line chemotherapy in patients with NSCLC.[35,36] No data, however, compared the activities of docetaxel and placebo in the second-line setting in Japan. On the basis of comparative studies of pemetrexed and docetaxel, pemetrexed is now employed more frequently in the US for treating patients with NSCLC in the second-line setting.[37] In Japan, however, pemetrexed has not been approved for use in patients with lung cancer because insufficient studies in Japanese populations have been carried out, even though a clinical phase II study has been completed.[38] There has also been a report describing the superiority of erlotinib in prolonging the survival of previously treated patients with NSCLC compared with best supportive care in the second-line or third-line setting.[39] This drug has just been approved for treatment of lung cancer in Japan.[40]

V15-32 was an open-label, randomized phase III study that compared 250 mg gefitinib with 60 mg/m^2 docetaxel in Japanese patients with NSCLC and a history of failure of one or two chemotherapy regimens (Figure 1).[41] The main purpose of this study was to demonstrate the

noninferiority of gefitinib over docetaxel for overall survival in these patients, according to predefined criteria (that is, upper threshold of the CI of the hazard ratio [HR] less than 1.25). A total of 484 patients were accrued, with 242 in each treatment arm; however, noninferiority of gefitinib for overall survival could not be established (HR 1.12; 95% CI 0.89–1.40), and no significant difference in overall survival was apparent between the two treatment groups ($P=0.330$). A Cox regression analysis, with adjustments for imbalances in the baseline characteristics of the patients, yielded an HR of 1.01 (95% CI of 0.80–1.27), $P=0.914$ (Table 2 and Figure 2).[41] Secondary end points included progression-free survival, time-to-treatment failure, response rate, and disease control rate. These end points were evaluated in the patients who had measurable target lesions at study entry. Gefitinib treatment was associated with a significantly improved overall response rate (22.5% versus 12.8%, $P=0.009$) and time-to-treatment failure (HR 0.63; 95% CI 0.51–0.77, $P<0.001$). No significant differences in progression-free survival (HR 0.90; 95% CI 0.72–1.12, $P=0.335$) or disease control rate (34% versus 33.2%, $P=0.735$) were seen between the two treatment groups.[41] Since cessation of chemotherapy in those without disease progression was included as an event for time-to-treatment failure, comparison of this end point between docetaxel and gefitinib-treated patients would not have much clinical relevance.

Additional analysis of V15-32

On behalf of the Drug Safety Policy Panel and FDA Safety Investigation Committee, Takeuchi stated the following on the basis of results of the V15-32 trial.[42] Firstly, the two groups were well balanced and met the requirements of randomization, which assured the comparability of the groups. Secondly, the hazard ratios in two comparative groups on Cox regression analysis should remain constant regardless of the passage of time. In the current study, it does not seem likely that this prerequisite was met; it is difficult, therefore, to evaluate the therapeutic results from the major outcome of the analysis, because the HRs were assumed to be constant regardless of the passage of time.

To understand how the therapeutic benefit in the gefitinib group, compared with the docetaxel group, changed in a time-dependent manner, Takeuchi conducted a retrospective, exploratory investigation of the effect at various time intervals, using survival rate as the evaluation index. In terms of the survival rate at an early stage of follow-up (that is, less than 1 year) the CI for the therapeutic effect indicated that docetaxel was superior to gefitinib. After about 24 months, however, the results showed a tendency for gefitinib to be superior to docetaxel. The CI was so wide that it was difficult to conclude that gefitinib was indeed superior to docetaxel at this stage (Figure 3).

Interpretation of the results of V15-32

The V15-32 study was the first comparative, large-scale, randomized trial conducted in previously treated patients with NSCLC in Japan. It is highly noteworthy that 490 patients were recruited within a period of about 2.5 years and accurate results were obtained. The median survival rates for docetaxel and gefitinib were 11.5 and 14.0 months, respectively. Despite the problems related to selection of patients, the results showed the high level of medical care in Japan. The initial hypothesis of noninferiority of gefitinib was not established. This finding implies that there might be a high probability of gefitinib being inferior to docetaxel for treating patients with NSCLC in the second-line setting. Docetaxel, therefore, remains the drug of first choice in these patients. As subset analyses could not identify subgroups of patients in whom gefitinib yielded better outcomes than docetaxel, a 'docetaxel-first' policy should be employed even in patients with a favorable risk profile (that is, females, adenocarcinoma histology and never-smokers). The response rates of patients to gefitinib were greater than 20%, and almost double that seen with docetaxel. Although the study was small, some of the patients treated with gefitinib have shown prolonged progression-free survival, and the survival curve of the gefitinib group crossed over the survival curve for the docetaxel group 18 months after treatment initiation.[41] These results strongly suggest that gefitinib could be beneficial in a subset of docetaxel-resistant and docetaxel-intolerant patients. The results of the primary analysis of gefitinib versus docetaxel have neither confirmed nor refuted these effects of gefitinib.[41] For the first 18 months after initiation of treatment, the survival rate was better in the docetaxel group than in the gefitinib group; the reasons for this finding may be hypothesized as follows: first, gefitinib might promote tumor proliferation; second, gefitinib might exert potent toxicity in some patients; and third, the antitumor activity of docetaxel might be superior in the overall population of patients. It is likely that the third reason could explain the better survival rate of the docetaxel group, and the late benefit of gefitinib would not have been expected if the first and second reasons are likely. One could speculate that docetaxel, a cytotoxic agent, would have some effect against the vast majority of the tumors, while gefitinib, a targeted agent, might be totally ineffective in patients not expressing the target. The differences in survival curves in the initial phase of follow-up might have reflected the effect of these 'relatively resistant' cases. Many patients, particularly from the docetaxel group, were actually crossed over to receive the other treatment. This made interpretation of the survival

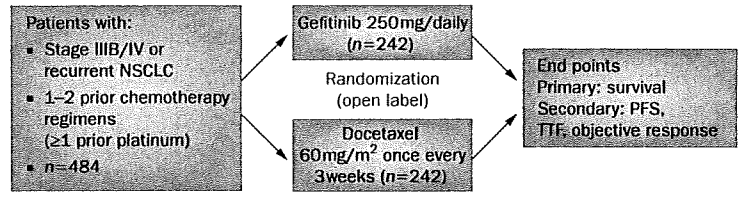
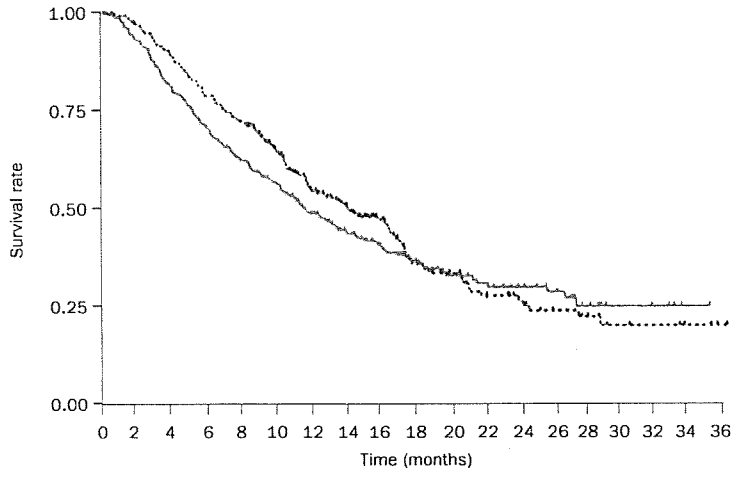


Figure 1 | Schematic diagram to show the randomization schema for the randomized phase III V15-32 trial. Abbreviations: NSCLC, non-small-cell lung cancer; PFS, progression-free survival; TTF, time-to-treatment failure. Data courtesy of AstraZeneca.

Table 2 | Overall survival data (intent-to-treat analysis) from the V15-32 study

Study outcomes	Gefitinib	Docetaxel
Number of patients	245	244
Number of events	156	150
Median (range) survival time (months)	11.5 (9.8–14.0)	14.0 (11.7–16.5)
1-year survival (%)[a]	48	54
Response rate (%)	22.5	12.8

[a]Hazard ratio 1.12 (95% CI 0.89–1.40; $P = 0.330$). Noninferiority could not be demonstrated.



Number of patients at risk:

Gefitinib	245	226	197	169	148	127	98	77	63	47	35	29	25	18	9	5	4	1	0
Docetaxel	244	233	214	189	173	140	105	87	69	44	35	25	18	14	10	7	6	3	0

Figure 2 | Table showing the overall survival data for patients treated in the randomized phase III V15-32 trial. Data courtesy of AstraZeneca.

results even more difficult. The decision to treat patients in the docetaxel arm with gefitinib as a post-protocol therapy was probably on the basis of clinical information available; that is, patients with clinical features known to be favorable for the effect of gefitinib were selected. This selection criterion might have offset the survival benefit of gefitinib in the later phase of follow-up.[43]

On 1 February 2007, the Ministry of Health, Labour and Welfare examined the results of the V15-32 trial presented to the Drug Safety Policy Panel, Safety Policy Investigation Committee, and Second Food and Drug Advisory Board of 2006. The results of this meeting were published.[44] First, the safety policy on interstitial pneumonia described in the package insert concerning the adverse events of gefitinib is to be continued. Second, there is no evidence to support the preference of gefitinib over docetaxel for second-line or third-line treatment. Third, to evaluate the clinical efficacy of gefitinib, the difference in the survival curves in the V15-32 study should be analyzed in detail and detailed subset analyses must be conducted. Forth, clinical factors that might affect the drug effects, and the effect of *EGFR* mutations on drug responsiveness, must be evaluated.

Results of the INTEREST trial

The INTEREST trial was a randomized, open-label, parallel-group, phase III trial of gefitinib versus docetaxel in patients with locally advanced or metastatic and/or recurrent NSCLC with a previous history of platinum-based chemotherapy.[45] The phase III study enrolled 1,466 patients from 149 centers in 24 countries. The primary end point was overall survival. The overall survival and 1-year survival rates were 7.6 months and 23%, respectively, in the gefitinib group. The corresponding survival rates were 8.0 months and 34%, respectively, in the docetaxel group. No significant differences in the outcomes between the two treatment arms were noted. The study demonstrated the noninferiority of gefitinib compared with docetaxel. Gefitinib was better tolerated, and the total outcome index of quality of life also favored gefitinib. On the basis of these data, AstraZeneca submitted a marketing authorization application to the European Medicines Agency for gefitinib as an agent for patients with locally advanced or metastatic NSCLC with a previous history of treatment with platinum-containing regimens. It is not known why the INTEREST trial demonstrated positive results, because the response rate to gefitinib is lower in Western patients compared with Japanese patients. The adjusted HR of gefitinib versus docetaxel in the V15-32 study was 1.01, which was almost identical to that in the INTEREST trial (HR = 1.02). This finding suggests that the efficacy of gefitinib was similar to that of docetaxel. Asian patients treated with docetaxel had a better outcome than Asian patients treated with gefitinib in the INTEREST trial. By contrast, Asian patients did not derive a benefit in the placebo arm in the ISEL trial. Since *EGFR* mutation was associated with better response to docetaxel in the INTEREST trial, it is possible that docetaxel worked better in the Asian patients, offsetting gefitinib efficacy in the comparisons and improving the overall outcomes of the Asian subset in the INTEREST and V15-32 studies.

Differences between TKIs and cytotoxic agents

Comparison of cytotoxic agents and molecular-targeted therapeutic drugs reveals that although the former show broader anticancer spectra, their maximal therapeutic quality in responders might be inferior compared with that of molecular-targeted therapeutic agents. Molecular-targeted agents can show narrow antitumor spectra but they can produce a profound effect. In general, the potency of the antitumor effect of the conventional cytotoxic agents is likely to be greater when clinical trials are conducted on large numbers of patients. Molecular-targeted agents exhibit antitumor activity only in those cells that possess the relevant molecular target, hence the effects of these drugs on overall tumor volume reduction would be lower than that of the conventional cytotoxic agents, even when both exert the same response rate.

Thus, the survival rate of patients treated with the molecular-targeted agents might not improve, even if the response rate is twice that of the conventional cytotoxic agents. The results of the V15-32 study indicate

this possibility. Waterfall plot figures have been used frequently for evaluation of the antitumor activities of drugs. The rates of variability in the responses of the tumors of each patient are plotted. If the value is positive the tumor is judged to have increased in size, and if the value is negative the tumor is judged to have reduced in size. The number of patients experiencing even the slightest tumor reduction is often expressed as a percentage. Waterfall plots have been suggested to be suitable for evaluation of the effects of cytotoxic antineoplastic agents against malignant tumors because they suppress tumor growth regardless of the molecular target of each agent. RECIST (Response Evaluation Criteria in Solid Tumors), commonly used all over the world for drug evaluation, have been introduced because it is impossible to measure the size of each tumor accurately. It would be unreasonable to expect highly reliable results from Waterfall plots, as it is not possible to measure tumor size accurately. These plots perhaps suffer from over or underestimation of the effects of drugs. There are occasional reports of analysis of the effects of molecular-targeted agents by the use of Waterfall plots. It has been suggested that cases demonstrating reduction of tumor size can be clearly separated from those not showing a size reduction in the evaluation of the antitumor effects of molecular-targeted drugs, because molecular-targeted drugs are effective only against tumors with expression of the molecular target (Figure 4). If we view the results of V15-32 with this information in mind, it is probable that the magnitude of the antitumor activity of docetaxel overall would be greater than that of gefitinib, which shows significant effect only in a small number or specific subsets of patients. In particular, it would be anticipated that differences in the antitumor activities between conventional cytotoxic agents and molecular-targeted agents would be marked in those patients who do not express the molecular targets.

Patients that may benefit from gefitinib

The high degree of sensitivity to gefitinib of NSCLCs that harbor *EGFR* mutations has been demonstrated in a prospective phase II study: the response rate to gefitinib was about 80%, and both progression-free survival and overall survival were prolonged.[28,31] NSCLC with *EGFR* mutations has also been suggested to be highly sensitive to cytotoxic antineoplastic agents, and it would be necessary to establish the superiority of gefitinib through comparative studies in this group of patients. It is unknown whether gefitinib should be the preferred drug in patients with tumors carrying *EGFR* mutations. According to a report from the National Cancer Center Central Hospital in Japan, the efficacy rates of gefitinib in those with *EGFR* mutations is 82% compared with only 11% in those without such mutations. Thus, the decision to employ gefitinib on the basis of the presence of *EGFR* mutations in the tumors would be incorrect—possibly in as many as 10–20% of the patients.[29] Moreover, determination of the presence of *EGFR* mutations is possible in only 25% of patients with advanced lung cancer, which

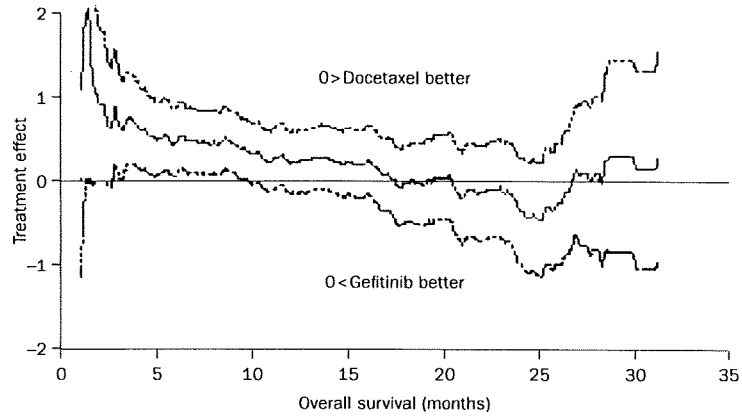


Figure 3 | Retrospective analysis of the survival data from the randomized phase III V15-32 trial. Permission obtained from Takeuchi © Takeuchi, M. *J. Lung Cancer* 7, 1–8 (2007)

poses a problem in selecting the most appropriate treatment.[8] The problem could be resolved if the methodology for the detection of *EGFR* mutations could be improved. It is known that progression-free survival and overall survival end points are favorable among patients who are Asian, are female, have adenocarcinoma histology and are nonsmokers, but the number of patients who meet all of these criteria is limited. Furthermore, when a group of patients who meet at least one of these criteria is selected, the incidence of false-positive and false-negative responses will increase. The results of the V15-32 study suggest that gefitinib should be administered as the drug of first choice only to patients with clear-cut targets, but currently there are no methods to distinguish these patients in a reliable manner.

Results of the IPASS trial

IPASS (IRESSA Pan Asia Study) was a phase III study designed to compare oral gefitinib monotherapy with intravenous carboplatin and paclitaxel chemotherapy as first-line treatment in chemotherapy-naive Asian patients with advanced NSCLC.[46] The eligibility criteria were: age ≥18 years, life expectancy ≥12 weeks, adenocarcinoma histology, never-smokers or light ex-smokers, performance status 0–2, stage IIIB/IV, and presence of measurable disease. A total of 1,217 patients were recruited between March 2006 and October 2007 from nine Asian countries, including China, Japan, Thailand, Taiwan, Indonesia, Malaysia, Philippines, Hong Kong and Singapore. Patients were randomly assigned to receive either 250 mg daily gefitinib ($n = 609$) or carboplatin (AUC 5 or 6) and paclitaxel (200 mg/m^2) ($n = 608$). The primary end point was noninferiority of these two arms for progression-free survival. The secondary end points were overall survival, objective response rate, quality of life, symptomatic improvement, and toxicity. Association of the efficacy with EGFR biomarkers was also analyzed as an exploratory end point. The study exceeded its primary end point and demonstrated the

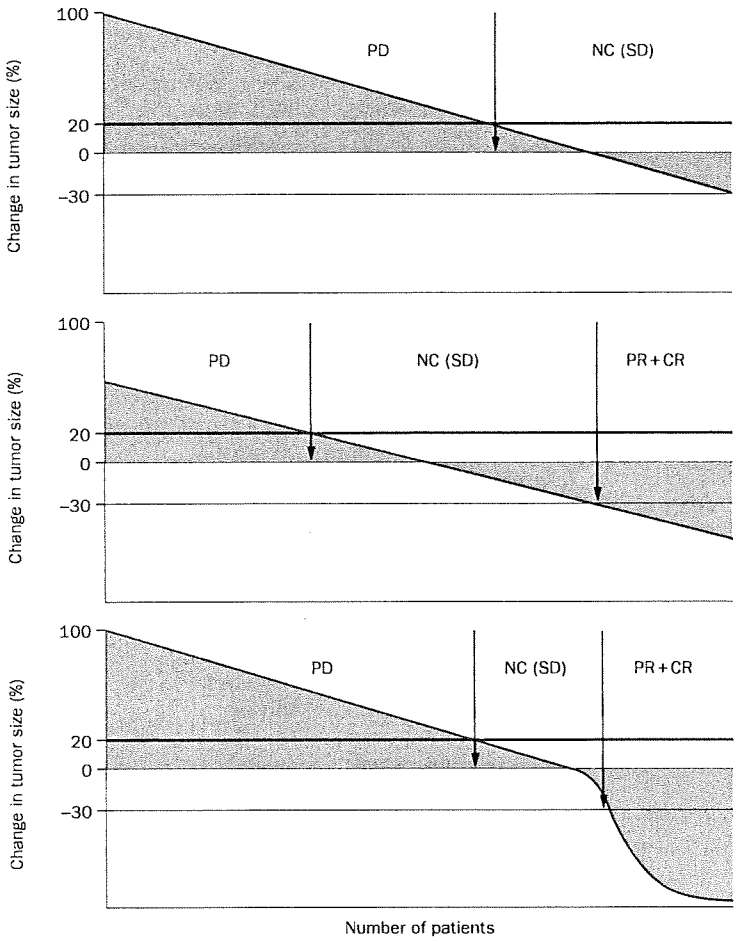


Figure 4 | Waterfall plots showing the differences in the effect of cytotoxic drugs and molecular-target drugs on tumor size. Abbreviations: CR, complete response; NC, no change; PD, progressive disease; PR, partial response; SD, standard deviation. Permission obtained from Takeuchi © Takeuchi, M. *J. Lung Cancer* 7, 1–8 (2007)

superiority of gefitinib over carboplatin and paclitaxel, in terms of progression-free survival, in the first-line setting. The risk of overall progression was reduced by 26% in gefitinib-treated patients compared with those who were administered chemotherapy.[16]

Interestingly, the treatment effect was not constant over time. The progression-free survival curves crossed at 6 months, favoring carboplatin and paclitaxel during the first 6 months and gefitinib thereafter. This evidence suggested there were two different populations of patients with regard to response to the chemotherapy doublet and gefitinib. In exploratory biomarker analyses, the progression-free survival was longer for patients with *EGFR* mutations who received gefitinib, compared to chemotherapy. By contrast, progression-free survival was longer for those in the carboplatin and paclitaxel arm than the gefitinib arm in patients with wild-type *EGFR*. A similar trend was observed in the exploratory analyses based on the *EGFR* copy number status. The target population in the IPASS trials was selected on the basis of clinical characteristics, such as presence or absence of adenocarcinoma histology and smoking history. About 60% of the patients had *EGFR* mutations in the tumor cells. In the 40% of patients without *EGFR* mutations, gefitinib showed no beneficial effect, whereas chemotherapy was effective. This is why the progression-free survival curves in the IPASS study crossed at 6 months after the start of treatment. The response rate to both gefitinib and chemotherapy was higher in those with *EGFR* mutations compared with those without such mutations; however, gefitinib had a greater beneficial effect than chemotherapy in patients with *EGFR* mutations. Another important finding of the IPASS trial was the extremely low response rate to gefitinib in patients with wild-type *EGFR*. The method used for the detection of these mutations was very sensitive, namely the scorpion ARMS method, so that all the mutation-positive patients could be identified. The overall survival data from the IPASS trial are awaited; however, the results of the IPASS trial have demonstrated that molecular-targeted drugs are effective only against tumors with the relevant molecular target, that is, *EGFR* mutations. Conversely, cytotoxic drugs have antitumor activity against tumors regardless of the presence or absence of *EGFR* mutations.

Lessons learned from EGFR-TKI data

Gefitinib has shown dramatic antitumor activity in phase I and II trials. As second-line treatment for Japanese patients with NSCLC, it produced response rates of almost 30%. In a placebo-controlled, comparative trial (ISEL)[14] the effect of gefitinib as second-line and third-line treatment for NSCLC in prolonging survival was proven among Asians, as demonstrated by the high response rates in the predefined subgroup analysis. By contrast, in non-Asians with low response rates, the survival curves of those treated with gefitinib versus no treatment were almost entirely superimposed. Paradoxically, in the BR-21 trial, the overall survival of patients treated with the EGFR-TKI, erlotinib, was significantly better than that of patients administered placebo, despite the low response rate.[39] In the subset analysis of BR-21, the efficacy of erlotinib in terms of survival benefit was reported to be observed in male patients or those with squamous histology, although these factors were associated with lower response rate. One could speculate that erlotinib might be effective in patients with wild-type *EGFR* tumors, although not to the extent to achieve major shrinkage of the tumor. If so, erlotinib could be regarded as a 'less-targeted drug' than gefitinib, since its efficacy is less affected by the target status of the tumor. Dosing strategies of gefitinib (administered at a third of the maximum tolerated dose) and erlotinib (administered at the maximum tolerated dose) are different, which could partly account for the discrepancy. This explanation should be tested in future clinical trials.

Four large, randomized trials, namely Intact 1 and 2, Talent, and Tribute, that compared the effect of a platinum doublet regimen with or without gefitinib or erlotinib yielded negative survival results, probably because of the limited effect of gefitinib or erlotinib.[9–13] The patients

accrued to these trials were not selected according to their *EGFR* mutational status or EGFR histology, smoking and gender status. Another reason might be a competitive cell-cycle effect of anticancer agents and molecular-targeted drugs. Two randomized, controlled trials, namely V15-32 and INTEREST, that compared gefitinib with docetaxel for second-line or third-line treatment NSCLC have been reported.[41,45] Although theV15-32 study did not demonstrate the noninferiority of gefitinib, the INTEREST trial established the noninferiority of this agent despite the low response rate observed in Western patients. It has long been believed that response is a good surrogate for progression-free survival or overall survival. The results of the V15-32 study do not support this hypothesis, and this finding poses a challenge when comparing the effect of molecular-targeted agents with that of cytotoxic antineoplastic agents on the basis of end points such as progression-free survival and overall survival.

The high response rate in the IPASS trial reflected the good progression-free survival for those treated with gefitinib. However, the progression-free survival curves crossed after 6 months, which suggests the existence of two different populations of patients with different effects of molecular-targeted drugs between the two groups. The IPASS trial was a clinical trial in a partially selected population of patients, which suggests the need for more-accurate selection of patients in future clinical trials. Nevertheless, results of the IPASS trial will have some influence on the interpretation of results of ongoing clinical trials. Comparative trials of gefitinib and platinum-based doublets for patients with advanced and/or recurrent disease who harbor *EGFR* mutations will need to be modified as it might be difficult to obtain informed consent from these populations, owing to the finding that progression-free survival is significantly longer in patients treated with gefitinib than in those receiving platinum-based chemotherapy.

Another issue relates to the antitumor activity of cytotoxic drugs against tumors with *EGFR* mutations. In the V15-32 trial, progression-free survival was better in patients with *EGFR* mutations who were treated with either gefitinib or chemotherapy. In the IPASS trial, progression-free survival in those who received gefitinib was quite different between patients with and without *EGFR* mutations. Conversely, progression-free survival tended to be better in patients with *EGFR* mutations than in those without such mutations who were administered platinum-based chemotherapy, although this difference was not significant despite the response rate to platinum-based chemotherapy being significantly higher in patients with *EGFR* mutations. The presence of *EGFR* mutations in the tumor is a predictive factor of response not only to EGFR-TKIs, but also to platinum-based chemotherapy. Thus, the role of *EGFR* mutations as a predictive factor of progression-free survival and overall survival remains unclear in patients treated with platinum-based chemotherapy. Although many randomized trials of EGFR-TKIs in unselected patients with NSCLC have been reported, the results are varied and it is quite difficult to interpret the outcomes of these clinical trials.[47–52]

Conclusions

The results of several randomized, controlled trials of targeted agents and cytotoxic therapies in patients with advanced NSCLC have produced confusing results, perhaps because of the following reasons. First, the modes of action of cytotoxic drugs and molecular-targeted drugs are different, although the differences remain to be precisely elucidated. Second, the majority of clinical trials have been conducted in unselected populations. The IPASS trial was conducted in a partially selected population; however, the additional analysis on the basis of *EGFR* mutations clearly identified the target populations that show response to EGFR-TKI and cytotoxic chemotherapy. Third, although biomarker studies are extremely important, the majority of biomarkers have not been validated and the techniques to assess the EGFR target have not been fully optimized. Data from classical biomarker studies might not be the best data to draw conclusions from because these studies were conducted without selecting patients on the basis of favorable profiles. For the field of personalized medicine with the use of targeted and cytotoxic agents to advance, the scientific and clinical significance of biomarkers should be analyzed more extensively.

Review criteria

Data for this Review were obtained by searching the PubMed database for articles published between 1 January 2000 to 1 November 2008. Only articles published in English were considered. The following search terms were used "non-small-cell lung cancer" "NSCLC", "epidermal growth factor receptor" "EGFR" and "tyrosine kinase inhibitor". When possible primary sources have been cited. Data from searches of the following conferences were also included: ASCO 2004–ASCO 2008 annual meetings, European Society of Medical Oncology 2008 annual meeting, and the 12th World Conference on Lung Cancer 2007.

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Short Communication

Close Association of *UGT1A9* IVS1+399C>T with *UGT1A1*28*, **6*, or **60* Haplotype and Its Apparent Influence on 7-Ethyl-10-hydroxycamptothecin (SN-38) Glucuronidation in Japanese

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ABSTRACT:

The anticancer prodrug, irinotecan, is converted to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by UDP-glucuronosyltransferase (UGT)1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. In a recent study, it was reported that the *UGT1A9* IVS1+399 (I399)C>T polymorphism is associated with increased SN-38 glucuronidation both in vitro and in vivo. However, its role in UGT1A9 expression levels and activity is controversial. Thus, we evaluated the role of I399C>T in SN-38 glucuronidation using 177 Japanese cancer patients administered irinotecan. I399C>T was detected at a 0.636 allele frequency. This polymorphism was in strong linkage disequilibrium (LD) with *UGT1A9*1b* (−126_−118T_9>T_{10}, |D'| = 0.99) and *UGT1A1*6* (211G>A, 0.86), in moderate LD with *UGT1A1*60* (−3279T>G, 0.55), but weakly associated with *UGT1A1*28* (−54_−39A(TA)$_6$TAA>A(TA)$_7$TAA, 0.25). Haplotype analysis showed that 98% of the I399C alleles were linked with low-activity haplotypes, either *UGT1A1*6*, **28*, or **60*. On the other hand, 85% of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*. Although I399T-dependent increases in SN-38 glucuronide/SN-38 area under concentration-time curve (AUC) ratio (an in vivo marker for UGT1A activity) and decreases in SN-38 AUC/dose were apparent ($P < 0.0001$), these effects were no longer observed after stratified patients by *UGT1A1*6*, **28*, or **60* haplotype. Thus, at least in Japanese populations, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either *UGT1A1*6*, **28*, or **60*.

Irinotecan is an important drug for treatment of various tumors including lung, colon, and gastric (Smith et al., 2006). The infused drug is metabolized to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by glucuronidation. At least four UDP-glucuronosyltransferase (UGT) isoforms, namely UGT1A1, UGT1A7, UGT1A9, and UGT1A10, are known to glucuronidate SN-38 (Gagné et al., 2002; Saito et al., 2007).

The UGT1A gene complex consists of 9 active first exons including *UGT1A10*, *1A9*, *1A7*, and *1A1* (in this order) and common exons 2 to 5. One of the 9 first exons can be used in conjunction with the common exons (Tukey and Strassburg, 2000). The UGT1A N-terminal domains (encoded by the first exons) determine substrate-binding specificity, and the C-terminal domain (encoded by exons 2 to 5) is important for binding to UDP-glucuronic acid. The 5′- or 3′-flanking region of each exon 1 is presumably involved in regulation of its expression. Substantial interindividual differences have been detected in mRNA and protein levels and enzymatic activity of the UGT1A isoforms (Fisher et al., 2000; Saito et al., 2007).

SN-38 glucuronidation is thought to be mediated mainly by UGT1A1, and its genetic polymorphisms affecting irinotecan pharmacokinetics and adverse reactions have been already identified. The TA-repeat polymorphism, −54_−39A(TA)$_6$TAA>A(TA)$_7$TAA (*UGT1A1*28* allele), is associated with lower promoter activity, resulting in reduced SN-38 glucuronidation (Beutler et al., 1998; Iyer et al., 1999). The single nucleotide polymorphism (SNP) 211G>A (Gly71Arg, **6* allele), found mainly in East Asians, causes reduced protein expression levels and SN-38 glucuronidation activity (Gagné et al., 2002; Jinno et al., 2003). Another SNP in the enhancer region of *UGT1A1*, −3279T>G (**60* allele), is also a causative factor for reduced expression (Sugatani et al., 2002). Allele frequencies have been reported for **28* (0.09–0.13), **6* (0.15–0.19), and **60* (0.26–0.32) in Japanese and Chinese populations and for **28* (0.30–0.39), **6* (~0), and **60* (0.44–0.55) in whites (Saito et al., 2007). In a previous study, in the Japanese population, we defined haplotype **28* as the haplotype harboring the **28* allele, haplotype **6* as that harboring the**6* allele, and haplotype **60* as that harboring the **60* allele (and without the **28* or **6* allele) (Sai et al., 2004; Saeki et al., 2006). Note that most of the **28* haplotypes concurrently harbored the **60* alleles, and that the **28* and **6* alleles were exclusively present on the different chromosomes (Sai et al., 2004; Saeki et al., 2006). We have also revealed that the haplotype **28*, **6*, or **60* was associated with reduced SN-38 glucuronide (SN-38G)/SN-38 area under concentration-time curve (AUC) ratios, an in vivo parameter for UGT1A activity (Minami et al., 2007).

In a recent study, an intronic SNP of *UGT1A9*, IVS1+399 (I399)C>T, has been shown to be associated with increased UGT1A9 protein levels and glucuronidation activities toward SN-38 and the UGT1A9 probe drug propofol (Girard et al., 2006). Elevation of

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ABBREVIATIONS: SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase; SNP, single nucleotide polymorphism; SN-38G, SN-38 glucuronide; AUC, area under concentration-time curve; I399, *UGT1A9* IVS1+399; LD, linkage disequilibrium.

SN-38 glucuronidation activity by this SNP is significant among subjects without *UGT1A1*28*. Sandanaraj et al. (2008) have also reported that I399C/C patients showed higher SN-38 AUC than C/T and T/T patients. With the same *UGT1A1* diplotypes, patients with I399T/T (and *UGT1A9* $-126\_-118T_{10}/T_{10}$) have shown higher SN-38G C_{max} than I399C/T (and T_9/T_{10}) patients. *UGT1A9*1b* (*UGT1A9* $-126\_-118T_9>T_{10}$) has been shown to have no affect on UGT1A9 expression levels (Girard et al., 2006; Ramírez et al., 2007; Sandanaraj et al., 2008). Thus, two groups did suggest that I399T allele was associated with higher glucuronidation activity. However, using human liver microsomes, Ramírez et al. (2007) showed that I399C>T had no significant effect on both UGT1A9 mRNA levels and glucuronidation activities for two UGT1A9 substrates. Therefore, the roles of I399C>T in UGT1A9 activities as well as SN-38 glucuronidation remain inconclusive.

In the present report, we reveal the linkage of I399C>T with *UGT1A1*, *UGT1A7*, and *UGT1A9* polymorphisms and analyze its association with the SN-38G/SN-38 AUC ratio and SN-38 AUC/dose (per dose) to clarify its role in SN-38 glucuronidation.

Materials and Methods

Patients. One hundred and seventy-seven patients (81 lung, 63 colon, 19 stomach, and 14 other cancer patients) administered irinotecan at the National Cancer Center were enrolled in this study as described previously (Minami et al., 2007). This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants. Eligibility criteria, patient profiles, and irinotecan regimens are summarized in our previous report (Minami et al., 2007). In brief, patients consisted of 135 males and 42 females with a mean age of 60.5 (26–78 years old), and their performance status was 0 (84 patients), 1 (89 patients), or 2 (4 patients). Irinotecan administrations were conducted according to the standard protocols in Japan as follows: i.v. 90-min infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly in irinotecan monotherapy; and 60 mg/m^2 weekly with cisplatin in most combination therapies.

Genotyping and Haplotype Analysis. Genomic DNA was extracted from whole blood of 177 irinotecan-administered patients (Saeki et al., 2006). *UGT1A9* IVS1+399C>T (rs2741049) was genotyped using the TaqMan SNP Genotyping Assay kit (C_9096281_10) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The *UGT1A1*28* allele [$-54\_-39A(TA)_6TAA>A(TA)_7TAA$], *UGT1A1*6* allele [211G>A (Gly71Arg)], *UGT1A1*60* allele (−3279T>G), *UGT1A7*2* haplotype [387T>G, 391C>A and 392G>A (Asn129Lys and Arg131Lys)], *UGT1A7*3* haplotype [387T>G, 391C>A, 392G>A, and 622T>C (Asn129Lys, Arg131Lys, and Trp208Arg)], and *UGT1A9*1b* allele ($-126\_-118T_9>T_{10}$) were determined previously (Saeki et al., 2006). Hardy-Weinberg equilibrium analysis of I399C>T, linkage disequilibrium (LD) analysis of the *UGT1A9*, *UGT1A7*, and *UGT1A1* polymorphisms, and haplotype estimation with an expectation-maximization algorithm were performed using SNPAlyze version 7.0 software (Dynacom, Chiba, Japan).

Pharmacokinetics. Pharmacokinetic data for the 176 irinotecan-treated patients (data for one patient was unavailable) were described previously (Minami et al., 2007). In brief, heparinized blood was collected before irinotecan administration and at 0, 0.33, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. SN-38 and SN-38G plasma concentrations were determined by high-performance liquid chromatography, and AUC was calculated using the trapezoidal method in WinNonlin version 4.01 (Pharsight, Mountain View, CA).

Statistical Analysis. Gene dose effects of I399C>T and *UGT1A1* haplotypes (**28*, **6*, *or *60*) were assessed by the Jonckheere-Terpstra test using StatExact version 6.0 (Cytel Inc., Cambridge, MA). Multiplicity adjustment was conducted with the false discovery rate. The significant difference was set at $p = 0.05$ (two-tailed).

Results

Linkages of *UGT1A9* IVS1+399 (I399)C>T with Other Polymorphisms. In our patients, I399C>T was detected at a 0.636 allele frequency, which is almost the same as those in the HapMap data (rs2741049) for Japanese (0.663) and Han Chinese (0.633) populations, but higher than those for Europeans (0.383) and Sub-Saharan Africans (Yoruba) (0.417). Genotype distribution for this SNP was in Hardy-Weinberg equilibrium ($p = 0.418$). LD analysis was performed between I399C>T and the previously determined genotypes, *UGT1A9*1b*, *UGT1A7*2* and **3*, and *UGT1A1*28*, **6*, and **60*, which were detected at >0.1 frequencies in Japanese populations (Saeki et al., 2006). When assessed by the ID'I value, I399C>T was in complete LD with *UGT1A7* 387T>G, 391C>A and 392G>A (*UGT1A7*2*, ID'I = 1.000); in strong LD with *UGT1A9* $-126\_-118T_9>T_{10}$ (*UGT1A9*1b*, 0.987), *UGT1A7* 622T>C (*UGT1A7*3*, 0.977), and *UGT1A1* 211G>A (*UGT1A1*6*, 0.864); and in moderate LD with *UGT1A1* −3279T>G (*UGT1A1*60*, 0.554), but weakly associated with *UGT1A1* $-54\_-39A(TA)_6TAA>A(TA)_7TAA$ (*UGT1A1*28*, 0.252). In r^2 values, the I399C>T was in strong LD with *UGT1A7*2* ($r^2 = 0.976$) and *UGT1A9*1b* (0.916), in moderate LD with *UGT1A7*3* (0.478), but in weak LD with *UGT1A1*6* (0.261) and *UGT1A1*60* (0.208), and in little LD with *UGT1A1*28* (0.018).

Haplotype Analysis. Haplotype analysis was performed using the 9 polymorphisms including I399C>T. As shown in Fig. 1, 95% (123/129) of the I399C alleles were linked with the *UGT1A9* -126_-118T_9 alleles, and 100% (225/225) of the T alleles were linked with the T_{10} alleles (*UGT1A9*1b*). The I399C alleles were completely (129/129) linked with the *UGT1A7* 387G, 391A, and 392A alleles, and most T alleles (223/225) were linked with the 387T, 391C, and 392G alleles. The 40% (51/129) and 60% (78/129) of the I399C alleles were linked with *UGT1A7*2* and *UGT1A7*3* haplotypes, respectively. We also found that 98% (126/129) of the I399C alleles were linked with the *UGT1A1*6* (211G>A), **28* [$-54\_-39A(TA)_6TAA>A(TA)_7TAA$], or **60* (−3279T>G). According to the *UGT1A1* haplotype definition by Sai et al. (2004), 42% (54/129), 36% (46/129), 19% (25/129), and 1% (1/129) of the I399C alleles were linked with the *UGT1A1* haplotypes **6a* (harboring **6* allele), **60a* (harboring **60* allele), **28b* (harboring **60* and**28* alleles), and **28d* (harboring **28* allele), respectively. On the other hand, 85% (191/225) of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*.

Association Analysis. The associations of I399C>T with irinotecan pharmacokinetic parameters were then analyzed using the estimated haplotypes. First, association with SN-38G/SN-38 AUC ratio, an in vivo parameter of UGT1A activity (Sai et al., 2004; Minami et al., 2007; Sandanaraj et al., 2008), was analyzed. *UGT1A7*2* had unchanged activity for SN-38 glucuronidation (Gagné et al., 2002), and neither *UGT1A9*1b* nor *UGT1A7*3* had significant effects on the SN-38G/SN-38 AUC ratio in our previous study (Minami et al., 2007). On the other hand, the *UGT1A1*6*, **28*, and **60* haplotypes were associated with the reduced SN-38G/SN-38 AUC ratios (Minami et al., 2007). Although effects of the haplotype **28* and **6* were more striking, haplotype *UGT1A1*60*, harboring only the **60* allele without the **28* allele, was weakly associated with the reduced ratio. To remove even this weak effect and clarify the real effect of I399C>T, *UGT1A1*60* was also considered as low-activity haplotype in this analysis. Namely, we analyzed the associations of I399C>T with the AUC ratio within the groups stratified by the *UGT1A1* haplotypes, *UGT1A1*28* (**28b* and **28d*), **6* (**6a*), and **60* (**60a*) (combined and shown as *UGT1A1*"+").

When stratified by the I399C>T genotype, a T allele-dependent

Gene		*UGT1A9*		*UGT1A7*[2]				*UGT1A1*[3]			Number	Frequency
Nucleotide change		-126_-118 T_9>T_{10}	IVS1+399 C>T	387 T>G	391 C>A	392 G>A	622 T>C	-3279 T>G	$(TA)_6$>$(TA)_7$	211 G>A		
Allele name		**1b*		**2, *3*	**2, *3*	**2, *3*	**3*	**60, *28*	**28*	**6*		
Haplotypes[1]	**1C-*3-*6a*			■	■	■	■			■	47	0.133
	**1C-*2-*60a*			■	■	■		■			44	0.124
	**1C-*3-*28b*			■	■	■	■	■	■		21	0.059
	**1C-*2-*28b*			■	■	■		■	■		4	0.011
	**1C-*3-*60a*			■	■	■	■	■			2	0.006
	**1C-*3-*28d*			■	■	■	■		■		1	0.003
	**1C-*2-*6a*			■	■	■				■	1	0.003
	**1bC-*3-*6a*	■		■	■	■	■			■	6	0.017
	**1C-*2-*1*			■	■	■					2	0.006
	**1C-*3-*1*			■	■	■	■				1	0.003
	**1bT-*1-*1*	■	■								190	0.537
	**1bT-*3-*1*	■	■	■	■	■	■				1	0.003
	**1bT-*1-*28b*	■	■					■	■		22	0.062
	**1bT-*1-*60a*	■	■					■			5	0.014
	**1bT-*1-*6a*	■	■							■	5	0.014
	**1bT-*1-*28d*	■	■						■		1	0.003
	**1bT-*2-*60a*	■	■	■	■	■		■			1	0.003
Alelle frequency		0.653	0.636	0.370	0.370	0.370	0.223	0.280	0.138	0.167	354	1.000

FIG. 1. Haplotypes assigned by using common *UGT1A9*, *UGT1A7*, and *UGT1A1* polymorphisms. [1]Haplotypes were shown as *UGT1A9* haplotypes − *UGT1A7* haplotypes − *UGT1A1* haplotypes. Major allele, white blocks; minor allele, gray blocks. **1C*, T_9 and I399C: **1bC*, T_{10} and I399C; **1bT*, T_{10} and I399T in *UGT1A9*. [2]*UGT1A7*2* and **3* are the haplotypes harboring the three and four *UGT1A7* alleles, respectively. [3]*UGT1A1* $(TA)_6$>$(TA)_7$ indicates −54_−39A$(TA)_6$TAA>A$(TA)_7$TAA.

increase in the SN-38G/SN-38 AUC ratio was observed ($p < 0.0001$, Jonckheere-Terpstra test) (Fig. 2A). However, this trend was obviously dependent on biased distributions of *UGT1A1* haplotypes; e.g., 96% of the I399C/C patients were homozygotes for *UGT1A1*28*, **6*, *or *60*; and "*UGT1A1*28*, **6*, or **60*"-dependent reduction of SN-38G/SN-38 AUC ratio was found within the I399T/T genotypes ($p < 0.05$). As shown in Fig. 2B, *UGT1A1*28*, **6*, or **60* (*UGT1A1*+)-dependent reduction in the SN-38G/SN-38 ratio was observed when patients were stratified by these three haplotypes. However, no significant effect of I399C>T was found within the stratified patients ($p > 0.05$ within the −/−, −/+, or +/+ patient group in Fig. 2B). As for SN-38 AUC/dose (SN-38 AUC values adjusted by the doses used), a similar *UGT1A1* haplotype dependence was observed. Although the I399T-dependent reduction of SN-38 AUC/dose was detected ($p < 0.0001$), biased distributions of the *UGT1A1*28*, **6*, or **60* were again evident, and the *UGT1A1* + haplotypes-dependent increase was significant within the I399 C/T and T/T patients ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 2C). Moreover, no significant effect of I399C>T on SN-38 AUC/dose was found when stratified by the *UGT1A1* haplotypes ($p > 0.05$ within the −/−, −/+, or +/+ patient group in Fig. 2D).

Discussion

In the present study, LD between I399C>T and *UGT1A1*, *UGT1A7*, or *UGT1A9* polymorphisms in Japanese populations was shown for the first time. Moreover, the apparent effect of I399C>T on SN-38 glucuronidation in Japanese cancer patients was suggested to result from its close association with *UGT1A1*28*, **6*, or **60*.

As for the influence of I399C>T on UGT1A9 activity, conflicting results have been reported. Girard et al. (2006) have shown that I399C>T was associated with increased UGT1A9 protein levels and enzyme activity toward an UGT1A9 probe drug propofol using 48 human liver microsomes derived mainly from whites. In contrast, using human liver microsomes from 46 white subjects, Ramírez et al. (2007) have revealed that the I399C>T had no significant effects on UGT1A9 mRNA levels and in vitro glucuronidation activities toward the two UGT1A9 substrates, flavopiridol and mycophenolic acid. Furthermore, another report has demonstrated that I399C>T had no influence on the pharmacokinetic parameters (such as AUC and C_{max}) of mycophenolic acid in 80 Japanese renal transplant recipients (Inoue et al., 2007). Thus, these latter two studies did suggest that the I399C>T polymorphism has no effect on UGT1A9 enzymatic activity. Note that, at least for Japanese populations, no study has reported that I399C>T affects UGT1A9 activity.

As for the influence of I399C>T on SN-38 glucuronidation, a possible enhancing effect has been suggested. Girard et al. (2006) have shown an increasing effect of I399C>T on SN-38 glucuronidation, and that this SNP did not show any close linkages with the *UGT1A1*28* or **60* allele ($r^2 < 0.06$). In addition, Sandanaraj et al. (2008) have reported that in 45 Asians consisting of Chinese (80%), Malay (18%), and others (2%), I399C/C patients had higher SN-38 AUC than C/T and T/T patients. Again, this SNP was not in LD with the *UGT1A1*28*, **6*, or **60* allele (r^2 were <0.09). Furthermore, association of I399T with increased SN-38G C_{max} has been observed even after stratified patients by *UGT1A1* genotypes, although the study sample size was small. These findings suggest that the I399T

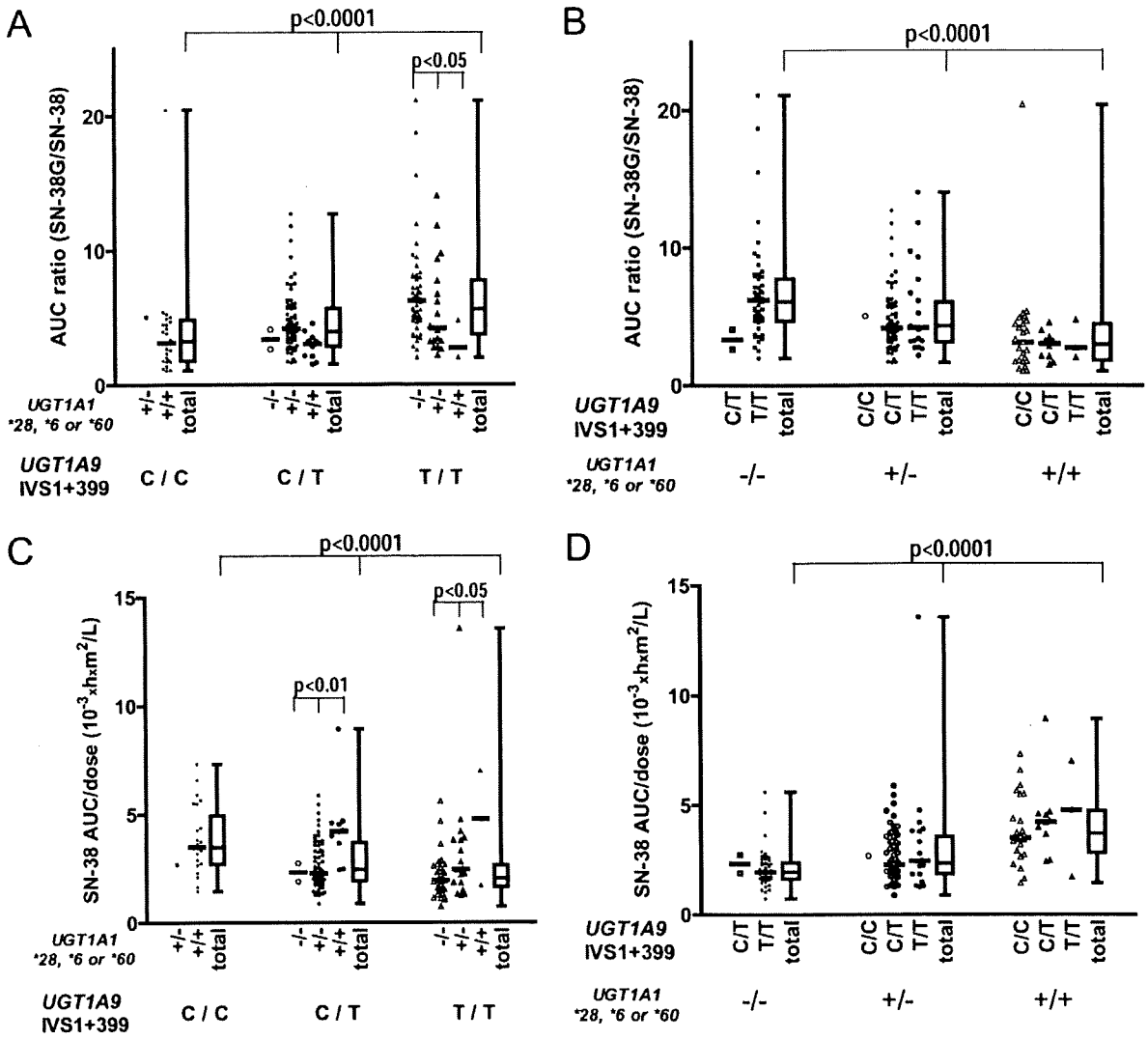

FIG. 2. Association analysis of *UGT1A9* IVS1+399 (I399)C>T with SN-38G/SN-38 AUC ratio (A and B) and SN-38 AUC/dose (C and D). A and C, I399 C/C, C/T, and T/T patients were further divided by the presence of *UGT1A1*28*, **6*, or **60* haplotypes: −/−, no *UGT1A1*28*, **6*, or **60*; −/+, heterozygotes for either *UGT1A1*28*, **6*, or **60*; +/+, homozygotes or compound heterozygotes for either *UGT1A1*28*, **6*, or **60*. B and D, *UGT1A1* −/−, −/+, and +/+ patients were further divided by I399 C/C, C/T, and T/T genotypes. Gene dose effects of I399C>T and the *UGT1A1* + haplotype were assessed by the Jonckheere-Terpstra test.

allele was associated with increased glucuronidation activity for SN-38 without linkages with the *UGT1A1* polymorphisms. Our data demonstrate that an increase in SN-38G/SN-38 AUC ratio (i.e., increased glucuronidation activity) was also found with I399C>T; however, after stratified patients by the *UGT1A1*6*, **28*, or **60* haplotypes (haplotype+) showing reduced SN-38 glucuronidation activity (Sai et al., 2004; Minami et al., 2007), any significant effect of the I399C>T was no longer observed. Thus, no direct effect of I399C>T on SN-38 glucuronidation was shown in the current study in Japanese populations. The discrepancy between our study and others might be derived from ethnic and/or population differences in haplotype distribution. In fact, in our Japanese population, 98% of the I399C alleles were linked with either *UGT1A1 *6*, **28*, or **60*, whereas 85% of the T alleles were linked with *UGT1A1*1*. On the other hand, in Sandanaraj's report (in Chinese + Malay), 84% of the I399C alleles were linked with *UGT1A1 *6*, **28*, or **60*, whereas only 67% of the T alleles were linked with *UGT1A1*1* (Sandanaraj et al., 2008).

In irinotecan therapies, genetic polymorphisms leading to increases in SN-38 AUC, which closely correlates with increased risk of severe neutropenia (Minami et al., 2007), are clinically important. The current study also demonstrated no significant influence of I399C>T on SN-38 AUC/dose after stratified patients by *UGT1A1* haplotypes. Consistent with this finding, no influence of this SNP was observed on the incidence of grade 3 or 4 neutropenia after irinotecan therapy in our population (data not shown). Recently, genetic testing of *UGT1A1*6* and **28*, which are related to severe neutropenia in Japanese populations, has been approved for clinical application in Japan. This study indicates that there is no clinical necessity for additional genotyping of I399C>T, at least in Japanese populations.

In conclusion of this study, the apparent influence of I399 (*UGT1A9* IVS1+399)C>T on SN-38 glucuronidation is attributable to its close association with *UGT1A1*6*, **28*, or **60* in the Japanese population. Furthermore, additional genotyping of I399C>T for personalized irinotecan therapy seems to be clinically irrelevant for Japanese populations.

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