and docetaxel, have been proven to improve the survival of patients with metastatic NSCLC compared with second-generation chemotherapies [8–10]. In addition, several phase I and II studies of third-generation chemotherapy with concurrent TRT for locally advanced NSCLC provided promising survival outcomes [11–17]. However, it seems to be difficult to deliver full doses of the above-described regimens because of dose-limiting toxicities.

Concurrent chemoradiotherapy with vinorelbine and cisplatin (NP) is one of the commonly used regimens for locally advanced NSCLC in Japan, yielding a reasonable response rate, median survival time (MST), and 3-year survival rate in a phase I trial and retrospective analysis [17,18]. Subsequently, in a recent phase III study comparing second- and third-generation regimens with concurrent TRT for unresectable stage III NSCLC, weekly paclitaxel, carboplatin, and TRT followed by 2 courses of triweekly consolidation provided good results in terms of both efficacy and toxicity [19]. And also weekly docetaxel, cisplatin and TRT provided better efficacy and hematological toxicities [20]. It has therefore been suggested that these regimens should be regarded as standard treatments for locally advanced NSCLC.

Although UFT, an oral preparation of uracil and tegafur, is seldom used for metastatic NSCLC, two phase II studies of UFT plus cisplatin (UP) in advanced NSCLC have exhibited efficacy almost equivalent to other potent regimens [21,22]. Moreover, a full dose regimen of UP chemotherapy with concurrent TRT for locally advanced NSCLC in a multi-institutional phase II trial has shown a promising outcome with low hematological toxicity [23].

The optimal combination chemotherapy with TRT for locally advanced NSCLC remains to be established. Thus, to select a proper candidate for a phase III study of chemoradiotherapy, we conducted a randomized phase II study comparing the UP arm with the NP arm.

2. Patients and methods

2.1. Patient eligibility

The study population consisted of patients between 20 and 75 years of age inclusive, with cytologically or histologically confirmed NSCLC with unresectable stage IIIA or IIIB disease. Mediastinoscopies were not performed and lymph node metastases were clinically diagnosed based on the results of computed tomography (CT) scan and/or positron emission tomography (PET) scan. Unresectable stage IIIA disease was defined by the presence of multiple/bulky N2 mediastinal lymph nodes on CT such that, in the judgment of the treating investigator, the patients were unsuitable as candidates for surgical resection. Patients were required to have lesions measurable with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0.

Other eligibility criteria included an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, no prior systemic chemotherapy, TRT or thoracic surgery. Laboratory requirements included a white blood cell count of 4000 mm^{-3} or more, a neutrophil count of 2000 mm^{-3} or more, a platelet count of 100,000 mm^{-3} or more, a hemoglobin level of 10 g/dL or more, a total bilirubin level of 1.5 mg/dL or less, an AST/ALT value of twice the upper normal limit or less, a creatinine level of 1.5 mg/dL or less, a creatinine clearance of 60 mL/min or more, and partial pressure of arterial oxygen of 60 torr or more.

Patients were ineligible if they had concomitant malignancies, active infectious diseases, serious complications such as ileus, uncontrolled diabetes mellitus, heart failure, renal failure, or hepatic failure, malignant pleural or pericardial effusion, interstitial pneumonitis or pulmonary fibrosis apparent on chest X-ray, and other medical problems regarded as making them ineligible for this study by physicians. Lactating, pregnant or possibly pregnant women, or those willing to become pregnant were also excluded. The study protocol was approved by the institutional review board of each hospital concerned and written informed consent was obtained from each patient.

Prestudy radiographic assessment to document tumor staging for eligibility included CT of the thorax including the upper abdomen, brain CT or magnetic resonance imaging and radioisotopic bone scan. PET scan was allowed to be substituted for radioisotopic bone scan.

2.2. Treatment schedules

Patients were randomly assigned to one of two treatment arms (UP arm and VP arm) as shown in Fig. 1 (CONSORT diagram), stratified by gender (male *v* female), age (59 or younger *v* 60–64 *v* 65–69 *v* 70–75), histology (adenocarcinoma *v* squamous cell carcinoma *v* large cell carcinoma *v* other), and clinical stage (IIIA *v* IIIB).

In the UP arm, oral UFT (400 mg/m^2/day) twice daily before meals from days 1 to 14 and from days 29 to 42 and cisplatin (80 mg/m^2) via intravenous infusion on days 8 and 36 were administered. According to body surface area (BSA), the actual dose of UFT was modified as follows: BSA less than 1.25 m^2, 500 mg/day (300 mg in the morning and 200 mg in the evening); BSA 1.25 m^2 or more, 600 mg/day (300 mg b.i.d.). Concurrent TRT was given in daily fractions of 2 Gy from day 1 up to a total of 60 Gy in 30 fractions over a 6-week period. In the NP arm, vinorelbine (20 mg/m^2) on days 1, 8, 29, and 36 and cisplatin (80 mg/m^2) on days 1 and 29 were administered intravenously. The schedule of TRT was the same as that of the UP arm.

Two cycles of additional treatment with the same dosage were optionally permitted in both arms as consolidation chemotherapy. There was no evidence that consolidation chemotherapy prolonged overall survival for locally advanced NSCLC. Therefore, consolidation chemotherapy was considered at the investigator's discretion.

2.3. Radiotherapy

Radiotherapy began on day 1 of chemotherapy in both arms with a linear accelerator photon beam of 4 MV or more.

In this study, both 2D and 3D treatment planning systems were allowed. Radiation doses were specified at the center of the target volume. 3D dose constraints for both planning target volume and normal-risk organs were not determined in the protocol. The doses were calculated assuming tissue homogeneity with correction for lung tissues in 3D treatment planning. As it turned out, 3D treatment planning was performed for all 66 patients who received radiotherapy.

The initial 40 Gy was delivered to the large field target volume, which included the primary tumor, ipsilateral hilum, and mediastinum. No prophylactic irradiation of the supraclavicular fossa area was given. The other 20 Gy was delivered to a pair of oblique fields to avoid excess irradiation of the spinal cord.

2.4. Treatment modifications

The administration of cisplatin was withheld on either arm if there was a decrease in the leukocyte count to below 3000 mm^{-3}, or the neutrophil count to under 1500 mm^{-3}, or the platelet count to less than 100,000 mm^{-3}, or if grade 2 or more nonhematological toxicities were observed, except for alopecia, anorexia, and malaise, until resolution of toxicity to grade 0 or 1. In the UP arm, UFT was stopped and then reduced in subsequent cycles from 600 mg or 500 mg to 400 mg or 300 mg, respectively if any grade 4 hematological toxicities, or grade 3 or worse nonhematological toxicities, except for alopecia, anorexia, or malaise, were observed. Whenever grade 2 diarrhea or stomatitis occurred, UFT was reduced. In the NP

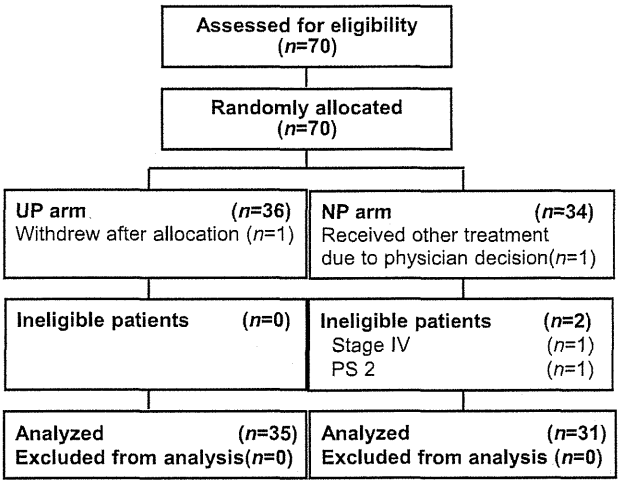

Fig. 1. CONSORT diagram. UP, uracil/tegafur and cisplatin; NP, vinorelbine and cisplatin.

arm, the administration of vinorelbine on days 8, 29, or 36 was omitted and a delay of up to 7 days was permitted if any grade 2 or worse hematological or nonhematological toxicities were observed. TRT was withheld on either arm in cases of any grade 4 hematological toxicities, grade 3 or worse esophagitis or dermatitis, grade 1 or worse fever, or any sign of pneumonitis. Any patient unable to receive a subsequent cycle within 7 days was removed from the protocol treatment, but was included in the study analysis.

2.5. Evaluation of efficacy and safety

All eligible patients who received any protocol treatment were regarded as evaluable for efficacy and safety. Complete blood cell counts and biochemistry tests were performed once a week during the treatment period. Thoracic CT was performed every 4 weeks during and after the treatment period until progressive disease was recognized.

The response was evaluated according to RECIST version 1.0 in the extramural review. Progression-free survival (PFS) was defined as the period from the date of randomization to the date when disease progression was first observed or death occurred. Overall survival (OS) was defined as the period between randomization and death from any cause. Toxicities were assessed according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

2.6. Statistical analysis

The primary endpoint was overall response rate (ORR), and secondary endpoints included PFS, OS, and the level of toxicity. Assuming that an ORR of 80% in eligible patients would indicate potential usefulness, while an ORR of 60% would be the lower limit of interest, with alpha = 0.05 and beta = 0.20, the estimated required accrual was 33 patients in each arm. Allowing for dropouts, the accrual goal was determined to be 35 patients in each arm.

Fisher's exact test was used to estimate the correlation among different variables between arms. Survival estimation was performed according to the Kaplan–Meier method.

3. Results

3.1. Patient characteristics

Between February 2006 and May 2009, 70 patients were enrolled from 5 institutions and were allocated to the UP arm ($n = 36$) and NP arm ($n = 34$). Of the 70 patients enrolled, 4 patients were excluded from final analysis, including one patient due to withdrawal of the informed consent before the allocated treatment, 2 patients due to ineligibility (stage IV and PS 2), and 1 patient who received a different regimen based on the physician's judgment instead of the protocol treatment. Eventually 66 patients (UP arm, $n = 35$ and NP arm, $n = 31$) were evaluable for efficacy and safety (CONSORT diagram, Fig. 1). No remarkable differences in demographic characteristics were found between the two treatment arms (Table 1).

Table 1
Characteristics of patients in each treatment arm.

Characteristic	UP arm ($n = 35$)		NP arm ($n = 31$)		P
	No.	%	No.	%	
Gender					0.6841
Male	28	80.0	26	83.9	
Female	7	20.0	5	16.1	
Age (years)					0.8326
Median	62		61		
59≥	12	34.3	14	45.2	
60–64	10	28.6	8	25.8	
65–69	7	20.0	5	16.1	
70–75	6	17.1	4	12.9	
PS (ECOG)					0.8281
0	16	45.7	15	48.4	
1	19	54.3	16	51.6	
Histology					0.4765
Adenocarcinoma	17	48.6	19	61.3	
Squamous cell carcinoma	15	42.9	11	35.5	
Large cell carcinoma	2	5.7	0	0	
Other	1	2.9	1	3.2	
Stage					0.8888
IIIA	13	37.1	11	35.5	
IIIB	22	62.9	20	64.5	

3.2. Treatment administered

As shown in Table 2, the median number of treatment cycles was 3 (range 1–4) in both arms. 94.3% of patients in the UP arm and 93.5% of patients in the NP arm underwent the projected two cycles of chemotherapy and concurrent TRT. As consolidation chemotherapy, 60% of patients in the UP arm and 58.1% of patients in the NP arm received the additional treatment with the same regimen as allowed by the protocol. Main reason for quitting chemotherapy after two cycles of the protocol treatment was investigator's discretion. Most of patients who received consolidation chemotherapy completed 4 cycle of treatment altogether as long as the progression was not observed.

In all except 1 patient in each arm, 60 Gy concurrent TRT was completed.

Table 2
Treatment delivery.

	UP arm ($n = 35$)		NP arm ($n = 31$)	
	No.	%	No.	%
Cycle number				
1	2	5.7	2	6.5
2	12	34.3	11	35.5
3	5	14.3	7	22.6
4	16	45.7	11	35.5
Median	3.0		3.0	
TRT (Gy)				
60	34	97.1	30	96.8
50–59	0	0	1	3.2
40–49	1	2.9	0	0
Median	60.0		60.0	

Table 3
Objective response rates.

	UP arm (n=35)		NP arm (n=31)	
	No.	%	No.	%
Complete response	2	5.7	1	3.2
Partial response	26	74.3	21	67.7
Stable disease	7	20.0	7	22.6
Progressive disease	0	0.0	2	6.5
Response rate (CR + PR[a])	28	80.0	22	71.0

[a] P=0.5659.

3.3. Efficacy

In the UP arm, the ORR was 80% (95% CI, 67–93%), including 2 patients (6%) with a complete response (CR), 26 (74%) with a partial response (PR), and no patient with progressive disease (PD). In the NP arm, the ORR was 71% (95% CI, 55–87%), including 1 patient (3%) with CR, 21 (68%) with PR, and 2 (6%) with PD (Table 3). Although the response rate in the UP arm was superior to that in the NP arm, this difference between two arms was not statistically significant (P=.566).

The PFS and OS data are shown in Fig. 2. With a median follow-up of 20.2 months, 40 patients had died. The median PFS were 8.8 months (95%CI, 6.7–11.1 months) in the UP arm, and 6.8 months (95%CI, 5.2–9.6 months) in the NP arm, respectively. The MST in the UP arm was 26.9 months (95% CI, 16.3–52.9 months) compared with 21.7 months (95% CI, 14.5–45.3 months) in the NP arm. The 2-/3-year survival rates were marginally higher in the UP arm (51.0/34.3%) than that in the NP arm (46.9/33.4%).

The sites of first failure among 31 recurrent cases in the UP arm, 12 (38.7%) were local and 19 (61.3%) were distant including 11 patients with brain metastasis, whereas among 26 recurrent cases in the NP arm, 14 (53.8%) were local, 12 (46.2%) were distant, including 6 with brain metastasis (data not shown).

3.4. Safety

Grade 3 or worse toxicities in each arm are shown in Table 4. Grades 3 and 4 neutropenia and febrile neutropenia were significantly more frequent in the NP arm than in the UP arm (P=.002 and .044, respectively). Although anorexia, nausea/vomiting, and diarrhea over grade 3 tended to be more frequent in UP arm, there were no statistically significant differences between the two arms in these categories. No one had grade 3 or worse esophagitis in either arm. Two patients in the NP arm died of radiation pneumonitis approximately five months after the completion of 60 Gy of TRT.

4. Discussion

We set out to compare oral fluoropyrimidine with third-generation anticancer agent in platinum-based chemoradiotherapy for locally advanced stage III NSCLC. The combined modality strategy with chemotherapy and concurrent TRT is considered as a standard treatment for locally advanced unresectable NSCLC. However, the optimal chemotherapy regimen remains to be determined. Two randomized phase III studies comparing third-generation regimen with second-generation regimen combined with concurrent TRT showed superior survival outcomes in third-generation regimen [19,20]. Moreover, based on recent randomized phase II and III studies, weekly carboplatin and paclitaxel with TRT has become a commonly used regimen, and is regarded as the reference arm for future phase III studies [16,19].

In this trial, we adopted the response rate as a primary endpoint because it is not influenced by post trial treatment. On the other hand, most of recent chemoradiotherapy phase II trials choose 2-year survival rate or PFS as primary endpoint. Thus, we also carefully followed-up for PFS and OS. Both the UP arm and NP arm showed a reasonably good response rate and survival outcomes in the present study. These data were comparable to those of third-generation regimens in other previous phase II and III trials. As to the primary endpoint, the ORR of the UP arm was better, but not with statistical significance, than that of the NP arm. In addition, the median PFS, the median OS, and the 2-year survival rate in the UP arm were better than those in the NP arm. There was a non-significant trend toward more favorable survival data in the UP arm than the NP arm although the small sample size in this study prevented reaching a definite conclusion.

It cannot be denied that epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib could contribute to long-term survival. EGFR mutation screening was not routinely performed in this study, because this examination was not available at the start of this study. Among 36 patients (UP arm, n=20 and NP arm, n=16) who subsequently underwent EGFR mutation screening, 5 patients in the UP arm and 3 patients in the NP arm harbored mutated EGFR (data not shown). Only 2 of them, both in the UP arm achieved PR and long-term survival. Hence, it is difficult to evaluate whether the use of EGFR-TKIs would have resulted in better survival outcomes.

It is noteworthy in the present study that hematological toxicities were very mild in the UP arm. In particular, the incidence of grade 3 or worse neutropenia in the UP arm was remarkably lower than not only in the NP arm of this study but also in the other regimens referred to above. Moreover, no febrile neutropenia was observed in the UP arm. Although grade 3 gastrointestinal toxicities which are common in fluoropyrimidines were more frequent in the UP arm than in the NP arm, they were all reversible and manageable thus this does not seem to be a critical impact. Therefore, UP

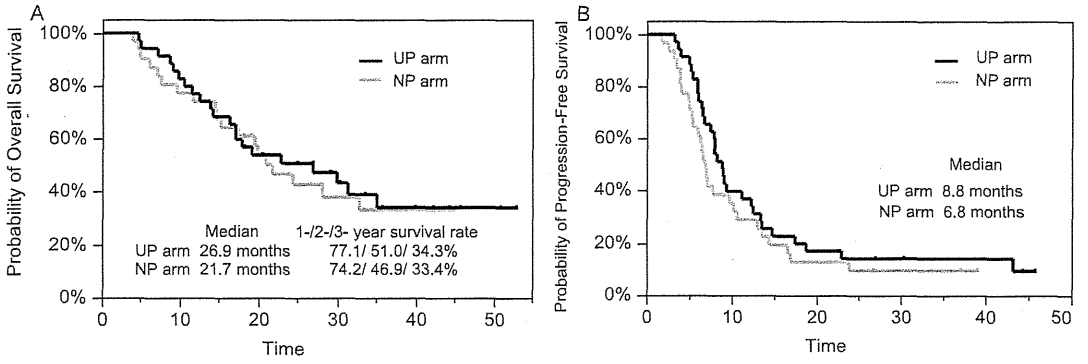

Fig. 2. (A) Overall survival and (B) progression free survival in each arm.

Table 4
Grade 3 or worse toxicity.

	UP arm (n=35)				NP arm (n=31)				P
	Grade		≥Grade 3		Grade		≥Grade 3		
	3	4	No.	(%)	3	4	No.	(%)	
Hematologic									
Leucopenia	7	1	8	22.9	14	5	19	61.3	0.0024
Neutropenia	3	4	7	20.0	8	10	18	58.1	0.0022
Anemia	2	0	2	5.7	2	0	2	6.5	1.000
Thrombocytopenia	1	0	1	2.9	1	0	1	3.2	1.000
Febrile neutropenia	0	0	0	0	4	0	4	12.9	0.0437
Non-hematologic									
Anorexia	5	0	5	14.3	3	0	3	9.7	0.7132
Nausea/vomiting	4	0	4	11.4	2	0	2	6.5	0.6762
Diarrhea	2	0	2	5.7	0	0	0	0	0.4942
Infection	0	0	0	0	2	0	2	6.5	0.2168
Pneumonitis	2	0	2	5.7	0	2[a]	2	6.5	1.000

[a] Both patients died of radiation pneumonitis.

might rise to a less toxic new standard regimen in comparison with the third-generation regimen for locally advanced NSCLC.

S-1 is a novel oral fluoropyrimidine agent designed for enhancing anticancer activity and reducing gastrointestinal toxicity. Indeed, it showed potent activity not only as a single agent but also in combination with CDDP for metastatic NSCLC [24,25]. In addition S-1 plus CDDP with concurrent TRT in a phase II study yielded high response rates, good survival data, and only mild toxicities [26,27]. Therefore oral fluoropyrimidines such as UFT and S-1 hereafter may play an important role in terms of concurrent chemoradiotherapy for locally advanced NSCLC.

As a limitation of this study, the radiation technique was old-fashioned. At the start of this multi-institutional study, 3D treatment planning system using CT was not available at all institutions. Therefore, both 2D and 3D treatment planning systems were allowed in the protocol. Because 3D dose constraints for both planning target volume and normal-risk organs were not determined by modern radiation technologies, the quality of radiotherapy in this study might have be rather lowered.

In conclusion, combined with concurrent TRT, UP achieved more favorable efficacy and safety than NP, suggesting it to be a promising candidate as a standard regimen with concurrent TRT for locally advanced NSCLC. Further evaluation of this regimen is warranted in a phase III setting in comparison with platinum-based third-generation chemotherapy with concurrent TRT.

Conflict of interest statement

None declared.

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REVIEW

Impact of EGFR Inhibitor in Non–Small Cell Lung Cancer on Progression-Free and Overall Survival: A Meta-Analysis

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Background The epidermal growth factor receptor (EGFR) signaling pathway is crucial for regulating tumorigenesis and cell survival and may be important in the development and progression of non–small cell lung cancer (NSCLC). We examined the impact of EGFR–tyrosine kinase inhibitors (TKIs) on progression-free survival (PFS) and overall survival (OS) in advanced NSCLC patients with and without EGFR mutations.

Methods Randomized trials that compared EGFR-TKIs monotherapy or combination EGFR-TKIs-chemotherapy with chemotherapy or placebo were included. We used published hazard ratios (HRs), if available, or derived treatment estimates from other survival data. Pooled estimates of treatment efficacy of EGFR-TKIs for the EGFR mutation–positive ($EGFRmut^+$) and EGFR mutation–negative ($EGFRmut^-$) subgroups were calculated with the fixed-effects inverse variance weighted method. All statistical tests were two-sided.

Results We included 23 eligible trials (13 front-line, 7 second-line, 3 maintenance; n = 14570). EGFR mutation status was known in 31% of patients. EGFR-TKIs treatment prolonged PFS in $EGFRmut^+$ patients, and EGFR mutation was predictive of PFS in all settings: The front-line hazard ratio for $EGFRmut^+$ was 0.43 (95% confidence interval [CI] = 0.38 to 0.49; $P < .001$), and the front-line hazard ratio for $EGFRmut^-$ was 1.06 (95% CI = 0.94 to 1.19; $P = .35$; $P_{interaction} < .001$). The second-line hazard ratio for $EGFRmut^+$ was 0.34 (95% CI = 0.20 to 0.60; $P < .001$), and the second-line hazard ratio for $EGFRmut^-$ was 1.23 (95% CI = 1.05 to 1.46; $P = .01$; $P_{interaction} < .001$). The maintenance hazard ratio for $EGFRmut^+$ was 0.15 (95% CI = 0.08 to 0.27; $P < .001$), and the maintenance hazard ratio for $EGFRmut^-$ was 0.81 (95% CI = 0.68 to 0.97; $P = .02$; $P_{interaction} < .001$). EGFR-TKIs treatment had no impact on OS for $EGFRmut^+$ and $EGFRmut^-$ patients.

Conclusions EGFR-TKIs therapy statistically significantly delays disease progression in $EGFRmut^+$ patients but has no demonstrable impact on OS. EGFR mutation is a predictive biomarker of PFS benefit with EGFR-TKIs treatment in all settings. These findings support EGFR mutation assessment before initiation of treatment. EGFR-TKIs should be considered as front-line therapy in $EGFRmut^+$ advanced NSCLC patients.

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The greatest changes in the treatment of advanced non–small cell lung cancer (NSCLC) have been novel molecular-targeted agents and the concomitant ability to personalize treatment. Controversy continues in many areas related to the incorporation of these changes into clinical medicine. How should such therapy be selected for individual patients? Is molecular testing required or is the use of demographic factors (such as histologic NSCLC type, sex, smoking history) sufficient for personalizing therapy? Questions remain concerning whether therapy with chemotherapy or with agents affecting the epidermal growth factor receptor (EGFR) influence progression-free survival (PFS) and/or overall survival (OS) in patients who do or do not harbor known mutations associated with EGFR. Is PFS a good surrogate for OS, or is PFS a useful endpoint on its own? Data directed at answering these controversies can guide oncologists in interpreting trials and in making more appropriate diagnostic and therapeutic choices for hundreds of thousands of patients each year.

The objective of this meta-analysis is to estimate better the treatment effect of EGFR–tyrosine kinase inhibitors (TKIs) on PFS and OS while examining for heterogeneity of treatment effects between groups of patients with and without EGFR mutations. The EGFR signaling pathway is crucial for regulating tumorigenesis and cell survival and may be overexpressed in the development and progression of NSCLC (1–3). Patients with activating somatic mutations in the region of the EGFR gene that encodes the tyrosine kinase domain are highly responsive to EGFR-TKIs (4–6). Previously published meta-analyses have been limited by studying the minority of patients with NSCLC—that is, the influence of EGFR-TKIs only in the population of patients harboring EGFR

mutations and predominantly in the front-line treatment setting (7–9). These meta-analyses have not demonstrated an OS advantage for patients with EGFR mutation treated with EGFR-TKIs. This analysis uses all trial data available to date and examines the effect of EGFR-TKIs treatment in major clinical settings—front-line, maintenance, and second-line or subsequent therapies. Additionally, the impact of EGFR-TKIs-chemotherapy combinations compared with EGFR-TKIs monotherapy is also explored. It is now recognized that as with EGFR mutations, other genetic alterations [such as EML-ALK abnormalities (10) and ROS-1 mutations (11)] are also more common in nonsmokers with adenocarcinoma, but these latter groups do not benefit from EGFR-TKIs–directed therapy. Such findings highlight the need for more specific molecular testing of patients and the need to include the most recent data from meta-analyses to understand better the treatment effects.

Individual trials and meta-analyses have clearly indicated that PFS and response rates are improved in patients with EGFR mutation who are treated with EGFR-TKIs, when compared with chemotherapy (7–9). The impact on OS is less clear, especially in patients treated beyond first-line therapy. Two separate trials have indicated that erlotinib is effective as second-line (12) and maintenance (13) therapy, with no statistically significant difference in treatment effect between those with EGFR mutation and wild-type tumors. However, a recent trial reported that chemotherapy was superior over erlotinib as second-line treatment for patients without EGFR mutations in exon 19 or 21 (14). Clearly, newer and larger meta-analyses are required to resolve these differences. Definitive analyses can provide stronger rationales for the choice of a specific therapy and can result in better utilization of health-care resources with these costly agents. For these reasons, we conducted this meta-analysis, which included the largest number of studies and patients to date with known EGFR mutation status and tested both PFS and OS as outcomes.

Methods

Study Eligibility and Identification

All randomized trials of EGFR-TKIs monotherapy vs any chemotherapy, EGFR-TKIs and chemotherapy vs the same chemotherapy alone, and EGFR-TKIs monotherapy vs placebo or best supportive care were eligible for inclusion.

Trials were identified from previous meta-analyses (7–9), and a search of Medline, Embase, CancerLit, and the Cochrane Central Register of Controlled Trials (CENTRAL) using the following terms: lung neoplasms, non–small cell lung cancer, gefitinib, erlotinib, EGFR, meta-analysis, systemic review, randomized, and clinical trials. Database searches were restricted to articles published in the English language between January 1, 2004, and June 6, 2012. Trials that enrolled patients with prior EGFR-TKIs treatment were excluded. Abstracts from conference proceedings of the American Society of Clinical Oncology, the European Society for Medical Oncology, and the World Lung Cancer Conference were searched to identify unpublished studies. Individual study sponsors (Hoffmann-La Roche and AstraZeneca) were contacted for relevant presentation slides and posters from these conferences when they were inaccessible from the websites. Individual investigators were also contacted if essential information relevant to this meta-analysis was unavailable from these sources.

Data Extraction

Information recorded from each trial included study name, year of publication or conference presentation, study design, line of treatment, and clinicopathological and demographic data. Mutational analysis data were also extracted, and the different methods of EGFR mutation assessment were recorded. We classified patients as EGFR mutation–positive ($EGFRmut^+$) based on the presence of a mutation as detected using molecular assessment tools such as Sanger sequencing, polymerase chain reaction clamp, and amplification refractory mutation system. Patients were classified as EGFR mutation–negative ($EGFRmut^-$) if no mutation was detected. We did not classify patients' EGFR mutation status based on immunohistochemistry and fluorescent in situ hybridization for EGFR gene copy numbers. Most trials analyzed exons 19 and 21 for EGFR mutations, and some trials also included exons 18 and 20.

Data were extracted independently by three authors (J. C.-H. Yang, C. K. Lee, and C. Brown), and discrepancies were resolved by consensus including a fourth author (V. Gebski).

Statistical Analyses

We extracted the hazard ratios (HRs) and the associated 95% confidence intervals (CIs) for PFS and OS outcomes to assess treatment efficacy within the $EGFRmut^+$ and $EGFRmut^-$ subgroups. Where available, we included the most updated OS data. If hazard ratios and confidence intervals were not reported, these were estimated where possible using the methods of Parmar (15).

Pooled estimates of the treatment efficacy of EGFR-TKIs for the $EGFRmut^+$ and $EGFRmut^-$ subgroups were calculated by using the fixed-effects inverse variance weighted method. We performed indirect comparisons to quantify the benefits of adding chemotherapy to EGFR-TKIs over EGFR-TKIs alone in both subgroups.

A sensitivity analysis was also conducted to examine the impact of the overall results from this study by limiting the analyses on front-line trials that were known to have determined EGFR mutation based on exons 19 and 21 only.

We used the $\chi 2$ Cochran Q test to detect for heterogeneity across the different studies and between subgroups defined by EGFR mutation status, study setting, and study design. The nominal level of significance was set at 5%. All 95% confidence intervals were two-sided.

Cochrane Review Manager (version 5, Cochrane Collaboration, Copenhagen, Denmark, http://ims.cochrane.org/home) was used for all analyses.

Results

The search strategy identified 40 studies, of which 23 (12–14,16–44) were eligible for inclusion in this meta-analysis (Figure 1). Trial data were obtained from published manuscripts and conference abstracts for 19 trials, and additional data on treatment efficacy by $EGFRmut^+$ and $EGFRmut^-$ subgroups were obtained directly from study investigators for four studies [ISEL (41), V-15-32 (31), TOPICAL (43), and IFCT-GFPC 0502 (32, 44)]. Treatment estimates for the TALENT study (37) were calculated on the basis of data extracted from presented survival curves. The hazard ratios for OS for ISEL (41), IFCT-GFPC 0502 (32,44), and V-15–32 (31) were estimated on the basis of the observed number of deaths. In all other studies, hazard ratios and associated variances were obtained directly from trial reports.

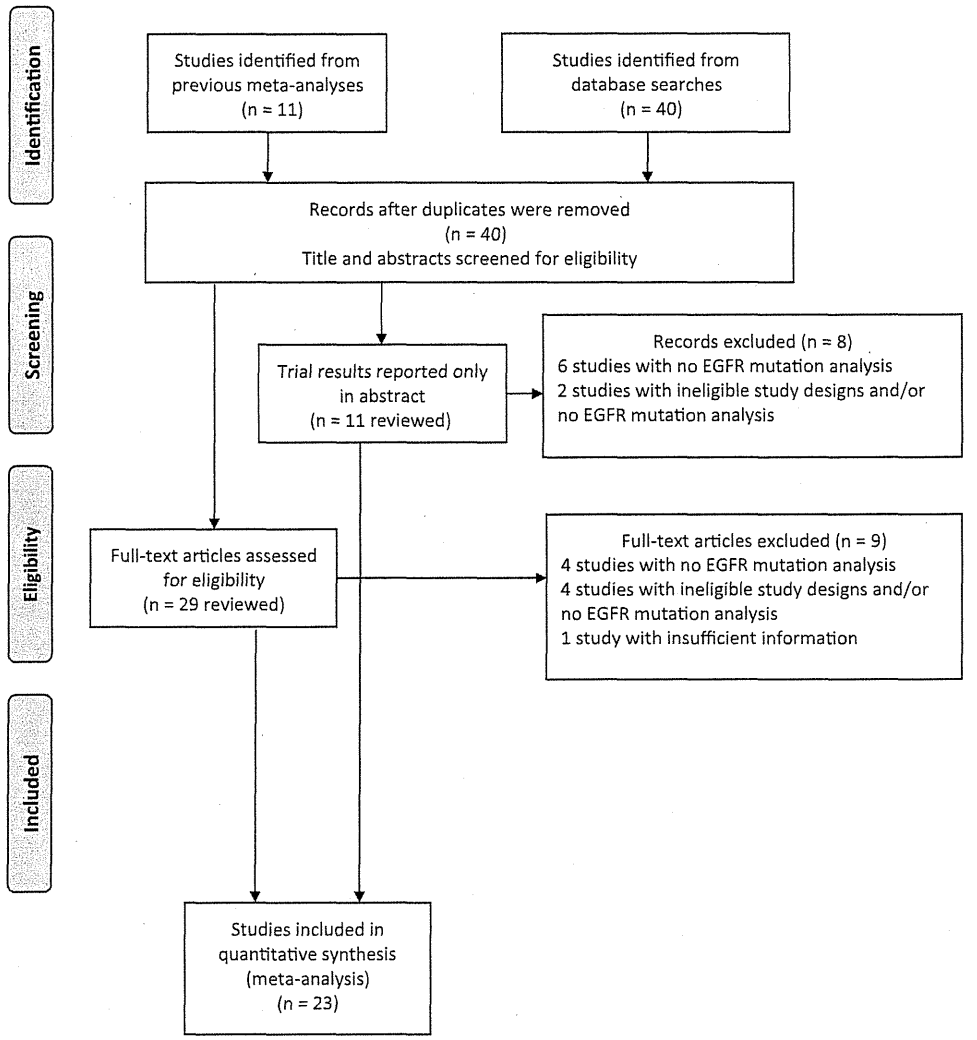

Figure 1. Flow diagram showing inclusion and exclusion of studies. EGFR = epidermal growth factor receptor.

A total of 14570 patients participated in these 23 trials. EGFR mutation status, as determined by mutation analysis only, was known for at least 31% (n = 4473) of trial patients. [In the TALENT study (37), the treatment comparisons for the subgroups were reported, but the number of patients in each subgroup was unknown.] Clinicopathological and demographic characteristics of patients enrolled in these studies are summarized in Table 1.

Trials investigated EGFR-TKIs for front-line therapy in treatment-naive patients (n = 13 trials), second-line or subsequent treatment after failure of chemotherapy (n = 7 trials), and maintenance treatment in patients with nonprogressive disease after front-line chemotherapy (n = 3 trials). Among the 13 front-line studies, eight compared EGFR-TKIs as monotherapy vs chemotherapy (16–21,23,27,33–35,38), four compared EGFR-TKIs with chemotherapy vs chemotherapy alone (22,24–26,37,45), and one was a placebo-controlled trial (36,43). Among the seven second-line and subsequent treatment trials, five compared EGFR-TKIs as monotherapy vs chemotherapy (12,14,28,29,31,42), and two were placebo-controlled studies (39–41). All three maintenance studies had a placebo arm (13,30,32,44).

Benefit of EGFR-TKIs on PFS in Different Settings

Data on PFS were available from 21 trials except ISEL (41) and BR21 (39). The treatment effect of EGFR-TKIs in different settings is shown in Figure 2. The test of interaction between treatment and EGFR mutation status was statistically significant (front-line setting: $P < .001$; second-line or subsequent treatment: $P < .001$).

In EGFRmut$^+$ patients, EGFR-TKIs treatment was associated with a lower risk of disease progression in the front-line setting (HR = 0.43; 95% CI = 0.38 to 0.49; $P < .001$) and second-line or subsequent treatment (HR = 0.34; 95% CI = 0.20 to 0.60; $P < .001$).

In EGFRmut$^-$ patients, EGFR-TKIs did not show a treatment advantage in the front-line setting or beyond. There was no statistically significant difference between EGFR-TKIs and chemotherapy in reducing the risk of disease progression in front-line therapy (HR = 1.06; 95% CI = 0.94 to 1.19; $P = .35$). EGFR-TKIs treatment was statistically significantly inferior to chemotherapy in second-line or subsequent therapy (HR = 1.23; 95% CI = 1.05 to 1.46; $P = .01$).

Maintenance therapy with EGFR-TKIs compared with placebo was effective in reducing the risk of disease progression in EGFRmut$^+$

Table 1. Demographic characteristics of patients*

Study name (year) (reference)	Treatment comparison	EGFR mutation assessment method	No. of EGFR+ patients (%)	No. of EGFR- patients (%)	No. of EGFR unknown patients (%)	Age, y, median	Asian, %	Males, %	Present/ former smokers, %	Adeno-carcinoma, %
Front-line treatment										
INTACT 1 (2004) (24,43)	Gefitinib + CisG vs CisG	Direct sequencing	32 (2)	280 (13)	1818 (85)	60	6	74	NK	46
INTACT 2 (2004) (25,43)	Gefitnib + CP vs CP					62	NK	60	NK	55
TRIBUTE (2005) (22)	Erlotinib + CP vs CP	Direct sequencing	29 (3)	198 (18)	851 (79)	63	3	61	89	61
TALENT (2007) (26,37)	Erlotinib + CisG vs CisG	NK	NK	NK	NK	61	4	77	NK	38
IPASS (2009) (19,20)	Gefitinib vs CP	ARMS	261 (21)	176 (15)	780 (64)	57	100	21	6	96
NEJ002 (2010) (17,38)	Gefitinib vs CP	PCR clamp	228 (100)	0	0	63‡	100	36	38	94
GTOWG† (2010) (27)	Erlotinib vs CV	Direct sequencing	10 (4)	75 (26)	199 (70)	76	NK	68	83	50
TOPICAL (2010) (36,43)	Erlotinib vs placebo	SequenomOncoCarta Panel	28 (4)	362 (54)	280 (42)	77	2	61	95	38
WJTOG3405* (2010) (21,33)	Gefitinib vs CisD	Direct sequencing, PCR clamp	172 (100)	0	0	64	100	31	31	97
OPTIMAL* (2011) (16,35)	Erlotinib vs CG	Direct sequencing	154 (100)	0	0	58	100	41	29	87
First-SIGNAL (2012) (23)	Gefitinib vs CisG	Direct sequencing	43 (14)	54 (17)	212 (69)	57	100	11	NK	NK
EURTAC* (2012) (18)	Erlotinib vs platinum-G or platinum-D	Direct sequencing	173 (100)	0	0	65	0	27	31	92
LUX Lung 3† (2012) (34)	Afatinib vs CisPem	TheraScreen EGFR29	345 (100)	0	0	61	72	35	32	100
Maintenance therapy										
IFCT-GFPC 0502* (2010) (32)	Erlotinib or G vs placebo	NK	8 (3)	106 (34)	196 (63)	58	0	73	90	65
SATURN (2010) (13)	Erlotinib vs placebo	Direct sequencing	49 (6)	388 (44)	452 (50)	60	15	74	83	45
INFORM (2011) (30)	Gefitinib vs placebo	NK	30 (10)	49 (17)	217 (73)	55	100	59	46	71
Second-line/subsequent treatment										
ISEL (2005) (41)	Gefitinib vs placebo	Direct sequencing, ARMS	26 (2)	189 (11)	1477 (87)	62	20	67	78	45
BR21 (2005) (39,40)	Erlotinib vs placebo	Direct sequencing, ARMS	34 (5)	170 (23)	527 (72)	61	13	65	75	50
INTEREST (2008) (28,29)	Gefitinib vs D	Direct sequencing	44 (3)	253 (17)	1169 (80)	61	22	65	80	54
V-15–32 (2008) (31)	Gefitinib vs D	Direct sequencing	31 (6)	26 (6)	432 (88)	NK	100	62	68	78
TITAN (2012) (12)	Erlotinib vs pemetrexed or D	Direct sequencing	11 (3)	149 (35)	264 (62)	59	13	76	83	50
TAILOR† (2012) (14)	Erlotinib vs D	Direct sequencing	0	219 (100)	0	67	0	68	77	69
KCSG-LU08-01 (2012) (42)	Gefitinib vs Pem	Direct sequencing	33 (24)	38 (28)	64 (48)	61	100	15	0	100

* ARMS = amplifcation refractory mutation system; CG = carboplatin-gemcitabine; CisD = cisplatin-docetaxel; CisG = cisplatin-gemcitabine; CisPem = cisplatin-pemetrexed; CP = carboplatin-paclitaxel; CV = carboplatin-venorelbine; D = docetaxel; EGFR+ = presence of epidermal growth factor receptor mutation; EGFR- = absence of epidermal growth factor receptor mutation; G = gemcitabine; NK = not known; PCR = polymerase chain reaction; PEM = pemetrexed.

* EGFR mutation based on exon 19 and exon 21 only.

† Trials reported in abstract format.

‡ Median age not available; mean age calculated instead.

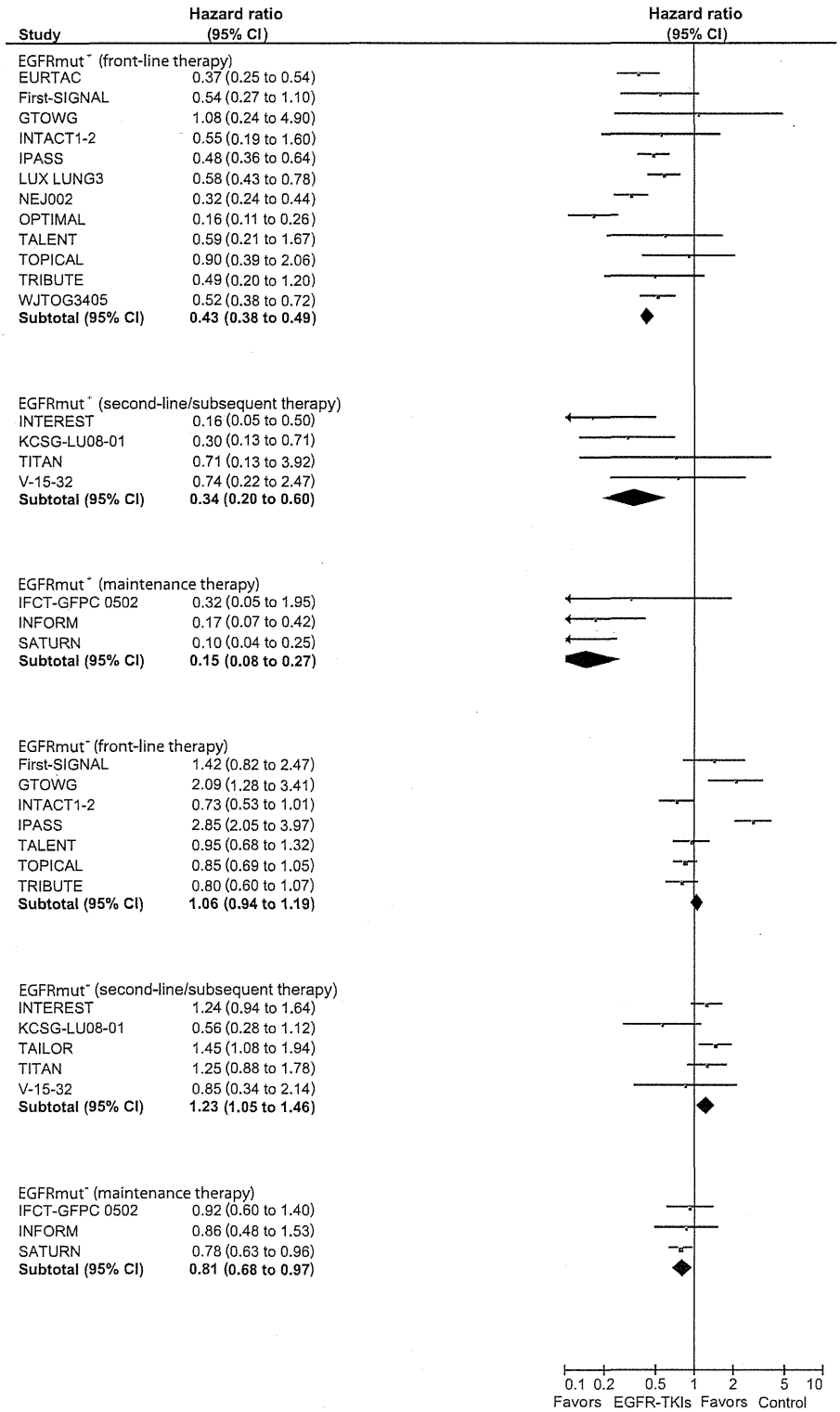

Figure 2. Forest plot of hazard ratios comparing progression-free survival in subgroups of epidermal growth factor receptor (EGFR) mutation–positive (EGFRmut+) and EGFR mutation–negative (EGFRmut-) patients who received EGFR–tyrosine kinase inhibitors (TKIs) vs control. Hazard ratios for each trial are represented by the **squares**, and the **horizontal line** crossing the square represents the 95% confidence interval (CI). The **diamonds** represent the estimated overall effect based on the meta-analysis fixed effect of the trials. All statistical tests were two-sided.

and EGFRmut$^-$ subgroups (EGFRmut$^+$: HR = 0.15, 95% CI = 0.08 to 0.27, P < .001; EGFRmut$^-$: HR = 0.81, 95% CI = 0.68 to 0.97, P = .02). The test of interaction between treatment and EGFR mutation status was statistically significant (P < .001).

Effect of EGFR-TKIs Combined With Chemotherapy on PFS

Data were available for four trials [INTACT 1 and 2 (45), TRIBUTE (22) and TALENT (37)] that combined EGFR-TKIs with chemotherapy. Combination EGFR-TKIs and chemotherapy compared with chemotherapy alone was effective in reducing the risk of disease progression in both subgroups (EGFRmut$^+$: HR = 0.54, 95% CI = 0.30 to 0.95, P = .04; EGFRmut$^-$: HR = 0.82, 95% CI = 0.68 to 0.98, P = .03; treatment-by-EGFR mutation status interaction: P = .17) (Figure 3). When EGFR-TKIs monotherapy was compared with chemotherapy, EGFR-TKIs treatment was associated with a reduced risk of disease progression in the EGFRmut$^+$ subgroup (HR = 0.42; 95% CI = 0.37 to 0.48; P < .001) but an increased risk in the EGFRmut$^-$ subgroup (HR = 1.56; 95% CI = 1.36 to 1.80; P < .001).

Within the EGFRmut$^+$ subgroup, an indirect comparison of data available from these trials indicates EGFR-TKIs treatment in combination with chemotherapy was not more effective than EGFR-TKIs alone in reducing the risk of disease progression (HR = 1.42; 95% CI = 0.80 to 2.53; P = .23). By contrast, within the EGFRmut$^-$ subgroup, EGFR-TKIs treatment in combination with chemotherapy was more effective in reducing the risk of disease progression than EGFR-TKIs alone (HR = 0.51; 95% CI = 0.43 to 0.62; P < .001).

Effect of EGFR-TKIs on OS in Different Settings

Data on OS were available from 19 trials except Lux Lung 3 (34), TAILOR (14), KCSG-LU08-01 (42), and INFORM (30). Subgroup analyses by treatment setting are summarized in Figure 4. The test interaction for treatment and EGFR mutation status was not statistically significant (front-line setting: P = .91; second-line or subsequent therapy: P = .37). For EGFRmut$^+$ patients, there was no treatment advantage of EGFR-TKIs in the front-line setting (HR = 1.01; 95% CI = 0.87 to 1.18; P = .86) or for second-line or subsequent therapy (HR = 0.74; 95% CI = 0.45 to 1.19; P = .21) in the risk of death. Similar results were observed in EGFRmut$^-$ patients.

Only two studies [SATURN (13) and IFCT-GFPC 0502 (32,44)] reported OS in the maintenance setting. There was no clear benefit of treatment with EGFR-TKIs over placebo in either EGFRmut$^+$ patients (HR = 0.78; 95% CI = 0.33 to 1.84; P = .57) or EGFRmut$^-$ patients (HR = 0.84; 95% CI = 0.69 to 1.04; P = .10). The test for interaction between treatment and EGFR mutation status was not statistically significant (P = .87).

Publication Bias

In this meta-analysis, the overall treatment effect was not statistically significant for the OS outcome. Any potential publication bias through the exclusion of non–statistically significant studies would therefore not have influenced these results.

Sensitivity Analysis

EGFR mutation, based on exons 19 and 21 only, was known to have been examined in three trials in a front-line setting (Table 1). One trial (34) provided the treatment estimate for PFS limited to patients with exons 19 and 21 only. In the front-line setting, similar qualitative results were obtained when the analyses were limited to only these four trials on PFS and OS outcomes for the EGFRmut$^+$ subgroup (Supplementary Figures 1 and 2, available online).

Discussion

This study extends the analysis beyond prior publications of the most clinically important molecular factor relevant to the treatment

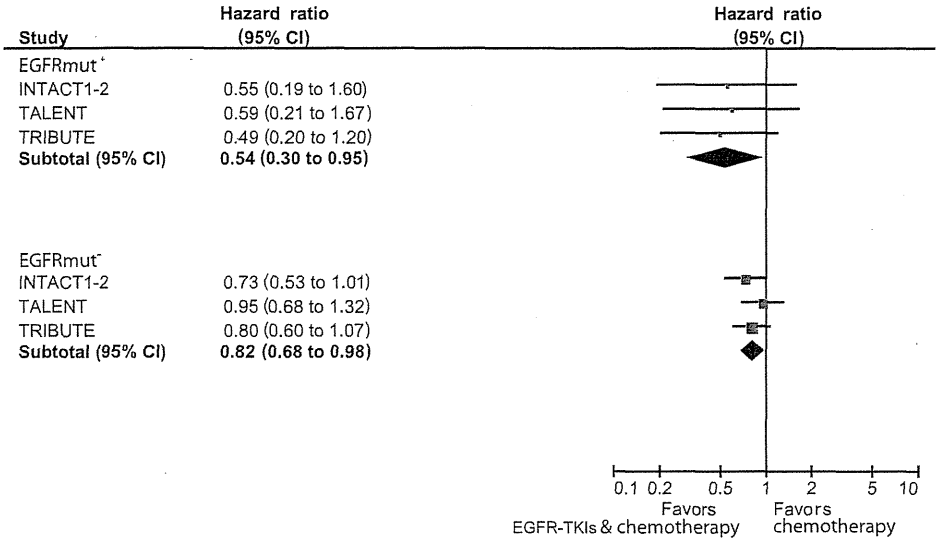

Figure 3. Forest plot of hazard ratios comparing progression-free survival in subgroups of epidermal growth factor receptor (EGFR) mutation–positive (EGFRmut$^+$) and EGFR mutation–negative (EGFRmut$^-$) patients who received EGFR–tyrosine kinase inhibitors (TKIs) and chemotherapy vs chemotherapy. Hazard ratios for each trial are represented by the **squares**, and the **horizontal line** crossing the square represents the 95% confidence interval (CI). The **diamonds** represent the estimated overall effect based on the meta-analysis fixed effect of the trials. All statistical tests were two-sided.

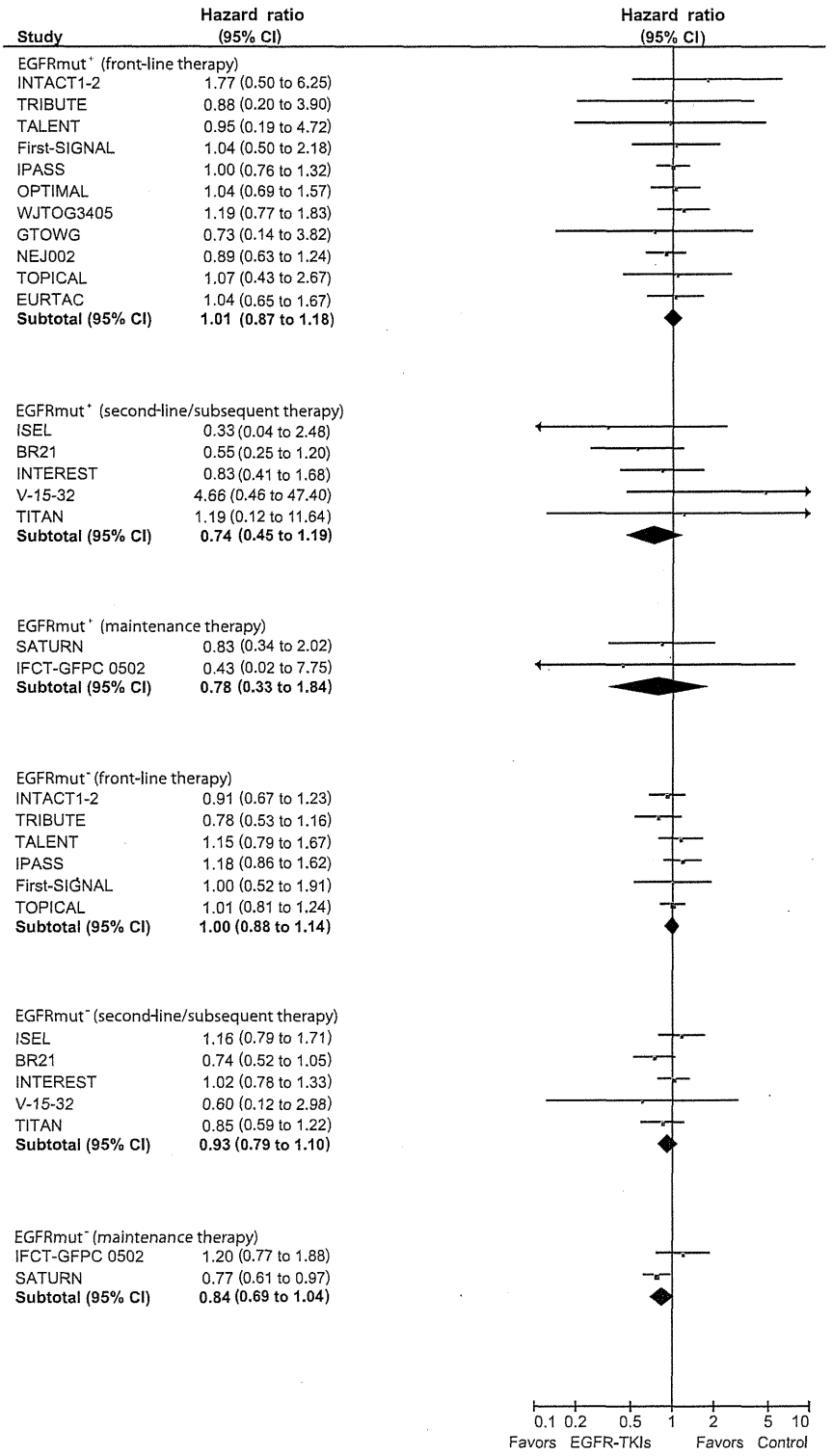

Figure 4. Forest plot of hazard ratios comparing overall survival in subgroups of epidermal growth factor receptor (EGFR) mutation–positive (EGFRmut+) and EGFR mutation–negative (EGFRmut-) patients who received EGFR–tyrosine kinase inhibitors (TKIs) vs control. Hazard ratios for each trial are represented by the **squares**, and the **horizontal line** crossing the square represents the 95% confidence interval (CI). The **diamonds** represent the estimated overall effect based on the meta-analysis fixed effect of the trials. All statistical tests were two-sided.

of NSCLC. Increased confidence in the findings is evident through the incorporation of results from 23 trials in nearly 15 000 patients with more than 4000 having molecular analysis. Additionally, this study approached issues not addressed in prior meta-analyses. As such, results from this study have implications for treatment and for study interpretation and design.

This meta-analysis summarizes the best available evidence to guide the use of EGFR-TKIs in patients with advanced NSCLC. EGFR-TKIs treatment is associated with 57% and 66% reduction in the risk of disease progression in EGFRmut$^+$ patients in front-line and second-line settings, respectively, but with no benefit in EGFRmut$^-$ patients (Figure 2). This study also demonstrates that EGFR mutation is an important predictive biomarker of TKIs treatment benefit in terms of PFS for all settings: front-line, maintenance, and second-line or subsequent therapy. This study demonstrates for the first time that the magnitude of effect on PFS for EGFRmut$^+$ patients is similar in patients receiving EGFR-TKIs in either the first- or second-line setting (HR = 0.43 and 0.34, respectively).

Even with mutational analyses in more than 4000 patients and with a large PFS benefit, this meta-analysis does not demonstrate OS advantage with EGFR-TKIs. Regardless of EGFR mutation status, the overall treatment effects on OS were similar. The frequently suggested reason for this lack of OS effect is the confounding effect of postprogression therapy between the randomization arms. None of the front-line trials prohibited patients from crossing over to the other treatment arm, and crossover was increasingly frequent over the decade during which these trials were conducted. For example, the NEJ002 trial randomly assigned patients to receive either gefitinib or chemotherapy. Not only did most patients receive subsequent treatment, but 94.6% of patients in the chemotherapy arm were reported to have received second-line gefitinib on disease progression (17). A recent systematic review of chemotherapy trials also indicated that PFS advantage is unlikely to be associated with an OS advantage with increasing impact of salvage therapy and that the prolongation of survival postprogression might limit the role of OS for assessing true efficacy derived from front-line therapy (46). Moreover, analysis of a recent trial indicated that compared with EGFRmut$^-$ patients, twice as many EGFRmut$^+$ patients responded to chemotherapy (28). Crossover effects, lack of blinding in experimental arms, and other factors that have been previously discussed can make PFS a difficult surrogate for OS (47–49). Ongoing work is still required to demonstrate the impact of other clinically meaningful benefits of EGFR-TKIs beyond survival and PFS for these patients.

Controversy continues regarding the role of the addition of EGFR-TKIs in patients receiving chemotherapy. For this reason, we analyzed this issue in four large, published, prospective, randomized trials in front-line treatment [INTACT 1 and 2 (45), TALENT (37), and TRIBUTE (22)]. Pooled results from these four front-line trials showed that combining EGFR-TKIs with chemotherapy over chemotherapy alone statistically significantly delayed disease progression in both the EGFRmut$^+$ and EGFRmut$^-$ subgroups. Preclinical studies (50,51) have demonstrated a synergistic effect of combining EGFR-TKIs with chemotherapy. However, indirect comparison of trial arms suggests that combined EGFR-TKIs treatment and chemotherapy is not more effective than EGFR-TKIs alone in reducing the risk of disease progression in EGFRmut$^+$ patients (HR = 1.42; 95% CI = 0.80 to 2.53; P = .23). A lack of additional benefit was confirmed in a prospective phase II trial (52) in which erlotinib monotherapy was compared with erlotinib chemotherapy combination in the EGFRmut$^+$ subgroup (median PFS 14.1 vs 17.2 months).

This meta-analysis provides information to define better the relative effectiveness of EGFR-TKIs for EGFRmut$^-$ patients. In front-line therapy, there was a non–statistically significant difference between EGFR-TKIs and control in reducing the risk of disease progression (pooled HR = 1.06; P = .35). This finding is consistent with previous in vitro studies that demonstrated a lack of sensitivity of wild-type EGFRmut$^-$ receptor lung tumor to EGFR-TKIs treatment (4–6). Although a small benefit of EGFR-TKIs over placebo in the EGFRmut$^-$ subgroup has been demonstrated in three maintenance studies [SATURN (13), INFORM (30), and IFCT-GFPC 0502 (32,44)] (pooled HR = 0.81; 95% CI = 0.68 to 0.97; P = .02), it must be realized that this benefit is markedly and both clinically and statistically significantly greater in EGFRmut$^+$ subgroups (pooled HR = 0.15; 95% CI = 0.08 to 0.27; P < .001), and the test of interaction between EGFR mutation status and treatment is highly statistically significant (P < .001).

This meta-analysis also examined the role of EGFR mutation in selecting patients for second-line or subsequent treatment. A 2012 editorial has illustrated the debate in this area (53). Although trials have differed in their results, one study (TAILOR) reported that chemotherapy was statistically significantly superior over erlotinib in terms of tumor response and PFS (OS results are not yet available) in patients without EGFR mutations in exon 19 or 21 undergoing second-line treatment, but the data remain premature and only available as a conference presentation (14). In the current meta-analysis, pooled results from trials of second-line and subsequent therapies demonstrated that treatment with EGFR-TKIs treatment, compared with chemotherapy, was associated with a 66% reduction in the risk of disease progression in the EGFRmut$^+$ subgroup (Figure 2). In contrast, EGFR-TKIs treatment, compared with chemotherapy, was 23% inferior (Figure 2) in delaying disease progression (but not OS) in EGFRmut$^-$ patients with good performance status who were suitable to receive chemotherapy. The test of interaction between EGFR mutation status and second-line or subsequent treatment was statistically significant (P < .001), suggesting that EGFR mutation is still an important treatment effect modifier and should be used to guide treatment decisions in this setting. Interestingly, updated results from the TOPICAL trial demonstrated that rash during the first cycle predicted PFS benefits with erlotinib in the EGFRmut$^-$ subgroup (43).

This meta-analysis has several strengths. We performed a comprehensive review, reported the most up-to-date published data, and contacted individual investigators to obtain relevant unpublished data. By examining both the EGFRmut$^+$ and EGFRmut$^-$ subgroups, the value of EGFR mutation status as a treatment effect modifier can be adequately assessed. This meta-analysis also overcomes the problem of inadequate power of individual studies to compare subgroups. For example, only six studies (16,18,19,21,34,38) included in this review had EGFRmut$^+$ results for more than 50 patients. Reliable interpretation of independent treatment effects in most of the individual studies in this review is not possible because of small sample sizes. Altogether, more than 4000 patients with mutational

analysis were included in this study. A major strength of this current meta-analysis is that the pooled results allow examination of second-line and maintenance treatment as well as elucidation of the effect of adding EGFR-TKIs to chemotherapy.

There are also limitations that should be noted from this analysis. Firstly, we assumed that all EGFR-TKIs, including gefitinib, erlotinib, and afatinib, have equivalent therapeutic efficacy for both the EGFRmut$^+$ and EGFRmut$^-$ subgroups. Secondly, EGFR mutation status was only assessed in 31% of patients enrolled in eligible trials, with treatment efficacy estimated from small numbers of EGFRmut$^+$ patients identified in many of these trials (Table 1). The potential influence on the results of restricting our analyses to this subset of patients is unknown. We further obtained efficacy data in the subgroups with known EGFR mutation status through personal communication with investigators of four trials (31,32,41,43). Although these subgroup data have not been published, the primary trial outcomes of these studies have been peer reviewed. Although nearly 15 000 patients were included in the analysis, the fact that only a minority had reported mutational analysis limits the ability to address several issues. Sequencing was the most commonly used method to detect EGFR mutation, and it has poor sensitivity in detecting EGFR mutant alleles in DNA samples extracted from tumors (54). These DNA samples may contain both malignant and nonmalignant (from adjacent normal or tumor stroma) cells and hence may impact the outcome of this meta-analysis through misclassification of patients' EGFR mutation status. Moreover, mutation of EGFR exons 19 and 21 are sensitizing mutations predictive of PFS benefit with EGFR-TKIs, whereas de novo mutations in exon 20 might reduce the effectiveness of EGFR-TKIs (55–57). In this meta-analysis, patients classified as EGFRmut$^+$ in some trials included those with mutations in exon 20. However, when we restricted our analysis to studies that classified patients as EGFRmut$^+$ based on presence of EGFR exon 19 and exon 21 mutations, we observed similar quantitative results. In front-line therapy, information on crossover and postprogression therapies was often not available, so adjustments could not be made to account for the lack of OS benefit in EGFRmut$^+$ patients treated with EGFR-TKIs.

Many reports have confirmed that EGFR mutations are more commonly found in patients with adenocarcinoma and in patients with low- and never-smoking histories. These factors have led to the debate as to whether knowledge of such demographic factors, rather than use of molecular studies, would be sufficient for treatment. The current meta-analysis, which examines multiple treatment settings, demonstrates that EGFR mutation status should guide personalization of treatment. Additionally, recent findings have reported that these same demographic features are more common in other genetic differences [such as those associated with EML-ALK translocations (10) and ROS 1 mutations (11)] that are not beneficially affected by EGFR-TKIs and for which specific therapy is available. Determining mutational status can avoid side effects of either EGFR-TKIs or chemotherapy and can lead to rational decision making. In that only the minority of all patients with NSCLC will have EGFR or other treatment-altering mutations, and because nearly all lung cancer therapy is costly, molecular analysis is increasingly important from clinical, scientific, and economic perspectives.

In conclusion, based on this meta-analysis, treatment with EGFR-TKIs statistically significantly delays disease progression in EGFRmut$^+$ patients but has no demonstrable impact on OS. EGFR mutation is a predictive biomarker of benefit with EGFR-TKIs treatment in delaying disease progression in front-line, second-line, and subsequent therapy and in maintenance settings. These findings support assessment of EGFR mutation status before initiation of EGFR-TKIs treatment and indicate that EGFR-TKIs should be considered as front-line therapy in EGFRmut$^+$ patients with advanced NSCLC.

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RESEARCH PAPER

Macrophage stimulating protein promotes liver metastases of small cell lung cancer cells by affecting the organ microenvironment

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Abstract The organ microenvironment significantly affects the processes of cancer metastasis. Elucidating the molecular mechanisms of interaction between tumor cells and the organ microenvironment is crucial for the development of effective therapeutic strategies to eradicate cancer metastases. Macrophage stimulating protein (MSP), an activator of macrophages, regulates a pleiotropic array of effects, including proliferation, cellular motility, invasiveness, angiogenesis, and resistance to anoikis. However, the role of MSP in cancer metastasis is still largely unknown. In this study, the action of MSP on the production of metastases was determined in a multiple-organ metastasis model. The murine MSP gene was transfected into two human SCLC cell lines, SBC-5 and H1048, to establish transfectants secreting biologically active MSP. MSP gene transduction did not affect cell proliferation and motility in vitro. Intravenously inoculated MSP transfectants produced significantly larger numbers of liver metastases than parental cells or vector control clones, while there were no significant differences in bone or lung metastases among them. Immunohistochemical analyses of liver metastases revealed that tumor-associated microvessel density and tumor-infiltrating macrophages were significantly increased in lesions produced by MSP transfectants. MSP could stimulate the migration of murine macrophages and endothelial cells in vitro. Consequently, MSP may be one of the major determinants that affects the properties of tumor stroma and that produces a permissive microenvironment to promote cancer metastasis.

Keywords Small cell lung cancer · Liver metastasis · Macrophage stimulating protein · Organ microenvironment

Abbreviations

MSP	Macrophage stimulating protein
RON	The recepteur d'origine nantais
SCLC	Small cell lung cancer
NK	Natural killer
SCID	Severe combined immunodeficient
MEM	Minimum essential medium
FBS	Fetal bovine serum
GFP	Green fluorescent protein
RT-PCR	Reverse transcription-polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGFA	Hepatocyte growth factor activator
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
TAM	Tumor-associated macrophage

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Introduction

Lung cancer is one of the major causes of malignancy-related death worldwide, and its incidence is increasing in

many counties. The manifestation of distant metastases to multiple organs is the most devastating complication and the main reason for poor prognosis in lung cancer. Unfortunately, more than 70 % of lung cancer patients have advanced stage disease at the time of diagnosis [1]. Although intensive efforts have been made to treat lung cancer, the eradication of lung cancer metastases is still a very challenging issue. Therefore, elucidating the molecular and biological mechanisms of lung cancer metastasis is essential to develop more effective therapeutic strategies.

Macrophage stimulating protein (MSP) was originally identified as a serum protein that elicited macrophage chemotaxis and activation [2]. MSP is secreted as an inactive single-chain precursor, pro-MSP [3–5], which becomes active after proteolytic cleavage by proteases [5, 6]. The recepteur d'origine nantais (RON) is a receptor tyrosine kinase with significant homology to c-Met, a potent proto-oncogene [7], and the only known ligand for RON is MSP [5, 8]. Upon binding by MSP, RON is activated via autophosphorylation within its kinase catalytic domain, resulting in a pleiotropic array of effects, including cellular motility, adhesion, proliferation, tubular morphogenesis, and apoptosis [9, 10]. Recently, much attention has been paid to the role of MSP in tumor progression and metastasis [11–13]. In a spontaneous metastasis model of mouse mammary tumors, MSP promoted distant metastases to various organs, especially to bone [12]. MSP was also reported to be a candidate gene that may affect bone tropism of human small cell lung cancer (SCLC) because the expression of MSP was up-regulated in bone metastatic lesions [13]. However, the role of MSP on cancer metastasis is not fully elucidated.

The goal of this study was to determine whether MSP affect the properties of tumor stroma and host microenvironment to promote cancer metastasis. As we sought to generate a model in which mouse MSP would be secreted and act on mouse organ environment, mouse MSP gene was overexpressed in human SCLC cell lines. Then, we examined the effect of MSP overexpression on the production of experimental metastases in natural killer (NK)-cell depleted severe combined immunodeficient (SCID) mouse model.

Materials and methods

Cell lines

The human SCLC cell line, SBC-5 [14] was kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan). The human SCLC cell line, H1048 was purchased from American Type Culture Collection (Manassas, VA). Human adenocarcinoma cell line, ACC-LC319 was kindly provided by Dr. T. Takahashi (Nagoya University, Nagoya, Japan) and its highly metastatic subline, ACC-LC319/bone2 was established as described previously [15]. Human adenocarcinoma cell line, PC14PE6, a highly metastatic variant of PC14, was kindly provided by Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX), and human adenocarcinoma cell line, A549 was purchased from IBL Japan (Ibaraki, Japan). Human lung squamous cell carcinoma cell line, H226 was kindly provided by Dr. J. D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). All cells were maintained in Eagle's minimum essential medium (MEM) and RPMI1640 medium respectively, each supplemented with 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (50 μg/ml). The murine macrophage-like cell line, RAW264.7, and the murine endothelial cell line, MS1, were purchased from American Type Culture Collection (Manassas, VA). A potent retrovirus packaging cell line, PLAT-E [16], was kindly provided by Dr. K. Yasutomo (The University of Tokushima, Tokushima, Japan). RAW264.7, MS1, and PLAT-E cells were cultured in Dulbecco's modified MEM supplemented with 10 % FBS, penicillin (100 U/ml), and streptomycin (50 μg/ml). All cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

Reagents

Anti-mouse interleukin-2 receptor β-chain monoclonal antibody, TM-β1 (IgG2b), was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan) [17]. Anti-mouse MSP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

MSP gene transduction

Replication-defective mouse stem cell viruses, pMIG which expressed green fluorescent protein (GFP) only, and pMIG-MSP which expressed MSP and GFP, were kindly provided by Dr. A. L. Welm (University of Utah, Salt Lake City, UT). The PLAT-E packaging cell line was transfected with pMIG or pMIG-MSP using Fugene 6 (Roche, Indianapolis, IN). SBC-5 and H1048 cells were infected with each viral supernatant. Then, 8 μg/ml of polybrene (Sigma, St. Louis, MO) was added. The mixture was spun at 2,500 rpm for 60 min at room temperature and then incubated for 72 h. Infected GFP-positive cells were sorted by flow cytometry (JSAN cell sorter; Bay bioscience, Kobe, Japan). After culturing the initial sorted population, GFP-positive cells comprised more than 85 % of the whole cell population. One clone and a vector control clone were established in each cell line, which were designated as SBC-5-MSP, SBC-5-Vector and H1048-MSP, H1048-Vector, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of mouse MSP mRNA was determined by RT-PCR. Total cellular RNA and RNA from liver metastatic lesions were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. Total RNA was reversely transcribed using a TaqMan® RNA-to-CT™ 2-Step kit (Applied Biosystems, Foster City, CA). The primers of mouse MSP and β-actin were as follows: MSP: 5′-GCT ACA CCA CAG ACC CGA AT-3′ and 5′-GGT ATT GGT TGT GCC TCG AT-3′; β-actin: 5′-AAG AGA GGC ATC CTC ACC CT-3′ and 5′-TAC ATG GCT GGG GTG TTG AA-3′. PCR was performed using Ampli Taq Gold (Applied Biosystems, Foster City, CA). Bands were visualized by ethidium bromide staining. PCR amplification of cDNA was performed under the following conditions: 30 cycles, 30 s at 94 °C; 30 s at 58 °C; 30 s at 72 °C. Before the first cycle, a denaturation step for 2 min at 94 °C was included, and after 30 cycles, the extension was prolonged for 7 min at 72 °C [18]. PCR products were analyzed by 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining with UV light.

Quantitative reverse transcription-PCR

Quantitative real-time RT-PCR for mouse cell lines and tissues were performed using SYBER Premix EX Taq system (TAKARA) and Applied Biosystems StemOnePlus (ABI). Amplified signals were confirmed to be single band by gel electrophoresis and were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data was analyzed using StepOneSoftware (ABI). The PCR primer sequences used are as follows : mouse GAPDH, 5′-CAA CTA CAT GGT CTA CAT GTTC-3′ (forward) and 5′-CGC CAG TAG ACT CCA CGAC-3′ (reverse); mouse hepatocyte growth factor activator (HGFA), 5′-TGA GGG ACC CCA AAG TGA GA-3′ (forward) and 5′-GCA CTT CCC TCA GAG GTA CA-3′ (reverse); mouse MSP, 5′-AGT TAA GGA ACC TGT TAC AC-3′ (forward) and 5′-ACC ATG GCT GCT CAT GTT GT-3′ (reverse); mouse RON, 5′-ATT GAA GAG GGT GTC GAA TA-3′ (forward) and 5′-TCA AAG GGA AGT AGT GGC AA-3′ (reverse). The expression of human RON and human β-actin were measured by quantitative real-time RT-PCR analysis on an ABI 7,700 Sequence Detection system (Applied Biosystems, Foster City, CA) with the following commercially available sets of primers and fluorogenic probes (TaqMan_Gene Expression Assays products): RON, Hs00234013_m1; β-actin, Hs99999903_m1.The quantitative RT-PCR experiments were done in triplicate, and the relative expression levels were calculated based on the comparative Ct method.

Western blotting

After incubation for 48 h, the supernatants of each cell line and homogenized mouse tissue samples were then harvested and their protein concentrations were determined using a protein assay (Bio Rad, Hercules, CA). For Western blot analysis, 30 μg of total proteins were resolved by SDS-PAGE (Invitrogen Life Technologies, Carlsbad, CA) and proteins were then transferred to PVDF membranes (Atto, Tokyo, Japan). After washing three times, membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, then incubated for 1 h at room temperature with anti-mouse MSP antibody (1:1,000 dilution; Santa Cruz, CA), anti-human and mouse RON antibody (1:200 dilution; Santa Cruz, CA) or anti-mouse pRONβ antibody (1:200 dilution; Santa Cruz, CA). Membranes were then incubated for 30 min at room temperature with species-specific horseradish peroxidase conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescent substrate (Pierce, Rockford, IL).

Cell proliferation assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method [19]. Tumor cells (2×10^3 cells/100 μl/well) were seeded into each well of a 96-well plate and incubated for 24–96 h. After incubation, 50 μl of stock MTT solution (2 mg/ml; Sigma, St. Louis, MO) was added to all wells, and cells were then further incubated for 2 h at 37 °C. Media containing the MTT solution was removed, and 100 μl of DMSO was added. Absorbance was measured with an MTP-32 Microplate Reader (Corona Electric, Ibaragi, Japan) at test and reference wavelengths of 550 and 630 nm, respectively.

Transwell migration assay

Human SCLC cells (SBC-5, H1048) and their subclones were incubated for 24 h in MEM with 10 % FBS, then were incubated for 48 h in MEM with 0.1 % FBS. Thereafter, culture supernatants were harvested and added to the bottom wells of a 24-well Cell Culture Insert (8.0 μm pore size; Becton–Dickinson, NJ). RAW264.7 cells or MS1 cells (1×10^5 cells/well) were seeded in the top wells and cultured in MEM with 0.1 % FBS for 17 h or 6 h, respectively. After incubation, cells that remained in the top chamber were removed with a cotton swab, migrated cells were fixed, and DNA was labeled with

Hoechst33342 (Dojindo Laboratories, Kumamoto, Japan). The numbers of migrating cells per field of view were counted on a fluorescent microscope under a 20-fold magnification.

To evaluate the migration activities of human SCLC cells (SBC-5, H1048) and their subclones, they were starved overnight in serum-free MEM. The resultant cells (1×10^5 cells/well) were seeded in the top wells of a 24-well Cell Culture Insert. MEM with 10 % FBS was filled in the bottom wells and incubated for 17 h. The numbers of migrating cells were determined in the same manner described above.

Animals

Male SCID mice, 5–6 weeks old, were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions throughout the study. All experiments were performed in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

In vivo metastasis model

To facilitate metastasis formation, SCID mice were pretreated with anti-mouse interleukin-2 receptor β-chain antibody to deplete NK-cells [20, 21]. Two days later, mice were inoculated with SBC-5, H1048, or other transfected clones (1.0×10^6 cells/mouse) into the tail vein. Four weeks (SBC-5 and subclones) or 8 weeks (H1048 and subclones) after tumor cell inoculation, mice were anesthetized by i.p. injection of pentobarbital and X-ray photographs of the mice were taken to determine bone metastasis. Mice were killed humanely under anesthesia, the major organs were removed and weighed, and the number of metastatic colonies on the surface of the organs was counted. The lungs were fixed in Bouin's solution (Sigma, St. Louis, MO) for 24 h. The number of osteolytic lesions on X-ray films was counted by two investigators independently.

Immunohistochemical analyses

For histological analyses, the major organs with metastasis were fixed in 10 % formalin. Frozen tissue sections (8 μm thick) were fixed with cold acetone and used for identification of endothelial cells using rat anti-mouse CD31/PECAM-1 antibody (1:250 dilution; BD Biosciences, Cowley, UK) or rat anti-mouse CD68 antibody (1:250 dilution; Abd Serotec, Oxford, UK). To evaluate the microvessel density and tumor-infiltrating macrophages, CD31- and CD68-positive cells in liver metastatic lesions were counted in five random fields per one section at a 400-fold magnification. For the quantification of CD31- and CD68-positive cells four sections from four mice (total 20 fields) and five sections from five mice (total 25 fields) were analyzed, respectively.

Statistical analysis

The significance of differences in in vitro and in vivo data was analyzed by a one-way ANOVA test. When P values for the overall comparisons were less than 0.05, post hoc pairwise comparisons were performed by a Newman–Keuls Multiple Comparison test. P values of less than 0.05 were considered to be significant. Statistical analyses were performed using the GraphPad Prism program Ver. 4.01.

Results

Generation of cell lines stably overexpressing MSP

First, we sought to establish cell lines which stably overexpressed mouse MSP. Human SCLC, SBC-5 cells were infected with pMIG or pMIG-MSP, and then infected cells were sorted for GFP using flow cytometry. After culturing the initial sorted population, GFP-positive cells comprised more than 85 % of the whole cell population (data not shown). The expression of MSP mRNA and protein was detected in SBC-5-MSP cells, but not in parental SBC-5 cells or SBC-5-Vector cells (Fig. 1a, b).

The culture supernatant of SBC-5-MSP cells, but not that of parental cells or the vector control clone, induced migration of RAW 264.7 (murine macrophage-like cells) and MS1 (murine endothelial cells) (Fig. 2a, b). As expected, both of these cell lines express mouse RON (Fig. 4b), and might be responsive for MSP-mediated migration. These results suggest that MSP secreted by transfectants was biologically active and that it has key roles in infiltration of macrophages and migration of endothelial cells.

MSP gene transduction did not affect the behavior of cancer cells in vitro

We next investigated the effect of MSP gene transduction on in vitro tumor cell behavior related with metastasis. There was no significant difference in cell growth between SBC-5-MSP cells and parental SBC-5 cells or SBC-5-Vector cells (Fig. 3a). Results of the two-chamber migration assay showed that MSP transduction also did not affect cell motility among these three cell lines (Fig. 3b). Willett et al. [22] demonstrated that several lung cancer cell lines expressed both human MSP and human RON, and that human MSP promoted the migration of human RON-expressing cells in an autocrine and/or paracrine manner. Thus, we examined the expression of human RON on