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Appendix 1

STUDY PARTICIPANTS

The following institutions and investigators participated in the trial:

Asahikawa Medical College (Yoshinobu Osaki), National Cancer Center Hospital East (Yutaka Nishiwaki, Kaoru Kubota, Nagahiro Saijo), National Cancer Center Hospital (Tomohide Tamura, Noboru Yamamoto, Hideo Kunitoh), Kanagawa Cancer Center (Kazumasa Noda, Fumihiro Oshita), Niigata Cancer Center Hospital (Akira Yokoyama, Yuko Tsukada), Kinki University Hospital (Kazuhiko Nakagawa, Isamu Okamoto) and Osaka City General Hospital (Koji Takeda, Haruko Daga).

Appendix 2

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CLINICAL INVESTIGATION

THE IMPACT OF RADIATION DOSE AND FRACTIONATION ON OUTCOMES FOR LIMITED-STAGE SMALL-CELL LUNG CANCER

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Purpose: To review the treatment outcomes of limited-stage small-cell lung cancer (LS-SCLC) patients and to compare the outcomes among three groups in which the total radiation doses were 45 Gy with accelerated hyperfractionation (AHF), <54 Gy with standard fractionation (SF), and ≥54 Gy with SF.

Methods and Materials: LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital were reviewed in this study. Of the 127 eligible patients, there were 37 patients in the AHF group, 29 in the SF <54 Gy group, and 61 in the SF ≥54 Gy group.

Results: Fifty-five patients (43%) were alive at the time of this analysis, and the median follow-up time of the surviving patients was 33 months. The median survival times were 30.0 months (95% confidence interval [CI] 16.3–43.7) for the AHF group, 14.0 months (CI 6.6–21.4) for the SF <54 Gy group, and 41.0 months (CI 33.9–48.1) for the SF ≥ 54 Gy group. As for the local control rates, and the overall and progression-free survival rates, all outcomes were significantly lower in the SF <54 Gy group than in the other two groups, although no significant difference was found between the AHF and SF ≥54 Gy groups.

Conclusions: These results suggest the importance of a high dose of radiation when using once-daily regimen. This study will support future prospective studies to establish optimal radiation doses and fractionation. © 2009 Elsevier Inc.

Small-cell lung cancer, Radiation therapy, Radiation dose, Fractionation, Accelerated hyperfractionation.

INTRODUCTION

Chemoradiotherapy is currently the standard treatment for limited-stage small-cell lung cancer (LS-SCLC) (1). Although thoracic radiotherapy (TRT) has been established as an integral component of the treatment platform for LS-SCLC, some questions regarding the optimal radiotherapy approach have also arisen. With regard to fractionation, Turrisi *et al.* determined that accelerated hyperfractionation (AHF) is superior to standard fractionation (SF) in an Intergroup Phase III study (2). However, despite the significant improvement in long-term survival, a pattern of care study found that only 10% of patients with LS-SCLC received a twice-daily regimen because of the inconvenience of twice-daily treatment sessions and the increased rate of severe esophageal toxicity seen with this regimen, whereas more than 80% received once-daily TRT (3). Although traditionally modest doses of TRT (45–50 Gy) are often used in once-daily 1.8- to 2-Gy fractions (4, 5), the optimal total dose for a once-daily regimen has not been proven. In addition, it is also still unclear whether twice-daily TRT of 45 Gy in 3 weeks is superior to a higher total dose than traditional modest doses delivered with a once-daily regimen. In this study, we reviewed the treatment outcomes of LS-SCLC patients that were treated with chemoradiotherapy at Aichi Cancer Center Hospital and compared the outcomes among three groups in which the total radiation doses were 45 Gy with a twice-daily regimen, less than 54 Gy with a once-daily regimen, and equal or greater than 54 Gy with a once-daily regimen.

METHODS AND MATERIALS

Patient selection

LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital and who met the eligibility criteria were enrolled into this retrospective study. The diagnosis of SCLC was confirmed by histologic or cytologic findings in all cases. Limited-stage was defined as disease confined to one hemithorax with or without bilateral supraclavicular node metastasis. The eligibility criteria consisted of no previous treatment and an Eastern Cooperative Oncology Group performance status

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of 0–2. Additional eligibility criteria were as follows: they had not undergone surgery for LS-SCLC nor had been treated with a radiation field, not including elective nodal irradiation, because the significance of omitting elective nodal irradiation remains unclear (6). Written informed consent was obtained from all patients before treatment. Each patient underwent the following studies: chest radiography and fiberoptic bronchoscopy, complete blood count and biochemical tests, a computed tomography (CT) scan of the thorax and abdomen, a CT scan or magnetic resonance imaging of the brain, and a radionuclide bone scan, or positron emission tomography. Positron emission tomography was used for a few patients (7%) who were treated after 2001 according to the physician's preference. Bone marrow aspiration or biopsy was performed in cases of neutropenia and thrombocytopenia.

Radiation therapy technique

TRT was carried out with linear accelerators, and the energy of 6–10 MV photons was used. The TRT fields were changed from anteroposterior-posteroanterior fields to parallel opposed oblique fields after 30 Gy in the twice-daily regimen and 36–40 Gy in the once-daily regimen. Most patients (83%) that were eligible for this study were treated using conventional fluoroscopic simulation techniques at the start of the TRT, and CT simulation techniques were used only for the planning of the boost fields. The other 17% were treated using CT simulation techniques throughout the entire TRT. The other planning techniques were similar to those in our previous report on non–small-cell lung cancer (7). TRT was administered twice daily (1.5 Gy per fraction, with a 6 h or more interval between fractions) for a total dose of 45 Gy in 3 weeks or once-daily (1.8–2.0 Gy per fraction) for a total dose of 39.6–66 Gy in 4–7 weeks. After the TRT, prophylactic cranial irradiation (PCI) was administered to the patients who had a complete or near-complete response (10). The PCI consisted of 24 Gy in 2 Gy per fractions or 25 Gy in 2.5 Gy per fractions once daily, 5 days per week.

All patients who entered the clinical trial were treated with the AHF regimen. However, there were no adequate rationale for a decision about a patient's TRT dose and fractionation. The TRT dose and fractionation was decided according to the physician's preference.

Chemotherapy

In principle, the patients were treated with four cycles of chemotherapy and received at least one cycle of chemotherapy concurrent with TRT. The chemotherapy was given in a 28-day cycle in the concurrent phase and a 21-day cycle in the sequential phase. The most commonly used regimens were cisplatin/etoposide, carboplatinum/etoposide, and cisplatin/irinotecan. As a general rule, the cisplatin/etoposide regimen consisted of cisplatin (80 mg/m^2 intravenously) on day 1 and etoposide (100 mg/m^2 intravenously) on Days 1, 2, and 3. The carboplatinum/etoposide regimen consisted of carboplatinum (area under the blood concentration-time curve: 5 intravenously) on Day 1 and etoposide (100 mg/m^2 intravenously) on Days 1, 2, and 3. The cisplatin/irinotecan regimen was only performed sequentially with TRT and consisted of cisplatin (80 mg/m^2 intravenously) on Day 1 and irinotecan (60 mg/m^2 intravenously) on Days 1, 8, and 15.

Study design and statistical analysis

All available radiation records and charts were reviewed to assess patient and tumor characteristics and the details of treatment and outcome. Tumor response was classified in accordance with the Response Evaluation Criteria in Solid Tumors criteria (9). Complications were graded in accordance with the National Cancer Institute's Common Toxicity Criteria, version 3.0 (10). The date of the last follow-up was defined as the last recorded information available for the patient. Only 3 patients were lost to follow-up. Survival was measured from the start date of any treatment to the date of the last follow-up or death from any cause. Local failure, defined as locoregional progression on CT (including the primary tumor and the bilateral mediastinal and ipsilateral hilar lymph nodes), was measured from the start date of any treatment to the date of the first evidence of locoregional disease progression. Concurrent local and distant failures were scored as local failures for the first failure sites. Progression-free survival was measured from the start date of any treatment until the date of local or distant failure.

Overall survival (OS), overall local, and overall progression-free survival were calculated using Kaplan-Meier estimates. Subgroup analysis was used to compare the outcomes among the three groups, in which the total radiation doses were 45 Gy with AHF, <54 Gy with SF, and ≥54 Gy with SF, using the log–rank test. Moreover, sex, age at diagnosis, performance status, disease stage (I, II, vs. III), PCI (yes vs. no), total chemotherapy cycles (<3 vs. ≥3), concurrent chemotherapy (yes vs. no), and the duration of TRT (<40 days vs. ≥40 days) were also assessed for their impact on OS using the log–rank test. Fisher's exact test was used for comparisons of categorical data. Cox's proportional hazards model was used for multivariate analysis. $p < 0.05$ was considered significant.

RESULTS

Patient and treatment characteristics

A total of 127 patients were enrolled into the study. The median total dose of TRT with the once-daily regimen was 54 Gy; therefore, we divided the patients that had been treated with the once-daily regimen into two groups using the median total dose of 54 Gy for the subgroup analysis. The characteristics of the 127 eligible patients are shown in Table 1. Fifteen patients (40%) from the AHF group entered a clinical trial, but no patients from the other two groups did. The baseline characteristics were balanced in terms of sex, performance status, stage, and chemotherapy cycles. However, there was a slight imbalance in age; the patients in the AHF group tended to be younger than those in the other two groups, and the rate of patients older than age 75 years was lower than in the other two groups, but these differences were not significant ($p = 0.15$). There were significant differences in the rate of patients that received concurrent chemotherapy and PCI among the three groups ($p = 0.012$, $p < 0.001$, respectively). Fifty-five (43%) patients were alive at the time of this analysis, and the median follow-up time of the surviving patients was 33 months (range, 2–118 months). The median follow-up time of the surviving patients was 34 months (range, 16–96 months) for the AHF group, 67 months (range, 12–91 months) for the SF <54 Gy group, and 22 months (range, 2–118 months) for the SF ≥54 Gy group. There were no significant differences in the median follow-up time of the surviving patients among the three groups ($p = 0.32$).

As a result, 84% received four or more cycles of chemotherapy. Eight percent received three cycles, and 8% received less than two cycles either because the patient refused continuation

Table 1. Patient and tumor pretreatment characteristics

Characteristic	Prescription group			p value*
	AHF group (n = 37)	SF <54 Gy group (n = 29)	SF ≥54 Gy group (n = 61)	
Age (y)	58 (40–68)	70 (51–82)	66 (29–81)	
≥75 (%)	0 (0%)	3 (10%)	5 (8%)	0.15
Sex (%)				0.59
Male	30 (81%)	25 (86%)	54 (82%)	
Female	7 (19%)	4 (14%)	7 (18%)	
Performance status				0.29
0	13 (35%)	7 (25%)	20 (33%)	
1	24 (65%)	20 (68%)	36 (59%)	
2	0 (0%)	2 (7%)	5 (8%)	
Stage				0.20
I	0 (0%)	1 (3%)	6 (10%)	
II	4 (11%)	0 (0%)	4 (7%)	
IIIA	22 (59%)	11 (38%)	24 (39%)	
IIIB	11 (30%)	17 (59%)	27 (44%)	
CHT cycles	3.9 (2–5)	3.7 (1–6)	3.9 (1–6)	0.72
≥3 cycles	33 (89%)	27 (93%)	56 (92%)	
Concurrent CHT	37 (100%)	23 (79%)	56 (92%)	0.012*
Total dose (Gy)	45 (45)	50 (39.6–52.2)	56 (54–63)	<0.001*
Duration of TRT (days)	21 (19–27)	41 (30–56)	43 (36–59)	<0.001*
PCI	24 (65%)	6 (21%)	18 (30%)	< 0.001*

Abbreviations: AHF = accelerated hyperfractionation; SF = standard fractionation; CHT = chemotherapy; TRT = thoracic radiotherapy; PCI = prophylactic cranial irradiation.

Age and total dose data are presented as the median value. CHT cycles data are presented as the mean value. The numbers in square brackets indicate the range of age and CHT cycles.

* Fisher's exact test.

of the chemotherapy or their leukocyte or platelet counts or renal function did not return to levels at which chemotherapy could be performed. Most patients (91%) received at least one cycle of concurrent chemotherapy with TRT, whereas the remaining 9% only received sequential chemotherapy because the radiation field sizes of these patients were too large as the primary tumor was located in the inferior lobe or the primary tumor was so bulky that concurrent chemoradiotherapy was considered to carry a high risk of severe radiation pneumonitis (11). All patients received at least one cycle of platinum-based agents/etoposide regimen regardless of the TRT regimen. The cisplatin/irinotecan regimen was only performed sequentially with TRT for 24% of patients in the AHF group and 16% of the SF ≥54 Gy group.

Tumor response

Table 2 shows the tumor response in each group. The overall response rate was 94% (95% confidence interval [CI] 91–98%, 58% complete response rate [CI 50–67%], and 36% partial response rate [CI 28–45%]) for all eligible patients. There was a significantly lower rate of complete response in the SF <54 Gy group than in the AHF and SF ≥54 Gy groups (p = 0.018 and 0.0062, respectively). There was a significantly higher rate of complete response in the AHF group than in the SF ≥54 Gy group (p = 0.042).

Table 2. Tumor response in each group

Response	Prescription group						p value*
	AHF (n = 37)		SF <54 Gy (n = 29)		SF ≥54 Gy (n = 61)		
	No.	%	No.	%	No.	%	
Overall response	34	92%	27	93%	59	97%	0.32
CR	27	73%	11	38%	36	59%	0.02*
PR	7	19%	16	55%	23	38%	
SD	0	0%	1	3%	2	3%	
PD	3	8%	1	3%	0	0%	

Abbreviations: AHF = accelerated hyperfractionation; SF = standard fractionation; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

* Fisher's exact test.

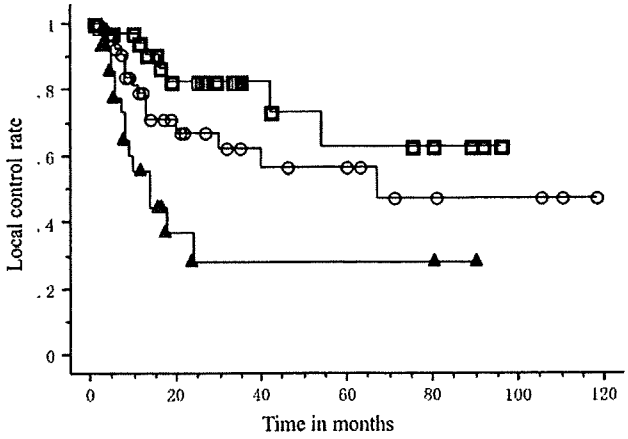

Fig. 1. The local control rates for patients with a total dose of 45 Gy with accelerated hyperfractionation (□), <54 Gy with standard fractionation (▲), and ≥54 Gy with standard fractionation (○).

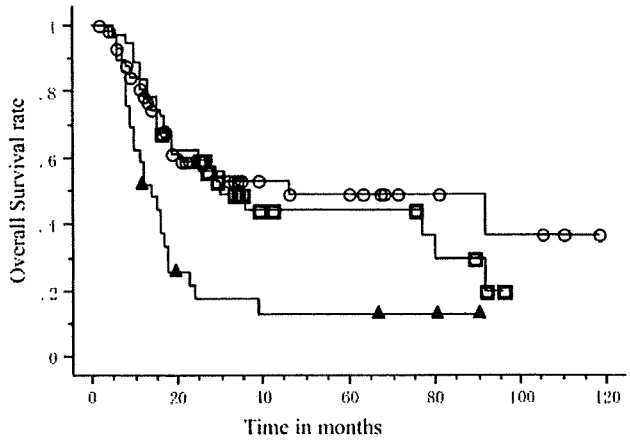

Fig. 2. Overall survival for patients with a total dose of 45 Gy with accelerated hyperfractionation (□), <54 Gy with standard fractionation (▲), and ≥54 Gy with standard fractionation (○).

Local control and progression-free survival

Figure 1 shows the local control rates for each group. The 3-year local control rates were 61.1% (CI 50.3–71.9%) for all eligible patients, 81.3% (CI 67.2–95.5%) for the AHF group, 27.7% (CI 5.0–50.4%) for the SF <54 Gy group, and 61.2% (CI 44.8–77.6%) for the SF ≥54 Gy group. The local control rate was also significantly lower for the SF <54 Gy group than the AHF and SF ≥54 Gy groups (p = 0.0016 and 0.011, respectively). Local control for the AHF group tended to be superior to that for the SF ≥54 Gy group, although no statistically significant difference was found (p = 0.096).

The 3-year progression-free survival rates were 28.1% (CI 19.5–36.7%) for all eligible patients, 37.5% (CI 21.5–53.5%) for the AHF group, 7.5% (CI 0–17.5%) for the SF <54 Gy group, and 33.2% (CI 19.7–46.7%) for the SF ≥54 Gy group. Progression-free survival was also significantly lower for the SF <54 Gy group than for the AHF and SF ≥54 Gy groups (p = 0.015 and 0.013, respectively). Progression-free survival was similar in the AHF group and the SF ≥54 Gy group (p = 0.80).

Overall survival

Figure 2 shows the survival curves for each group. The median survival time of all eligible patients was 24.0 months (CI 18.1–29.9 months). The median survival times were 30.0 months (CI 16.3–43.7 months) for the AHF group, 14.0 months (CI 6.6–21.4 months) for the SF <54 Gy group, and 41.0 months (CI 33.9–48.1 months) for the SF ≥54 Gy group. The 3-year survival rates were 41.2% (CI 31.6–50.8%) for all eligible patients, 44.1% (CI 26.5–61.7%) for the AHF group, 13.8% (CI 0–27.3%) for the SF <54 Gy group and 53.1% (CI 38.6–67.6%) for the SF ≥54 Gy group. There was a significantly lower rate of OS in the SF <54 Gy group than in the AHF and SF ≥54 Gy groups (p = 0.0018 and 0.00036, respectively). OS for the SF ≥54 Gy group seemed to be slightly superior to that for the AHF group, although no statistically significant difference was found (p = 0.64).

Factors associated with overall survival

Table 3 shows the effects of patient characteristics, disease factors, and treatment parameters on OS according to univariate analysis. To evaluate further the independent effects of disease stage, chemotherapy cycles, concurrent chemotherapy, PCI,

Table 3. Factors associated with overall survival according to univariate analysis

Factors	No. of patients	3-year OS (95% CI)	p value
Sex			0.367
Male	109	40.8% (33.1–48.5)	
Female	18	41.8% (36.1–47.5)	
Age (y)			0.652
<75	119	40.6% (33.2–48.0)	
≥75	8	48.6% (43.6–53.6)	
PS			0.546
0, 1	120	42.0% (34.6–49.4)	
2	7	28.6% (23.6–33.6)	
Stage			0.016*
I, II	15	80.0% (78.3–81.7)	
III	112	35.7% (28.3–43.1)	
CHT cycle			0.033*
<3	11	43.4% (35.8–51.0)	
≥3	116	20.0% (14.2–25.8)	
Concurrent CHT			0.026*
Yes	116	43.7% (36.1–51.3)	
No	11	20.0% (17.3–22.7)	
Treatment group			<0.001*
AHF	37	44.1% (26.5–61.7)	
SF ≥54 Gy	29	53.1% (38.6–67.6)	
SF <54 Gy	61	13.8% (0–27.3)	
Duration of RT (days)			0.821
<40	66	41.3% (31.0–51.6)	
≥40	61	40.0% (29.8–50.2)	
PCI			0.089
Yes	48	48.1% (36.6–59.6)	
No	79	35.6% (31.6–39.6)	

Abbreviations: CI = confidence interval; PS = performance status; CHT = chemotherapy; NA = not applicable; AHF = accelerated hyperfractionation; SF = standard fractionation; RT = radiation therapy; PCI = prophylactic cranial irradiation.

* Statistically significant.

Table 4. Factors associated with overall survival according to multivariate analysis

Factors	Hazard ratio of death (95% CI)	*p* value
Stage (I, II vs. III)	0.24 (0.074–0.78)	0.017*
CHT cycle (<3 vs. ≥3)	0.50 (0.24–1.07)	0.073
Concurrent CHT (yes vs. no)	0.61 (0.29–1.31)	0.20
Treatment group (AHF vs. SF ≥54 Gy SF 54 Gy)	NA	0.033*
PCI (yes vs. no)	0.75 (0.43–1.31)	0.31

Abbreviations: CHT = chemotherapy; NA = not applicable; AHF = accelerated hyperfractionation; SF = standard fractionation.
* Statistically significant.

and treatment group on OS, a multivariate Cox proportional hazards regression analysis was performed. This analysis included those factors that had displayed a p value <0.10 in the univariate analysis. As a consequence, disease stage and treatment group remained significant factors in the multivariate analysis (Table 4).

Toxicity

Documentation concerning toxicity data was not available for 6 patients (2 in each group), which left 121 patients assessable for toxicity. Only late toxicity ≥Grade 2 was assessed from the available information of each chart. There were only 2 treatment-related deaths (one in the SF <54 Gy group and the other in the SF ≥54 Gy group). Both patients died of radiation pneumonitis. Five patients developed Grade 2 radiation pneumonitis, 4 in the SF <54 Gy group and 1 in the SF ≥54 Gy group. Apart from the toxicities described, no other information about late toxicity was noted in the charts.

DISCUSSION

In our study, the comparison of overall, progression-free, and local control survival rates and the rate of complete response suggested that TRT administered with a total dose of <54 Gy by once-daily regimen was more disadvantageous than TRT treated with a total dose of ≥54 Gy in a once-daily regimen or a total dose of 45 Gy administered using the AHF regimen, and the difference was statistically significant for all outcomes. These results clearly demonstrate that radiation intensification improves the complete response rate and local control and that improved local control translates into improved OS. Furthermore, these results also suggest the importance of a high dose of radiation when using a once-daily regimen.

Because SCLC has high radiation sensitivity (12), recent pattern of care studies have shown that the traditional modest doses of TRT that are used in once-daily 1.8- to 2-Gy fractions are also widely used for LS-SCLC in Japan and Turkey (4, 5). However, although response rates are high, local control rates have been poor in this TRT setting. Intensifying the radiotherapy effect by accelerating its delivery was one of the initial strategies explored in prospective trials. Turrisi *et al.* (2) randomly assigned 471 LS-SCLC patients to either 45 Gy in 5 weeks (1.8 Gy once-daily for 25 fractions) or 45 Gy in 3 weeks (1.5 Gy twice-daily for 30 fractions) beginning with the first of four cycles of PE. The 5-year survival rate was 26% with accelerated TRT compared with 16% for the conventional TRT ($p = 0.04$), and the accelerated TRT arm was also superior to conventional TRT in local tumor control ($p = 0.06$). These data strongly suggest that attempts designed at improving local tumor control can favorably impact on the long-term outcome of patients with LS-SCLC. However, despite the significant improvement in long-term survival, only 10% of patients with LS-SCLC received a twice-daily regimen (3). Moreover, a second trial performed by the North Central Cancer Treatment Group reported negative results with a twice-daily regimen, although overall treatment times and total radiation doses were identical in each arm of the North Central Cancer Treatment Group trial (13). Therefore, different strategies were considered that might increase the local control rate with chemoradiotherapy. Accelerated fractionation via the concomitant boost technique uses once-daily irradiation early in the course of treatment and then twice-daily irradiation toward the end. Komaki *et al.* (14) reported a Phase I study (Radiation Therapy Oncology Group 97-12) using this regimen to improve local control by increasing the dose of TRT given with concurrent cisplatin/etoposide without causing acute severe esophagitis. They found that 61.2 Gy was the maximum tolerated dose, and there was a suggestion of improvement in the estimated short-term survival rate (18 months) by dose escalation from 50.4 Gy to 61.2 Gy (25% and 82%, respectively). Roof *et al.* (15) also showed a clear dose–response curve between 54 and 63 Gy with a once-daily regimen, although they did not find a significant difference in outcome because of their small sample size of 54 patients.

Obviously, there are problems that limit the interpretation of a single institutional retrospective review. We recognize the imbalance among our three groups. The rates of patients receiving PCI and concurrent chemotherapy were significantly different among the three groups (Table 1), although the multivariate analysis proved that these factors were not associated with OS. Another difficulty associated with retrospective reviews is the accurate assessment of toxicity. The rate of Grade 2-4 toxicities in our study was lower than those reported elsewhere. Although the patient charts were thoroughly scrutinized, the documentation concerning complications may not have been as thorough as it would have been in a prospective trial.

Whether twice-daily TRT to 45 Gy in 3 weeks is superior to a higher total dose than traditional modest doses delivered with a once-daily regimen is still unclear. Our results did not find a significant difference in outcome between the AHF group with a total dose of 45 Gy and the SF group with a total dose of ≥54 Gy. However, there was a significantly higher

rate of complete response in the AHF group compared with the SF ≥54 Gy group ($p = 0.042$), and the local control for the AHF group tended to be superior to that for the SF ≥54 Gy group. These results indicate that in the once-daily regimen much more than 54 Gy is necessary to achieve local control at the same level as 45 Gy with the AHF regimen. Despite the significantly higher rate of complete response and local control in the AHF group compared with the SF ≥ 54 Gy group, progression-free survival and OS were similar between the AHF group and the SF ≥54 Gy group ($p = 0.80$ and 0.64, respectively). We think that the reason was our small sample size. On the other hand, these data indicated that patients in the AHF group died from systemic disease as did those in the SF ≥54 Gy group, despite the better local control in the AHF group compared with the SF ≥54 Gy group. Therefore, another chemotherapy strategy such as integrating newer chemotherapy agents (16) or dose-intense regimens using either growth factors or stem-cell support (17) may be necessary for the platform of the curative approach to LS-SCLC.

Further intensification of TRT regimens such as a Phase III trial is under development by the Cancer and Leukemia Group B and the Radiation Therapy Oncology Group (18). This randomized trial is designed to compare three TRT approaches with four cycles of PE, with the three regimens being 45 Gy in 3 weeks (1.5 Gy twice-daily for 30 fractions), 70 Gy in 7 weeks (2.0 Gy once-daily for 35 fractions), and 61.2 Gy in 5 weeks (1.8 Gy accelerated fractionation via concomitant boost). We think that this trial will demonstrate the optimal method of radiation dose intensification. However, at this time, 45 Gy twice-daily TRT should be considered as the standard treatment for LS-SCLC because there are no Phase III once-daily trials that have shown better outcomes than the twice-daily regimen.

In conclusion, this analysis suggests that disease stage and treatment groups that are stratified according to 45 Gy with AHF, <54 Gy with SF, and ≥54 Gy with SF are independent factors associated with improved OS in patients with LS-SCLC and the potential importance of a high dose of radiation when using a once-daily regimen. However, there are problems that limit the interpretation of our single institutional retrospective review. There were some prognostic differences in the three groups compared, especially in the rates of patients receiving PCI and concurrent chemotherapy. A future prospective study of TRT regimens in the setting of chemoradiotherapy for LS-SCLC is needed to establish optimal radiation doses and fractionation, and such a study is under development by the Cancer and Leukemia Group B and Radiation Therapy Oncology Group.

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ORIGINAL ARTICLE

Relationship of mRNA expressions of RanBP2 and topoisomerase II isoforms to cytotoxicity of amrubicin in human lung cancer cell lines

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Abstract
Purpose RanBP2 is a small ubiquitin-like modifier ligase for DNA topoisomerase II (TopoII) and plays a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis. Engineered-mice with low amounts of RanBP2 have been reported to form lung adenocarcinomas. Furthermore, in the murine embryonic fibroblasts, formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII, has been reported. In this study, we tested whether the association between mRNA expression of the RanBP2 gene and chemosensitivity of a TopoII inhibitor, amrubicin could be seen.
Methods Using a panel of 20 lung cancer cell lines, the mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes were examined by quantitative real-time reverse transcription PCR. The in vitro cytotoxicity of amrubicin was assessed using a tetrazolium-based colorimetric assay (MTT assay).
Results Although RanBP2 mRNA expression was infrequently downregulated in human lung cancer cell lines, significantly higher RanBP2 transcripts were observed in small cell lung cancer than non-small cell lung cancer. There were no correlations between chemosensitivity of amrubicin and mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes.
Conclusions Our in vitro results suggest that mRNA expressions of RanBP2 and TopoII isoforms are unlikely to be a predictive biomarker for the sensitivity to amrubicin.

Keywords SUMO ligase · Topoisomerase II inhibitor · Predictive biomarker · Chromosomal instability · Lung cancer

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Introduction

Lung cancer is a leading cause of cancer mortality in the United States and in Japan [13, 29]. Lung cancer has two main types: small cell lung cancer (SCLC) and non-SCLC (NSCLC). The major histological subtypes of NSCLC include adenocarcinoma, squamous carcinoma and large cell carcinoma. About 15% of lung cancers are SCLC. SCLC spreads rapidly and widely forming additional large tumors in lymph nodes, bones, adrenal glands, liver and brain. Because of its aggressive nature the overall survival of SCLC is worse than that of NSCLC and only 5–10% at 5 years. Survival of patients with either SCLC or NSCLC is strongly correlated with the stage of disease. For patients with advanced tumors, the prognosis is dismal because the available treatment regimens such as chemotherapy and radiation therapy are essentially palliative and primarily serve only to prolong survival. In fact, combination chemotherapy with etoposide plus cisplatin or irinotecan plus cisplatin for extensive-stage (ES) SCLC as well as the common first-line platinum-based combination regimens for advanced NSCLC only produced a median survival time of about 1 year [18, 19, 23]. Thus, new treatment approaches are clearly required.

Amrubicin, developed and approved in Japan for the treatment of SCLC and NSCLC, is a totally synthetic

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anthracycline anticancer agent and a potent TopoII inhibitor [14]. Amrubicin monotherapy with 45 mg/(m^2 day) for 3 consecutive days by intravenous administration produced response rates of 75.8 and 27.9% for previously untreated patients with ES-SCLC and advanced NSCLC, respectively. A phase II study of the combination of 60 mg/m^2 cisplatin and 40 mg/(m^2 day) amrubicin for 3 days has been reported to show response rate of 87.8%, the MST of 13.6 months and 1-year survival rate of 56.1% against ES-SCLC. Based on this result, Japan Clinical Oncology Group (JCOG) is conducting a randomized phase III study to compare the combinations of cisplatin plus amrubicin and cisplatin plus irinotecan for previously untreated ES-SCLC.

To improve clinical outcomes in advanced lung cancer, clinical integration of molecular biomarkers that predict responses to chemotherapeutic agents may be indispensable [16]. Recently, RanBP2 has been reported to act as a small ubiquitin-like modifier (SUMO) ligase for DNA TopoII and play an important role in targeting TopoII to centromeres during mitosis and in maintaining chromosome stability [5]. Embryonic fibroblasts derived from the engineered mutant mice with low expression of RanBP2 have been reported to show formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII [4], suggesting that low expression of RanBP2 may have an analogous effect of TopoII inhibitors. In addition, RanBP2 has a tumor suppressor function since these mutant mice succumbed to a range of cancers, primarily lung carcinomas, and were also susceptible to chemically-induced tumorigenesis. Based on these observations, we hypothesized that RanBP2 expression might be involved in chemosensitivity of a TopoII inhibitor, amrubicin.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for individualizing the most beneficial chemotherapy. As one of the multiple approaches to establishing predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the RanBP2 gene as well as the TopoII-alpha and beta genes and chemosensitivity to amrubicin using human lung cancer cell lines.

Materials and methods

Cell lines and drug

Fifteen NSCLC and five SCLC cell lines used were described previously [24]. These cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Amrubicin was kindly provided by Sumitomo pharmaceuticals Company, Osaka, Japan.

Cytotoxicity assay

Cytotoxicity was evaluated using an MTT assay as described previously [11]. Suspensions of exponentially growing cells were dispensed into wells of 96-well tissue-culture plates. After incubation at 37°C for 24 h, the solutions of amrubicin at various concentrations were added, and then incubated for 3 days. The effects of treatment were expressed as percent growth inhibition using untreated cells as the uninhibited control and assessed by IC50 (drug concentrations inducing a 50% reduction of cell survival) which was calculated from dose–response curves.

RNA preparation and RT-PCR amplification

Total RNA was extracted and further purified as described previously [24]. The RNAs were stored at −80°C until use. Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase-PCR (RT-PCR) for absolute quantitating mRNA levels of the RanBP2, TopoII-alpha, TopoII-beta and beta-actin genes as described previously. The PCR primers used were as follows.

RanBP2-S:	5′-CAATGGAAATGGGGAAGACTTT-3′
-AS:	5′-CATCACTTCAGTCCCACCTGTA-3′
TopoII-alpha-S:	5′-GGTGTGGAACTAGAAGGCCTAA-3′
-AS:	5′-TGAATCAGACCAGGGATTTCTC-3′
TopoII-beta-S:	5′-TTTTTCACCATCATTTGGTCTG-3′
-AS:	5′-GGGCTTAGGGACTGTATCTGAA-3′
Beta actin-S:	5′-TTCTACAATGAGCTGCGTGTG-3′
-AS:	5′-CAGCCTGGATAGCAACGTACA-3′

Linear regression analysis of standard-curves demonstrated a strong correlation for all the genes ($R^2 > 0.99$). The relative gene expression levels were normalized with a house keeping gene, beta-actin.

Western blot analysis

Western blot analysis was done as described previously [11], using the following primary antibodies: anti-RanBP2 (ab2938, Abcam), anti-TopoII-alpha (ab45175, Abcam), anti-TopoII-beta (ab58442, Abcam) and anti-actin (A2268, Sigma-Aldrich) antibodies.

Statistical analyses

The strength of the association between the expression levels of RanBP2, TopoII-alpha and TopoII-beta and chemosensitivity data was calculated by either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. For comparison of IC50 values of amrubicin and each gene expression level among histological subtypes, we employed one-way

analysis of variance (ANOVA) followed by Bonferroni post-test. All analysis was performed with the use of Stat View software version 5.0.

Results

Chemosensitivity of amrubicin was examined using 20 human lung cancer cell lines including 15 NSCLC cells and 5 SCLC cells. Cytotoxicity following a 72 h continuous exposure of amrubicin was measured by MTT assay. The IC 50 value of amrubicin in SK-LC-3 was about 9 μM, while the IC 50 values in the other cell lines were less than 1 μM as shown in Table 1. There was no significant difference between histological types (Fig. 1).

The mRNA quantifications of the RanBP2, TopoII-alpha, TopoII-beta genes were carried out in real-time PCR and the expression levels were normalized with beta-actin as an internal control (Table 1). Among 20 cell lines tested, the level of RanBP2 mRNA expression in an H460 cell line was about 20-fold lower than those in non-tumorous lung tissues obtained from two patients with lung cancer. There were statistically significant differences in the RanBP2 expression between SCLC and the other histological subtypes ($p < 0.05$) (Fig. 2a). We checked RanBP2 protein expression in two lung cancer cell lines, SK-LC-2 and H460, representing high and low expression of the RanBP2 gene, and found similar mRNA and protein expression patterns (Fig. 2d). We also found statistically higher expression levels of TopoII-alpha in SCLC and adenocarcinoma cell lines compared with those in normal lung tissues,

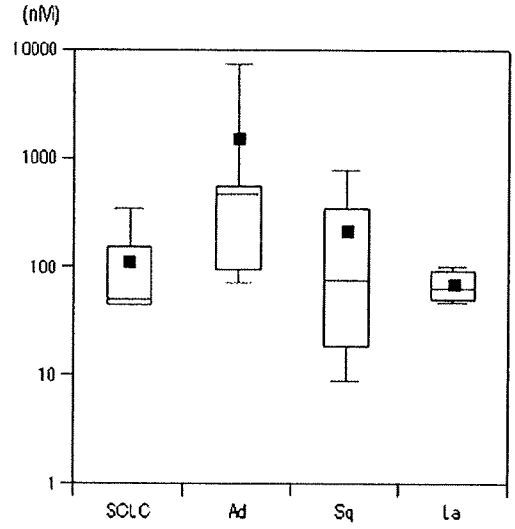

Fig. 1 IC50 values of Amrubicin in lung cancer cell lines. *Box plots* show relationships between IC50 values of Amrubicin and the four histological subtypes of lung cancer. The *horizontal line* within each *box* represents the median value and the closed box shows the mean value, respectively

Table 1 IC50 values for amrubicin and relative mRNA expression for RanBP2, TopoII alpha and TopoII beta in lung cancer cell lines

Cell line	Histology	Amrubicin (μM)	RanBP2	TopoIIa	TopoIIb
ACC-LC-94	Ad	0.0668	0.621	1.324	1.174
ACC-LC-319	Ad	0.579	1.108	1.682	1.437
SK-LC-3	Ad	8.99	2.634	2.009	1.899
A549	Ad	0.131	1.191	1.553	1.665
SK-LU-1	Ad	0.492	1.307	2.930	1.479
VMRC-LCD	Ad	0.0835	4.134	2.660	2.942
RERF-LC-MT	Ad	0.469	0.661	0.8719	0.753
Calu1	Sq	0.203	1.280	2.173	1.750
SK-MES-1	Sq	0.0768	1.160	0.883	0.807
PC-1	Sq	0.009	1.937	1.739	2.888
RERF-LC-A	Sq	0.0222	0.717	1.454	1.036
PC-10	Sq	0.77	0.713	1.049	1.170
NCI-H460	La	0.101	0.043	1.518	2.098
Calu6	La	0.0469	1.467	1.116	1.828
SK-LC-6	La	0.0632	2.362	2.508	4.383
ACC-LC-48	SCLC	0.0512	1.957	1.672	2.044
ACC-LC-49	SCLC	0.0866	2.592	1.975	3.523
ACC-LC-80	SCLC	0.0459	3.953	1.361	1.993
ACC-LC-172	SCLC	0.0439	2.387	4.450	4.207
SK-LC-2	SCLC	0.337	3.662	3.510	4.264
NL 1	Normal lung	NA	1.006	0.158	2.447
NL 2	Normal lung	NA	0.913	0.179	1.937

NL 1 and NL 2: non-tumorous lung tissues obtained from two patients with lung cancer.
Ad adenocarcinoma,
La large cell carcinoma,
SCLC small cell lung cancer,
Sq squamous cell carcinoma.
NA not available

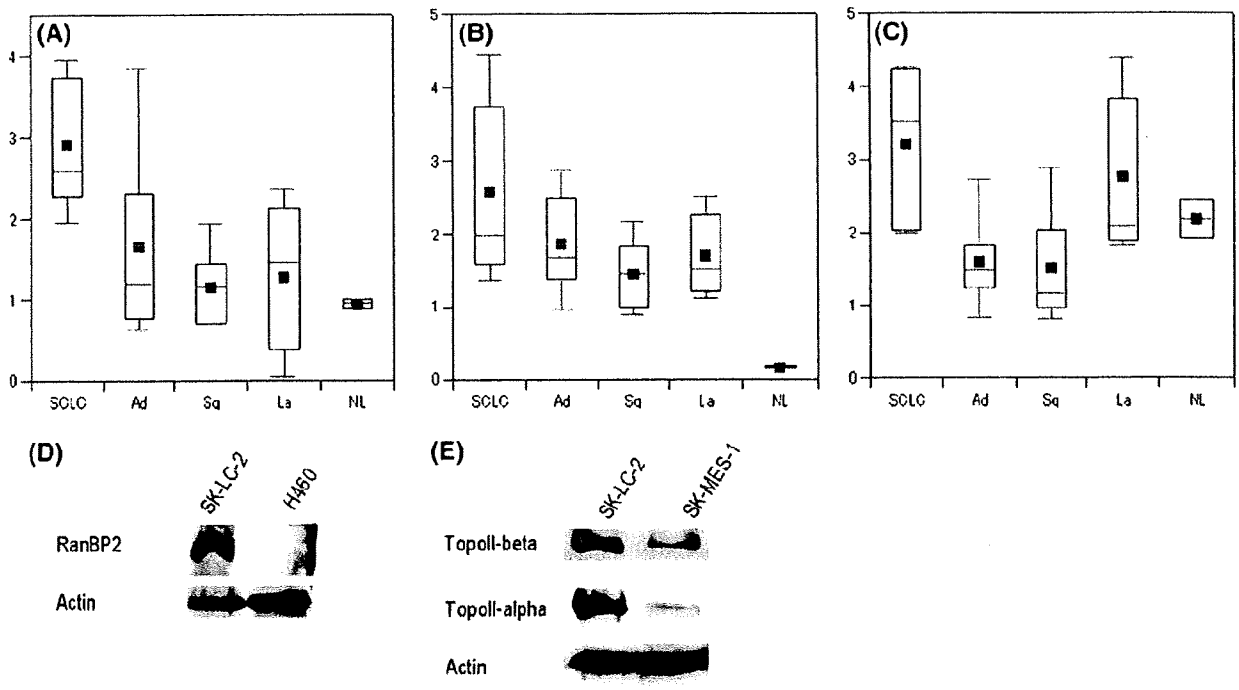

Fig. 2 Relative mRNA expression for (a) RanBP2, (b) TopoII-alpha and (c) TopoII-beta among histological subtypes and normal lung tissues, and protein expression for (d) RanBP2 and (e) TopoII isoforms in representative cell lines. a RanBP2 mRNA expression in SCLC was higher than those in the other histological subtypes of lung cancer. b TopoII-alpha mRNA expression levels in lung cancer cell lines were relatively higher compared to those in normal lung tissues. c The expression levels of TopoII-beta in lung cancer cell lines were similar to those in normal lung tissues. d, e Western blot analyses for RanBP2 and TopoII isoforms in two lung cancer cell lines representing high and low expression, respectively. The expression patterns of protein and mRNA were not different

although there were no significant differences in TopoII-alpha mRNA expression levels among four histological subtypes of lung cancer (Fig. 2b). On the other hand, the expression levels of TopoII-beta in lung cancer cell lines were similar to those of normal lung tissues, although relatively higher expression levels were observed in SCLC and large cell carcinoma (Fig. 2c). In addition, we checked TopoII-alpha and TopoII-beta protein expressions in two lung cancer cell lines, SK-LC-2 and SK-MES-1, representing high and low expression of the two TopoII isoforms, and found that protein expression patterns of these genes were not different with mRNA expression patterns (Fig. 2e).

There were weak but significant positive correlations between RanBP2 and TopoII-alpha mRNA expressions, between RanBP2 and TopoII-beta mRNA expressions and between TopoII-alpha and TopoII-beta mRNA expressions among 20 lung cancer cell lines ($r = 0.532$; $P < 0.05$, Fig. 3a and $r = 0.623$; $P < 0.05$, Fig. 3b, $r = 0.647$; $P < 0.01$, Fig. 3c, respectively). Chemosensitivity data were analyzed in relation to the mRNA expression levels of the RanBP2, TopoII-alpha, TopoII-beta genes using linear regression analysis. No significant associations were observed between the IC50 values of amrubicin and the mRNA expression levels of RanBP2 (Fig. 4a), TopoII-alpha (Fig. 4b) and TopoII-beta (Fig. 4c) among 20 cell lines.

Discussion

RanBP2 has been reported to be involved in both nucleocytoplasmic transport and mitosis and also act as a SUMO ligase for DNA TopoII and play a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis [5]. In addition, RanBP2 hypomorphic mice are particularly sensitive to spontaneous and carcinogen-induced lung tumors, indicating that RanBP2 might play a potential tumor suppressor role in human lung cancer. Two previous studies reported that RanBP2 mRNA expression levels are substantially reduced in human non-SCLC [2, 8]. However, the present study showed that RanBP2 transcript levels were infrequently downregulated in human lung cancer cell lines compared with normal lung tissues, although there were statistically significant differences in the RanBP2 expression between SCLC and NSCLC. Consistent with our results, several lines of evidence from publicly available human gene expression data of the Oncomine

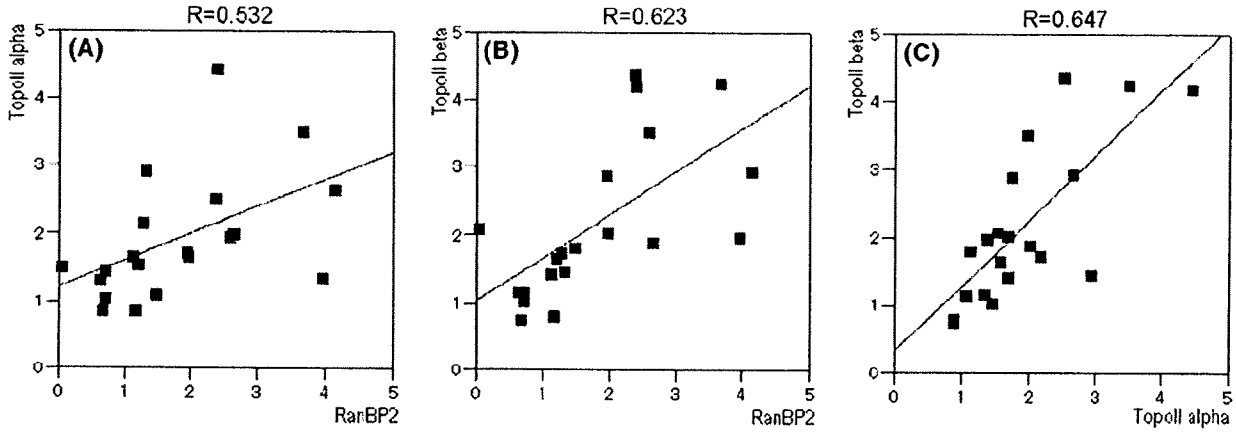

Fig. 3 Correlations between a RanBP2 and TopoII alpha mRNA expression, b RanBP2 and TopoII beta mRNA expression and c TopoII alpha and TopoII beta mRNA expression in lung cancer cell lines

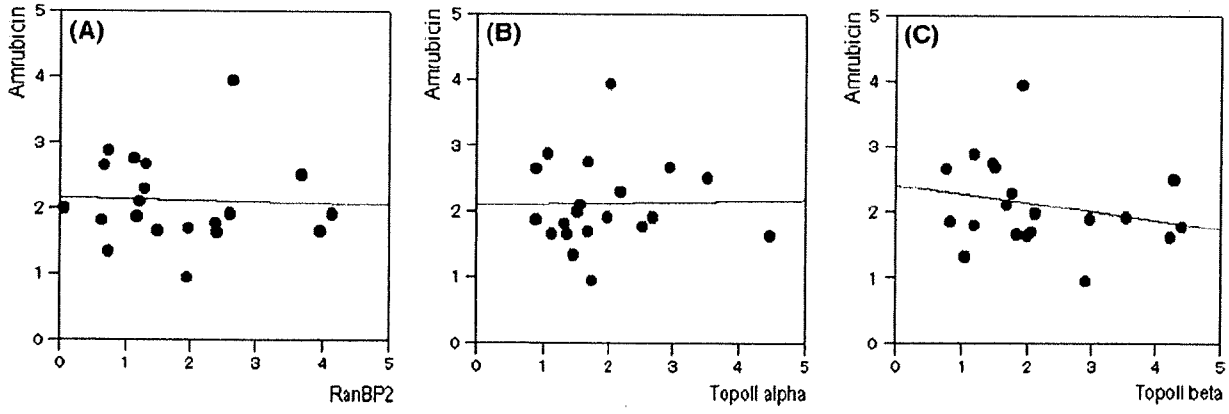

Fig. 4 Associations between relative mRNA expression for (a) RanBP2, (b) TopoII alpha and (c) TopoII beta and chemosensitivity of Amrubicin Log(IC50 in nM)

database (http://www.oncomine.com) and GEO profiles (http://www.ncbi.nlm.nih.gov/geo/) reported that RanBP2 mRNA expression levels are not reduced in NSCLC compared with normal lung tissues [3, 22, 26–28, 30]. In addition, there is a microarray study showing that RanBP2 expression levels are similar to those of our data in four overlapping lung cancer cell lines [9]. The concordance and discordance between our findings and previous works might be caused by the difference between cell lines and resected human lung tumors as well as the different experimental conditions used. Thus, further studies are warranted to establish the role of RanBP2 as a tumor suppressor gene in human lung carcinogenesis.

In RanBP2 hypomorphic murine embryonic fibroblasts (MEFs), formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by mutation or chemical inhibition of TopoII-alpha [4], was observed, while spindle structure, kinetochore–microtubule interactions, and localization of kinetochore and spindle assembly checkpoint proteins appeared normal [5]. Therefore, the low expression of RanBP2 may have an analogous effect of TopoII inhibitors, although the inhibitors are able to cause an inevitable consequence of DNA damage at high doses [4, 21]. Then, we speculated that there might be an association between RanBP2 mRNA expression and chemosensitivity of a TopoII inhibitor, amrubicin and tested whether we could see it using human lung cancer cell lines. However, we did not find any associations, suggesting that cytotoxicity of amrubicin might come mainly from DNA damage response induced at high doses and that formation of chromatin bridges in anaphase caused by low expression of the RanBP2 gene might not have additional effects on amrubicin-induced DNA damage response.

The two isozymes, TopoII-alpha and TopoII-beta function to unknot and decatenate covalently closed circles of DNA, although functional differences of these isozymes

and their differential spliced variants as well as precise role of their homodimerization and heterodimerization are unknown [20, 21]. There are several lines of evidence indicating a close relationship between TopoII-alpha levels and drug sensitivity in cell lines made resistant to TopoII inhibitors [7, 17, 25], cell lines with reduced expression of TopoII [1] and a VP-16-resistant breast cancer cell line infected with adenovirus containing TopoII-alpha [32]. Another study has shown the relationship between TopoII expression and multidrug sensitivity including TopoII inhibitors using eight human lung cancer cell lines [10]. There is also some evidence that TopoII-beta may be related with resistance to TopoII inhibitors [6, 15]. However, we did not find any association between expression levels of TopoII isoforms and chemosensitivity of amrubicin. Consistent with our results, a previous report of unselected human lung cancer cell lines also showed no clear association between TopoII-alpha protein expression and in vitro sensitivity to TopoII inhibitors [31]. Another study also failed to show importance of the enzyme using a panel of cell lines [12]. Although the behavior of cell lines in vitro may differ from the in vivo situation, and depend on the experimental conditions, these contradictory findings may require further investigation.

Amrubicin is highly active and one of the most potent anticancer drugs against SCLC and NSCLC [14]. Among the toxicities, hematologic adverse events such as leukopenia and thrombocytopenia are frequent and dose-limiting factors. Although identification of molecular biomarkers with the potential to predict treatment outcomes is essential to eliminate the use of any ineffective agents and to avoid toxic side effects [16], the cellular response to amrubicin is still poorly understood. To predict drug response in lung cancer patients, integrated analyses such as array-based mRNA expression profile, epigenome profiles, proteome analysis would be needed.

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ORIGINAL ARTICLE

hOGG1 Ser326Cys polymorphism and risk of lung cancer by histological type

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Human 8-oxoguanine DNA glycosylase 1 (*hOGG1*) has a major role in the repair of 8-hydroxyguanine, a major promutagenic DNA lesion. The genetic polymorphism rs1052133, which leads to substitution of the amino acid at codon 326 from Ser to Cys, shows functional differences, namely a decrease in enzyme activity in *hOGG1*-Cys326. Although several studies have investigated the association between rs1052133 and lung cancer susceptibility, the effect of this locus on lung cancer according to histology remains unclear. We therefore conducted a case–control study with 515 incident lung cancer cases and 1030 age- and sex-matched controls without cancer, and further conducted a meta-analysis. In overall analysis, the homozygous Cys/Cys genotype showed a significant association with lung cancer compared to Ser allele carrier status (odds ratio (OR)=1.31, 95% confidence interval (CI)=1.02–1.69). By histology-based analysis, the Cys/Cys genotype showed a significantly positive association with small-cell carcinoma (OR=2.40, 95% CI=1.32–4.49) and marginally significant association with adenocarcinoma (OR=1.32, 95% CI=0.98–1.77). A meta-analysis of previous and our present study revealed that this polymorphism is positively associated with adenocarcinoma, although suggestive associations were also found for squamous- and small-cell lung cancers. These results indicate that rs1052133 contributes to the risk of adenocarcinoma of lung.
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Keywords: *hOGG1*; lung cancer; polymorphism

INTRODUCTION

Cancer is linked to environmental exposure to various carcinogens, of which tobacco smoke is a well-known example. Exposure leads to various types of DNA damage, such as oxidative damage. Genetic variations in DNA repair genes are associated with DNA repair capacity, suggesting a consequent association with cancer risk.[1]

8-Hydroxyguanine, produced by reactive oxygen species in tobacco smoke, is a major form of DNA damage.[2] This alteration to the DNA structure causes G:C to T:A transversions, and may thus be responsible for mutations that lead to carcinogenesis.[3] Human 8-oxoguanine DNA glycosylase 1 (*hOGG1*) has been extensively studied as the main enzyme involved in the repair of 8-oxoG DNA adducts. Although it has a major role in the repair of 8-hydroxyguanine, however, its role in carcinogenesis has not been well elucidated.[4] Genetic polymorphisms of *hOGG1* have been documented, and the polymorphism Ser326Cys (rs1052133) is associated with complementation activity for *Escherichia coli* mutants that are defective in the repair of 8-hydroxyguanine. Activity in the repair of 8-hydroxyguanine is greater with the *hOGG1*-Ser326 protein than the *hOGG1*-Cys326 protein,[5] and the possible contribution of this locus to the risk of a variety of human cancers has been reported.[6]

A number of studies[7–14] and systematic approaches[15–17] have examined the role of the Ser326Cys polymorphism in lung cancer susceptibility. One meta-analysis showed that the overall odds ratio (OR) of homozygotes for the *hOGG1*-326Cys allele against those for the *hOGG1*-326Ser allele was 1.24 (95% confidence interval (CI)=1.01–1.53), suggesting that the locus is involved in susceptibility to overall lung cancer.[17] In contrast, another meta-analysis reported no significant association.[15] A recent pooled analysis from the International Lung Cancer Consortium involving a substantial number of cases and controls showed a suggestive association for this polymorphism in Caucasians.[16] One question that remains unanswered is whether the impact of rs1052133 differs according to histological subtype of lung cancer.

Here, we evaluated the role of the *hOGG1* Ser326Cys polymorphism in lung cancer susceptibility among a Japanese population in

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consideration of histology. We also conducted a meta-analysis of the literature to evaluate the impact of this polymorphism by histology.

MATERIALS AND METHODS

Subjects

The case subjects were 515 patients who were newly and histologically diagnosed with lung cancer and who had no history of cancer. Controls were randomly selected from among the 2395 cancer-free individuals and matched by age (±3 years) and sex to cases in a 1:2 case/control ratio. All subjects were recruited within the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC), as described elsewhere,[18,19] and were exactly the same cohort we reported on in a previous paper.[20] In brief, information on lifestyle factors was collected using a self-administered questionnaire from all first-visit outpatients at Aichi Cancer Center Central Hospital aged 18–79 who were enrolled in the HERPACC between January 2001 and November 2005. Response was checked by a trained interviewer. Outpatients were also asked to provide blood samples. Each patient was asked about their lifestyle when healthy or before the current symptoms developed. Approximately 95% of eligible subjects completed the questionnaire and 60% provide blood samples. The data were loaded into the HERPACC database and routinely linked with the hospital-based cancer registry system to update the data on cancer incidence. All participants gave written informed consent and the study was approved by institutional ethical committee of Aichi Cancer Center.

Genotyping of *hOGG1*

DNA from each sample was extracted from the buffy coat fraction using a BioRobot EZ1 with an EZ1 DNA Blood 350 μl kit or QIAamp DNA Blood mini kit (Qiagen KK, Tokyo, Japan). Polymorphisms of *hOGG1* Ser326Cys were examined based on TaqMan assays by Applied Biosystems (Foster City, CA, USA). The principle of the TaqMan real-time polymerase chain reaction (PCR) assay system using fluorogenic probes and 5′ nuclease has been described by Livak.[21] All of the assays were carried out in 96-well PCR plates using a 7500 Fast Real-Time PCR System (Applied Biosystems) coupled with the 7500 Fast System SDS software. Amplification reactions (5 μl) were carried out in duplicate with 30 ng of template DNA, 2× TaqMan Universal Master Mix buffer (Applied Biosystems) and 20× primer and probe mix (Applied Biosystems). Thermal cycling was initiated with a first denaturation step of 20 s at 95 °C, and then by 40 cycles of 3 s at 95 °C and 30 s at 62 °C. Genotyping quality was statistically assessed using the Hardy–Weinberg test in our laboratory; when allelic distributions for controls departed from the Hardy–Weinberg frequency, genotyping was assessed using another method.

Consumption of tobacco, alcohol, fruits and vegetables

Cumulative smoking dose was evaluated as pack-years (PY), the product of the number of packs consumed per day and the number of years of smoking. Smoking habit was entered in the four categories of never, former, and current smokers of <40 and ≧40 PY. Former smokers were defined as those who quit smoking at least 1 year before the survey. Drinking habit was categorized in the three categories of never, former and current drinkers. Former drinkers were defined as those who quit drinking at least 1 year before the survey. Consumption of fruits and vegetables was determined using a semiquantitative food frequency questionnaire (SQFFQ), described in detail elsewhere.[22] Briefly, the SQFFQ consisted of 47 single food items with frequencies in eight frequency categories. We estimated average daily intake by multiplying the frequency of intake by the serving size of food (in grams). Energy-adjusted intake of fruits and vegetables was calculated by the residual method.[23] The SQFFQ was validated using a 3-day weighed dietary record as standard, which showed that reproducibility and validity were acceptable.[24]

Statistical analysis

To assess the strength of associations between *hOGG1* polymorphism and risk of lung cancer, we estimated ORs with 95% CIs, using conditional logistic models adjusted for potential confounders. For stratified analyses exploring interactions, we applied unconditional logistic regression models because matching was not retained after stratification by smoking and drinking habit and carotene intake in conditional models. Fruit and vegetable intake was categorized into three levels by applying thresholds of tertiles among controls. Potential confounders considered in the multivariate analyses were age, sex, smoking habit (never smokers, former smokers, current smokers of less than 40 or 40 or more PY), drinking habit (never, former and current drinkers), total energy intake (as a continuous variable), and dietary fruit and vegetable intake (g per day, tertiles). Missing values for each covariate were treated as dummy variables and were included in the model. Trend for genotype was assessed by application of a score test value for each genotype (0, homozygous for reference allele or combined reference genotypes; 1, heterozygote or one reference genotype and 2, homozygous nonreference allele or nonreference genotype). Differences in categorized demographic variables between cases and controls were tested by the χ^2-test. Mean values for age and total energy intake were compared for cases and controls by Wilcoxon's signed-rank test. Accordance with the Hardy–Weinberg equilibrium was checked for controls using the χ^2-test and the exact *P*-value was used to assess any discrepancies between

Table 1 Characteristics of case and control subjects

	Cases (n=515) n (%)	*Controls* (n=1030) n (%)	P-value
Age			
<50	53 (10.3)	108 (10.5)	
50–59	142 (27.6)	283 (27.5)	
60–69	193 (37.5)	389 (37.8)	
70–79	127 (24.7)	250 (24.3)	1.00
Mean age (range)	61.9 (23–79)	61.8 (26–79)	0.87
Sex			
Male	381 (74.0)	762 (74.0)	
Female	134 (26.0)	268 (26.0)	1.00
Smoking (Pack-years)			
<5	136 (26.4)	424 (41.2)	
5–19.9	31 (6.0)	118 (11.5)	
20–39.9	88 (17.1)	208 (20.2)	
>40	258 (50.1)	275 (26.7)	<0.001
Unknown	2 (0.4)	5 (0.5)	
Drinking status			
Never	196 (38.1)	378 (36.7)	
Former[a]	15 (2.9)	56 (5.4)	
Current	304 (59.0)	596 (57.9)	0.08
Fruit/vegetable consumption (g per day)			
Tertile 1 (<118.4)	199 (38.8)	342 (33.2)	
Tertile 2 (118.4–211.3)	140 (27.3)	341(33.1)	
Tertile 3 (>211.4)	166 (32.4)	341(33.1)	0.03
Unknown	8(1.6)	6 (0.6)	
Total energy intake (kcal, s.d.)[b]	1670 (371)	1676 (352)	1.00
Histology			
AD	316 (61.4)		
SQ	91 (17.7)		
SM	55 (10.7)		
LA	40 (7.8)		
Others	13 (2.5)		

Abbreviations: AD, adenocarcinoma; LA, large-cell carcinoma; SM, small-cell carcinoma; SQ, squamous-cell carcinoma.
[a]Former drinkers were defined as subjects who had quit drinking at least 1 year previously.
[b]Energy-adjusted.

genotypes and allele frequencies, with a *P*-value of less than 0.05 considered statistically significant. All analyses were performed using STATA version 10.1 (Stata, College Station, TX, USA).

Meta-analysis

We conducted a meta-analysis of relevant articles reporting associations between the *hOGG1* polymorphism and lung cancer in consideration of the histological subtypes adenocarcinoma, squamous-cell carcinoma and small-cell carcinoma. Medline was searched for papers published between January 1995 and March 2009 and indexed with the terms (lung neoplasms AND (hOGG1 OR OGG1)). Inclusion criteria were (1) reporting of ORs or risk ratios calculated by comparing the Ser/Ser to the Cys/Cys or Cys allele carrier according to histological subtype; (2) a cohort, nested case–control, population-based case–control or hospital-based case–control study design and (3) use of cancer-free controls. All potentially relevant papers were independently reviewed by at least two investigators (TO and KM) and any disagreements were resolved by consensus. The reference lists of studies identified through the search process were also checked. Among the 65 papers identified through this process, 7 were considered eligible.[5,7–11,17] Two investigators (TO and KM) abstracted the data independently. We used OR from a random-effect model as a summary statistic for association.[25] Heterogeneity among the studies was examined based on the *Q* and I^2 statistics. The latter indicates the proportion of variation in summary estimates attributable to heterogeneity.[26] We determined which model to use to calculate summary OR and its 95% CI, a random- or fixed-effect model, based on significance in the *Q* statistics. The meta-analysis was conducted using the 'metan' command[27] in STATA version 10.1.

RESULTS

Characteristics of the 515 cases and 1030 controls are shown in Table 1. Age and sex were appropriately matched. Smoking habits differed remarkably between cases and controls, with the proportion of current smokers of 40 PY or more significantly higher in cases. Former drinkers tended to be more common among cases, albeit without statistical significance. Consumption of fruits and vegetables was significantly lower among cases. The distribution of histological type among cases was as follows: adenocarcinoma, 61.4% (n=316); squamous-cell carcinoma, 17.7% (n=91); small-cell carcinoma, 10.7% (n=55); large cell carcinoma, 7.8% (n=40) and others, 2.5% (n=13).

Table 2 presents the frequency distribution of *hOGG1* genotypes and ORs with 95% CI for lung cancer cases compared with controls. No significant dissociation from the Hardy–Weinberg equilibrium was observed among controls. In overall analysis, Cys/Cys showed a significantly positive association with lung cancer. The confounder-adjusted OR for Cys/Cys relative to Ser/Ser+Ser/Cys was 1.31 (1.02–1.69, P=0.036). In histology-based analysis, those with the Cys/Cys genotype were at significantly increased risk of small-cell carcinoma and marginally significantly increased risk of adenocarcinoma, compared to those with the Ser/Cys and Ser/Ser genotypes combined. No significant associations were observed for squamous-cell carcinoma.

Table 3 shows associations between *hOGG1* Ser326Cys polymorphism combined with smoking and lung cancer risk. In overall analysis, the effect of cumulative smoking dose was stronger in those with Cys/Cys. In analyses by histology, a similar trend was observed for adenocarcinoma and small-cell carcinoma but not for squamous-cell carcinoma. This trend was more prominent for small-cell carcinoma. Adjusted ORs for heavy smoking (PY≧40) were 26.3 (5.34–129.6) for the Ser allele carrier and 72.3 (14.6–358.2) for those with the Cys/Cys.

To further examine the impact of *hOGG1* Ser326Cys polymorphism according to histology, we conducted a meta-analysis. Table 4 shows a summary of studies that have investigated the association between *hOGG1* Ser326Cys polymorphism and lung cancer risk, including the present study. As shown in Figure 1, *hOGG1* Ser326Cys polymorphism summary ORs showed a significant association with adenocarcinoma (OR=1.44, 95% CI=1.18–1.77) with no significant heterogeneity. Although squamous-cell carcinoma showed a similarly increased risk (OR=1.81, 95% CI=1.06–3.07), the significant heterogeneity across studies (I^2=58.5) was a limitation. Although without significance and from a limited number of studies, the pooled estimate was 2.05 (0.91–4.63), suggesting an increased risk for small-cell carcinoma.

Table 2 *hOGG1* genotype distribution and ORs for lung cancer

Genotype	*Cases* n=*515*	*Controls* n=*1030*	*OR1 (95% CI)*[a]	*P-value*	*OR2 (95% CI)*[b]	*P-value*
Overall						
Ser/Ser	117	250	1.00 (reference)		1.00 (reference)	
Ser/Cys	257	544	1.01 (0.77–1.32)		0.96 (0.72–1.26)	
Cys/Cys	141	236	1.28 (0.94–1.73)	0.054	1.27 (0.93–1.75)	0.047
Ser/Ser+Ser/Cys	374	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	141	236	1.27 (1.00–1.62)	0.05	1.31 (1.02–1.69)	0.036
Adenocarcinoma						
Ser/Ser+Ser/Cys	227	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	89	236	1.29 (0.97–1.72)	0.085	1.32 (0.98–1.77)	0.066
Squamous-cell carcinoma						
Ser/Ser+Ser/Cys	72	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	19	236	0.99 (0.58–1.70)	0.98	1.10 (0.63–1.94)	0.73
Small-cell carcinoma						
Ser/Ser+Ser/Cys	34	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	21	236	2.22 (1.26–3.92)	0.006	2.40 (1.22–4.12)	0.009

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex, smoking habit, drinking habit, total energy intake and energy-adjusted fruit/vegetable intake.

Table 3 Associations between *hOGG1* Ser326Cys polymorphisms and smoking by PY on lung cancer risk

	Ser (+)		*Cys/Cys*	
Histology	*Case/Control*	*OR (95% CI)*[b]	*Case/Control*	*OR (95% CI)*[b]
Overall[a]				
Smoking (pack-years)		Ser (+)		Cys/Cys
<5	95/317	1.0 (reference)	41/107	1.35 (0.88–2.09)
5–19.9	22/89	1.26 (0.72–2.21)	9/29	1.29 (0.55–3.03)
20–39.9	66/160	2.38 (1.53–3.68)	22/48	2.54 (1.39–4.63)
>40	191/223	5.26 (3.54–7.78)	67/52	7.44 (4.53–12.2)
Adenocarcinoma				
Smoking		Ser (+)		Cys/Cys
<5	89/317	1.0 (reference)	39/107	1.36 (0.87–2.13)
5–19.9	15/89	0.95 (0.50–1.79)	5/29	0.70 (0.23–2.13)
20–39.9	39/160	1.62 (0.98–2.66)	13/48	1.75 (0.86–3.54)
>40	84/223	2.75 (1.77–4.28)	30/52	3.99 (2.25–7.08)
Squamous-cell carcinoma				
Smoking		Ser (+)		Cys/Cys
5–19.9	3/406	1.0 (reference)	3/136	3.22 (0.64–16.3)
20–39.9	13/160	6.99 (1.93–25.4)	5/48	8.99 (2.04–39.5)
>40	56/223	19.5 (5.87–64.3)	11/52	16.6 (4.37–63.0)
Small-cell carcinoma				
Smoking		Ser (+)		Cys/Cys
5–19.9	2/406	1.0 (reference)	1/136	1.50 (0.13–16.7)
20–39.9	8/160	12.6 (2.39–66.2)	4/48	18.8 (3.09–114.3)
>40	24/223	26.3 (5.34–129.6)	16/52	72.3 (14.6–358.2)

Abbreviations: CI, confidence intervals; OR, odds ratios.
[a]Five controls and two cases are excluded from analysis because of smoking information unknown.
[b]ORs were adjusted for age, sex, smoking habit, drinking habit, total energy intake and energy-adjusted fruit/vegetable intake.

Table 4 Summary of published studies examining association between *OGG1* polymorphism and lung cancer risk according to histology

		Subjects in each study						*Odds ratio (95% CI) for Cys/Cys relative to Ser/Ser*		
		Case								
Author	*Year*	*Total*	*Adeno*	*Squamous*	*Small*	*Control*	*Ethnicities*	*Adeno*	*Squamous*	*Small*
Sugimura *et al.*[7]	1999	241	1974	78	118	197	Japanese	1.34 (0.53–3.39)	2.27 (0.92–5.60)	0.51 (0.09–2.87)
Wikman *et al.*[8]	2000	105	50	50	NA	105	Caucasian	1.84 (0.41–14.41)	1.76 (0.24–13.1)	NE
Ito *et al.*[9]	2002	138	138	0	0	241	Japanese	0.81 (0.44–1.52)	NE	NE
Le Marchand *et al.*[10]	2002	298	141	66	43	405	Caucasian, Japanese and Hawaiian	2.1[a] (1.1–3.9)	3.7[a] (1.7–8.3)	3.4[a] (1.1–10.4)
Park *et al.*[11]	2004	179	63	56	32	358	Caucasian	4.20 (1.10–15.8)	4.8 (1.1–21.0)	NE
Hung *et al.*[17]	2005	2188	499	902	0	2198	Caucasian	1.66 (1.04–2.66)	1.02 (0.63–1.64)	NE
Kohno *et al.*[12]	2006	1097	1097	0	0	394	Japanese	1.47 (1.02–2.13)	NE	NE
Our study	2009	515	316	91	55	1030	Japanese	1.32 (0.98–1.77)	1.10 (0.63–1.94)	2.40 (1.22–4.12)

Abbreviations: CI, confidence intervals; NE, not estimated; OR, odd ratios.
[a]ORs are calculated as that of the homozygous Cys/Cys genotype compared to those with the Ser/Ser and Ser/Cys genotype combined.

DISCUSSION

In this case–control study, we found that the *hOGG1* 326Cys/Cys genotype, which results in weaker activity, was associated with a significantly increased risk of lung cancer overall. By subtype we found a significant association of the Cys/Cys genotype with small-cell carcinoma and a marginally significant association with adenocarcinoma. Moreover, in our subsequent meta-analysis of epidemiological studies based on histology, we observed that this genotype was associated with an increased risk of adenocarcinoma. Although results for squamous- and small-cell carcinoma were not conclusive,

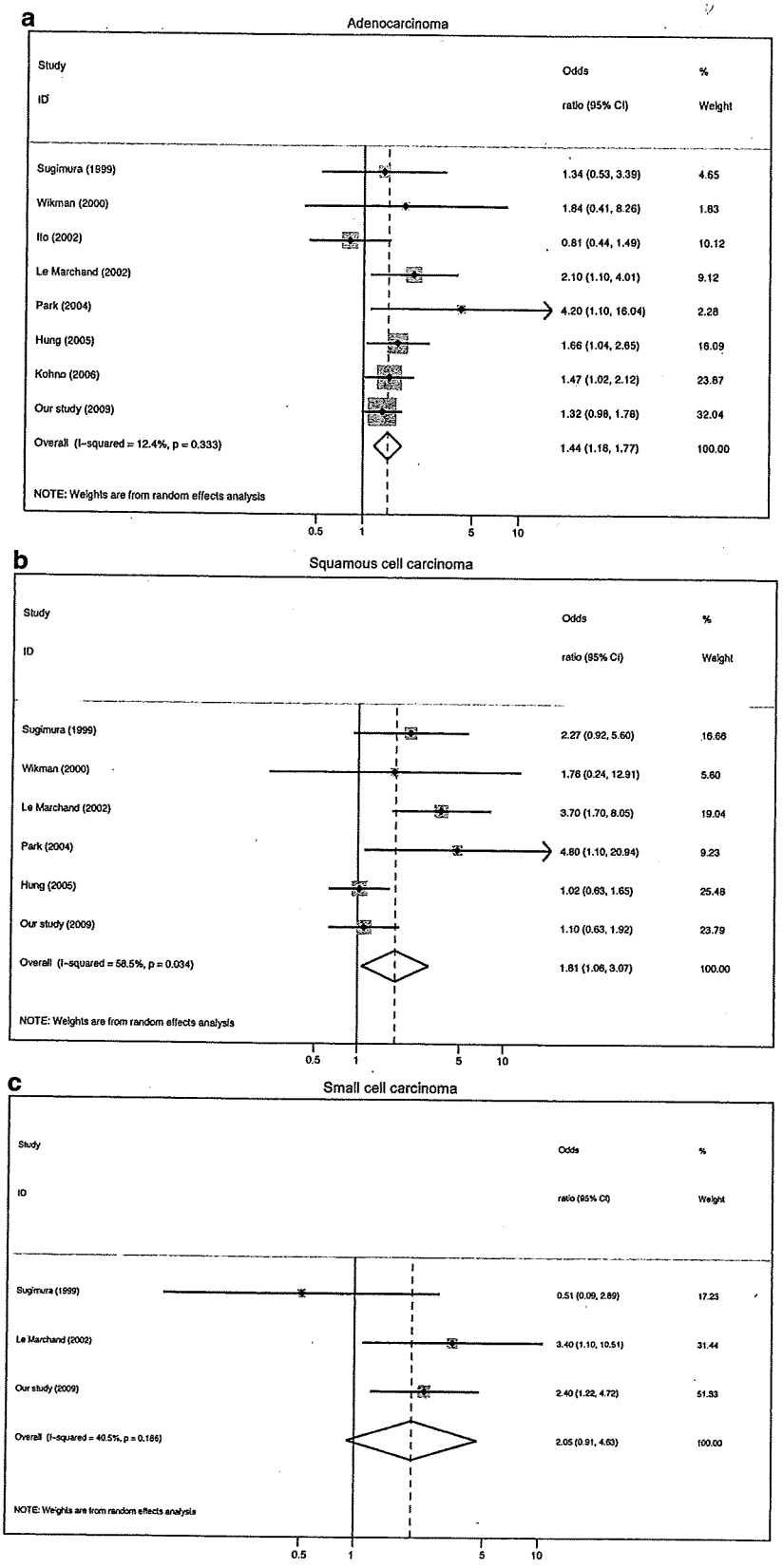

Figure 1 Meta-analysis for human 8-oxoguanine DNA glycosylase 1 (hOGG1) polymorphism according to histological subtype. A meta-analysis was conducted of the studies listed in Table 4. We extracted the ORs and 95% CIs for the Cys/Cys homozygotes of *hOGG1* relative to the Ser/Ser according to histological subtype from each study. We applied a random-effect model. An OR >1.0 indicates a higher risk with the Cys/Cys genotype than with Ser/Ser homozygotes. I^2 indicates the proportion of variation in summary estimates attributable to heterogeneity. All analyses were performed using the 'metan' command in STATA (version 10.1).

we also identified a potentially increased risk for these types of lung cancer.

Results of a number of studies examining the role of the *hOGG1* Ser326Cys polymorphism in lung cancer susceptibility conducted to date have been inconsistent.[7–12,17] Our case–control study showed a significant association between *hOGG1* Ser326Cys polymorphism and lung cancer overall, supporting the potential effect of this polymorphism on lung cancer susceptibility. Because the question of whether the effect of this polymorphism differed by histology remained unanswered, we also conducted a meta-analysis with consideration to histology. To the best of our knowledge, this is the first report to summarize the association between *hOGG1* polymorphism and susceptibility by histological type. Results of our meta-analysis indicated that the effect is consistent for adenocarcinoma, but not for squamous- or small-cell carcinoma. This inconsistency might be due to the heterogeneity of populations and distribution of subtypes across studies. The subjects included in the analyses were mainly Japanese and Caucasian. The most common subtype was adenocarcinoma in Japanese but squamous-cell carcinoma in Caucasians. Given that the magnitude of effect of smoking on risk differs by histological subtype,[28] the magnitude of effect of the *hOGG1* polymorphism might also differ across subtypes and populations. Even within the same histological subtype, the effect of smoking differs with the presence of certain gene mutations in cancer.[29] A comprehensive understanding of the *hOGG1* polymorphism will thus require further study, with particular focus on squamous- and small-cell carcinomas.

Our case–control study had several potential limitations. One methodological issue was the selection of hospital-based patients without cancer as controls. However, because cases and controls were selected from the same hospital and almost all patients lived in the Tokai area of central Japan, the internal validity of this case–control study is likely acceptable. External validity (generalizability of the results) has been confirmed in our previous study.[30] In addition, to dilute any bias that might have resulted from the inclusion of a specific diagnostic group that is related to the exposure, we did not set eligibility criteria for control diseases. As for allele frequencies in the subjects, given that our frequencies were comparable to those previously reported in public databases such as HapMap JPT,[31] bias in the distribution of selected polymorphisms was negligible. Second, the self-reported values for lifestyle factors considered as potential confounders may be inaccurate. If present, however, any such misclassification would likely be nondifferential, and would likely underestimate the causal association. The meta-analysis was based on published data, and the potential for publication selection bias could not be ruled out even if heterogeneity across the studies was limited for adenocarcinoma.

In conclusion, we found a positive association between lung cancer and Cys/Cys individuals in a Japanese population. The association was clear for small-cell carcinoma and adenocarcinoma of the lung in this population. Further systematic evaluation revealed that associations with the locus were conclusive for adenocarcinoma. Further studies are needed to clarify the effect of genotype on squamous-cell carcinoma and small-cell carcinoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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