vomiting and omission of day 8 at level 2. This patient also presented grade 3 hyperbilirubinemia suspected to be drug-induced hepatitis, and died 16 days after the start of the treatment. The worst value of his laboratory data was 6.6 mg/dl in total bilirubin on day 12, 40 IU/l in AST on day 7 and 103 IU/l in ALT on day 7. He had a past history of drug-induced hepatitis related to aspirin. The excluded patient was administered GEM at 800 mg/body. Despite the dose was approximately two-thirds of the planned dose, he experienced grade 3 nausea/vomiting and the treatment was discontinued. The median number of administered cycle was 1. The actual administered cycles were one in seven patients, two in one patient, three in two patients and four in two patients. The reasons for the discontinuation in seven patients who terminated the treatment at one cycle were toxicity for three patients, patient refusal for two patients, treatment delay for one patient and both toxicity and disease progression was for one patient.

Anti-tumor Activity

There were seven stable diseases and five progressive diseases (PD). No partial or complete response was observed (Table 4). Four patients received second-line chemotherapy after GC: docetaxel for two patients and gefitinib for two patients. One patient received gefitinib experienced partial response; however, remaining three patients had PD also in the second-line treatment. The MST was 3.8 months (Fig. 1).

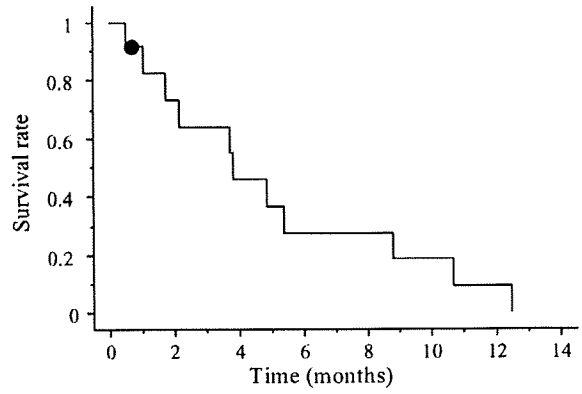

Figure 1. Kaplan–Meier curve of overall survival. Median overall survival time was 3.8 months.

DISCUSSION

This is the first PS 2-specific Phase I study of GC in Japanese patients, and the MTD and recommended dose were determined to be GEM 1000 mg/m^2 and CBDCA AUC of 4.

The recommended dose of CBDCA was lower than other studies conducted in the USA (19,20). With respect to the dose of CBDCA, the method of measuring serum creatinine values is critical. In Japan, most institutions use the enzymatic method, whereas the Jaffe method remains the mainstream in the USA (21). According to the study comparing these two methods, serum creatinine values are higher in the Jaffe method than in the enzymatic method by ~0.2 mg/dl (21). Therefore, at the same AUC, higher CBDCA dose is administered in Japan than in the USA. Incidentally, based on the Calvert formula, a difference of 0.2 mg/dl of creatinine leads to the difference of AUC = 1. In short, the AUC = 4 in Japan roughly corresponds to the AUC = 5 in the USA. For global clinical trials, the difference of methods for measurement of laboratory data also should be paid attention to.

Table 4. Drug delivery and anti-tumor efficacy

Dose level	Number of patients	Median course (range)	Overall response			
			CR	PR	SD	PD
1	6	1 (1–4)	0	0	4	2
2	6	2 (1–4)	0	0	3	3

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

PS is one of the most powerful and reliable prognostic factors in advanced NSCLC (6–8), and a worse PS is characterized by lower response rate to chemotherapy and shorter survival (9,10). Median survival of patients with PS 2 is substantially shorter than that of patients with PS 0 or 1. Moreover, patients with PS 2 are at higher risk for severe toxicity than those with better PS. According to the population-based surveys, up to 30–40% of all advanced NSCLC is characterized PS 2 (22,23). Namely, patients with PS 2 constitute a distinctive, non-trivial subgroup in NSCLC. However, little attention has been paid to this special patient population until recently.

The guidelines from the American Society of Clinical Oncology support the single use of third-generation non-platinum agents for patients with PS 2 (24). This recommendation is mainly based on the results of Phase III trials comparing single-agent chemotherapy with best supportive care alone, in which good tolerability and significant survival benefit or improvement of QOL with single-agent chemotherapy have been demonstrated (25–28). However, PS 2 patients accounted for a small proportion of patients in those trials and any conclusive evidence cannot be drawn for the treatment of patients with PS 2. At present, available data from PS 2-specific clinical trials are quite limited. In this context, no consensus has been developed on the standard chemotherapy in patients with PS 2.

The role of adding platinum to third-generation single agents is still unclear. Recently, the Norwegian Lung Cancer Study Group reported the results of a retrospective study that compared the outcome of patients with PS 2 to that of patients with PS 0 or 1 who had participated in randomized trials comparing two third-generation, CBDCA-based

regimens (29). According to the retrospective study, although MST of patients with PS 2 was significantly shorter than that of patients with PS 0 or 1 (4.5 vs. 8.9 months; $P < 0.01$), toxicity was acceptable for patients with PS 2 and they achieved better symptom improvement compared with patients with PS 0 or 1. ECOG conducted the first PS 2-specific randomized trial (19). In the randomized Phase II trial, two platinum-based chemotherapy regimens, PTX + CBDCA (PC) and GP, have been compared, and both regimens were proved feasible with acceptable toxicity. However, survival time was quite limited in both treatment arms: MST was 6.2 months for PC and 6.9 months for GP, respectively. A Greece Group performed a randomized Phase II trial comparing non-platinum single-agent chemotherapy with CBDCA-based chemotherapy (30). In the study, patients were randomly assigned to either GC or GEM alone and MST was 6.7 months for GC and 4.8 months for GEM alone, respectively ($P = 0.49$), whereas neutropenia ($P = 0.007$) and thrombocytopenia ($P < 0.001$) were more common in GC arm. In contrast, according to a subgroup analysis of the Cancer and Leukemia Group B study 9730 comparing PC with PTX alone, patients with PS 2 (107 patients, 18% of the population) achieved significantly better survival when they were treated with PC than those treated with PTX alone (20). Thus, the role of platinum-based chemotherapy for patients with PS 2 is still controversial.

The results could vary even between PS 2-specific trials due to two major reasons. First, determining PS score is inevitably subjective, there is considerable inter-observer variation even between healthcare professionals (31). Second, there can be significant heterogeneity in the PS 2 patient population: the reasons for impaired PS may be due to tumor-related (such as pain, fatigue and weight loss), to pre-existing co-morbidities (such as chronic obstructive pulmonary disease, cardiovascular disease and age-related decline in functional status) or both, furthermore (32). There is a clear need for a more objective classification system that takes into account the individual effects of disease-related symptoms and co-morbidities. The common co-morbidity scales are the Cumulative Illness Rating Scale-Geriatric (CIRS-G) and the Charlson scale. Their prognostic impacts have been validated prospectively (33,34). Moreover, they are more objective than PS. Although our study did not, all future studies for PS 2 patients should use such co-morbidity scales to stratify patients more accurately.

Recently, molecular-targeted agents, especially epidermal growth factor receptor tyrosine kinase inhibitors such as gefitinib or erlotinib, have been tested in clinical trials for patients with poor PS. Inoue et al. (35) conducted a Phase II trial of gefitinib in patients with NSCLC whose tumor harboring EGFR gene mutation. In the study, all patients were not feasible for cytotoxic chemotherapy due to poor PS: 26 of 29 patients were PS 2–4. Overall response rate and MST were 66% and 6.5 months, respectively. In addition, PS improvement rate was 79%, and no treatment-related deaths were observed. These excellent results strongly suggest that stratification with molecular status should be required in the future trial of PS 2 or more.

In this study, we determined the MTD and the recommended dose of GC in Japanese patients with PS 2. Response rate and overall survival of the regimen were disappointing. However, some previous studies clearly support the use of platinum agent in PS 2 patients (19,20). Future clinical trials for PS 2 patients should use more objective criterion such as co-morbidity scales in addition to PS in order to measure patients' risk more accurately. Such studies may reveal that which patients should be treated and not be treated with platinum-based chemotherapy among PS 2 patients.

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Conflict of interest statement

None declared.

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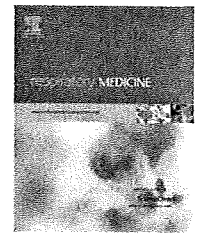

Trends in chemotherapy for elderly patients with advanced non-small-cell lung cancer

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KEYWORDS
Non-small-cell lung cancer;
Elderly;
Chemotherapy;
Third-generation;
Second-line

Summary
Background: In approximately the year 2000, the results of a number of important studies of non-small-cell lung cancer (NSCLC) were published.
Methods: Between July 1992 and December 2003, 223 patients with NSCLC aged ≥70 years received chemotherapy alone as their initial treatment at the National Cancer Center Hospital East. These patients were divided into 2 groups: those that began treatment between 1992 and 1999 (group A) and between 2000 and 2003 (group B). The details of chemotherapy regimens and outcomes were compared.
Results: In group A, 83% of patients received platinum-based chemotherapy, two-thirds of these regimens comprised platinum plus second-generation combination chemotherapy. In contrast, although 55% of patients received platinum-based chemotherapy in group B, 41% of patients received non-platinum-based chemotherapy. Among patients in group B, performance status was significantly associated with the selection of platinum-based or non-platinum-based chemotherapy; age was marginally associated with this selection. Median survival time (MST), 1-year survival rate, and 2-year survival rate were 6.7 months, 14%, and 7%, respectively, in group A, and 8.1 months, 35%, and 20% in group B ($p = 0.0109$). Multivariate analysis revealed that clinical stage and administration of salvage chemotherapy were independent prognostic factors.
Conclusions: In and after the year 2000, chemotherapy regimens changed greatly and survival of elderly patients significantly improved in our institute, and this improvement appears to be attributable mostly to the effect of salvage chemotherapy. These results suggest that even elderly patients should be offered salvage chemotherapy regardless of age, if possible.

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Introduction

Lung cancer is the leading cause of cancer-related death in many industrialized countries. Non-small cell lung cancer (NSCLC) accounts for approximately 80%–85% of all lung cancers, and the majority of patients have metastatic disease at the time of diagnosis.[1] Previous data indicate that more than 50% of advanced NSCLC are diagnosed in patients aged ≥65 years and about 30%–40% are diagnosed in patients ≥70 years of age.[2] NSCLC can therefore be regarded as a disease of the elderly, and the proportion of older adults among NSCLC patients is expected to progressively increase due to the aging of the populations of most developed countries.

Platinum-based combination chemotherapy improves survival and quality-of-life (QOL) in patients with advanced NSCLC, and is now widely accepted as the standard in chemotherapy.[3,4] However, platinum-based chemotherapy is often contraindicated in elderly patients because of patient deficits in functional status and organ function. In addition, elderly patients have approximately twice as many comorbidities as the general population.[5,6] Past studies on the effect of age on treatment choices for advanced NSCLC have revealed that elderly patients were less likely to receive active treatments.[7–9] Until recently, clinical trials for NSCLC have not specifically examined the importance of chronological age or the desirability of an upper age limit for treatment. Indeed, it has been reported that elderly patients are under-represented in those trials. In fact, according to a survey conducted by the Southwest Oncology Group, only 39% of patients enrolled in lung cancer trials between 1993 and 1996 were older than age 65, even though such patients represent 66% of the overall cancer patient population.[10]

The lack of clinical data on elderly NSCLC patients encouraged physicians to carry out elderly-specific clinical trials. In the 1990s, third-generation cytotoxic agents, such as vinorelbine (VNR), gemcitabine, docetaxel (DOC), paclitaxel, and irinotecan, were developed, and single-agent chemotherapy regimens using these agents were investigated in many phase II trials. In 1999, the results of the first elderly-specific phase III trial comparing VNR to best supportive care were published.[11] In that study, a significant survival benefit was seen in patients receiving VNR. Furthermore, VNR was well tolerated and QOL scores were better in patients treated with VNR. Then, in 2000, 2 phase III trials revealed a survival benefit for DOC when used as second-line chemotherapy agent, which was the first evidence for the effectiveness of second-line chemotherapy for NSCLC.[12,13] In addition, in 2002, as compared to other countries, approval for gefitinib, epidermal growth factor tyrosine kinase inhibitor (EGFR-TKI), was granted early in Japan.

These results should have a significant impact on clinical practice relating to advanced NSCLC in the elderly. In this study, we reviewed data on chemotherapy regimens used in the treatment of elderly NSCLC patients at our institute, and compared regimens and patient outcomes before and after year 2000.

Materials and methods

Patients

Between July 1992 and December 2003, 223 NSCLC patients aged ≥70 years received chemotherapy alone as their initial treatment at the National Cancer Center Hospital East. These patients were divided into 2 groups: those that began treatment between 1992 and 1999 (group A) and those that began treatment between 2000 and 2003 (group B). Chemotherapy regimens and outcomes were then compared between groups. Group B patients were then subdivided into 2 groups—the platinum-based chemotherapy group and the non-platinum-based chemotherapy group—and the clinical factors responsible for treatment choice were then analyzed. In addition, Group B patients were subdivided into another two groups; EGFR-TKI treated group or not-treated group. All patient data were obtained from our database.

Tumor evaluation and statistical analysis

Survival time was measured from the start of chemotherapy to either the time of death from any cause or the date patients were last known to be alive. The survival curve was estimated using the Kaplan-Meier method, and compared by using the log-rank test. Comparisons between individual clinical factors were performed using the χ^2 test. Multivariate analysis was conducted according to the Cox proportional hazards model. $P < 0.05$ was considered to denote statistical significance. All statistical analyses were performed using StatView, Version 5.0 (Abacus Concepts, Berkley, CA).

Results

Patient characteristics

Patient characteristics are listed in Table 1. There were 74 patients in group A and 149 in group B. Median age was almost identical, but the proportion of patients aged ≥75 years was significantly higher in group B ($p = 0.0182$). Other clinical factors, including sex, performance status (PS), tumor histology, disease stage, and smoking history, were not significantly different between the groups.

First-line chemotherapy

Details of first-line chemotherapy are shown in Table 2. In group A, 83% of patients received platinum-based chemotherapy; two-thirds of these were platinum-based plus second-generation combination chemotherapy regimens. In group B by contrast, although 55% of patients received platinum-based chemotherapy, 41% received non-platinum-based chemotherapy and 4% received epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI). EGFR-TKI as first-line treatment was all clinical trial settings. In group B, second-generation agents were no longer used, and the most frequently administered third-generation single-agent chemotherapy was VNR.

Table 1 Patient characteristics ($n = 223$).

	Group A ('92–'99)	Group B ('00–'03)	P
No. of patients	74	149	
Age			
Median	73	74	
<75	55 (75%)	86 (58%)	0.0182*
≥75	19 (25%)	63 (42%)	
Sex			
Male	62 (84%)	115 (78%)	0.29
Female	12 (16%)	34 (22%)	
ECOG PS			
0–1	69 (93%)	136 (91%)	0.8
2	5 (7%)	13 (9%)	
Histology			
Ad	52 (70%)	84 (56%)	0.06
Non-Ad	22 (30%)	65 (44%)	
Stage			
≤IIIb	25 (34%)	55 (37%)	0.66
IV	49 (66%)	94 (63%)	
Smoking history			
Current/former	62 (84%)	119 (80%)	0.59
Never	12 (16%)	30 (20%)	

ECOG, Eastern Clinical Oncology Group; PS, performance status.

Clinical factors influencing selection of platinum-based and non-platinum-based chemotherapy

In group B, 143 patients (96%) received platinum-based or non-platinum-based chemotherapy; only 6 patients (4%) received EGFR-TKI. In order to determine the clinical factors that affected the selection of platinum-based and non-platinum-based chemotherapy, relevant clinical factors were individually compared between these 2 patient subgroups. As shown in Table 3, only PS significantly differed between groups: patients with a PS of 0 or 1 tended to receive platinum-based chemotherapy; those with a PS of 2 tended to receive non-platinum-based chemotherapy ($p = 0.004$). Other clinical factors did not significantly differ between the groups; however, the proportion of patients aged ≥75 years was marginally higher in the non-platinum-based chemotherapy group ($p = 0.0596$).

EGFR-TKI treatment

In group B, 34 patients received EGFR-TKI treatment during entire treatment period, while no patients in group A. In group B, the proportion of patients who received EGFR-TKI was significantly higher in female, adenocarcinoma, and never-smoked patients (Table 4).

Second-line chemotherapy and beyond

The characteristics of patients who underwent multiple-line chemotherapy are shown in Table 5. In group A, second-line chemotherapy was administered to only 4 patients (5%) and no patients underwent third-line chemotherapy. In group B by contrast, second-line and third-line chemotherapy was administered to 62 (42%) and 22 (15%) patients, respectively. DOC was the agent most frequently used in second-line chemotherapy; EGFR-TKI was the most common agent for third-line chemotherapy.

Table 2 First-line chemotherapy.

	Group A ('92–'99)	Group B ('00–'03)
Platinum-based		
Platinum + 2nd-generation	41 (56%)	0 (0%)
CDDP + VDS + MMC	26	0
CDDP + VDS	14	0
254S + VDS	1	0
Platinum + 3rd-generation	20 (27%)	83 (55%)
CDDP + VNR	3	44
CDDP + DOC	15	15
CBDCA + PTX	0	12
CDDP + VNR + GEM	0	5
CDDP + GEM	0	4
CDDP + CPT-11	2	3
Non-platinum-based		
2nd-generation	3 (4%)	0 (0%)
ETP	3	0
3rd-generation (mono)	7 (10%)	29 (20%)
VNR	2	23
DOC	0	5
GEM	0	1
PTX	3	0
CPT-11	2	0
3rd-generation (doublet)	2 (3%)	31 (21%)
GEM + VNR	2	31
EGFR-TKI	0 (0%)	6 (4%)
Gefitinib	0	6

CDDP, cisplatin; VDS, vindesine; MMC, mitomycin C; 254S, nedaplatin; VNR, vinorelbine; DOC, docetaxel; CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; CPT-11, irinotecan; ETP, etoposide.

Survival

Median survival time (MST) was 6.7 months in group A and 8.1 months in group B ($p = 0.0109$). The 1-year survival rate and 2-year survival rate were 14% and 7%, respectively, in group A, and 35% and 20% in group B. Survival curves are shown in Fig. 1. The relationships between clinical variables and survival are shown in Table 6. Univariate analysis revealed that female, stage ≤IIIB, never-smoker, EGFR-TKI treatment, and the administration of salvage chemotherapy were associated with better survival. However, multivariate analysis demonstrated that only clinical stage and the administration of salvage chemotherapy were independent prognostic factors.

Discussion

In approximately the year 2000, the results of large phase III trials of treatments for advanced NSCLC were published. The findings of these studies were of great importance in understanding how to treat elderly patients with NSCLC. In

Table 3 Patient characteristics of Group B (platinum vs non-platinum).

	Platinum-based	Non-platinum-based	*P*
No. of patients	83	60	
Age			
Median	73	74	
<75	55 (66%)	28 (47%)	0.0596
≥75	30 (34%)	32 (53%)	
Sex			
Male	66 (80%)	47 (78%)	>0.9999
Female	17 (20%)	13 (22%)	
Performance status			
0–1	81 (98%)	50 (83%)	0.0040*
2	2 (2%)	10 (17%)	
Histology			
Ad	51 (61%)	29 (48%)	0.1283
Non-Ad	32 (39%)	31 (52%)	
Stage			
≤IIIb	33 (40%)	21 (35%)	0.6032
IV	50 (60%)	39 (65%)	
Smoking history			
Current/former	66 (80%)	49 (82%)	0.8326
Never	17 (20%)	11 (18%)	

Ad, adenocarcinoma.

the present retrospective study, we attempted to evaluate the impact of those trials by reviewing the records of 223 patients aged ≥70 years who began chemotherapy between 1992 and 2003 at our institute and comparing treatment details between those who began chemotherapy between 1992 and 1999 (group A) and those who began treatment between 2000 and 2003 (group B).

Table 4 Patient characteristics of Group B (EGFR-TKI treated vs not EGFR-TKI treated).

	EGFR-TKI (+)	EGFR-TKI (−)	*P*
No. of patients	34	115	
Age			
Median	73	74	
<75	25 (74%)	61 (53%)	0.0473*
≥75	9 (26%)	54 (47%)	
Sex			
Male	19 (56%)	96 (83%)	0.0019
Female	15 (44%)	19 (17%)	
Performance status			
0–1	30 (88%)	106 (92%)	0.4944
2	4 (12%)	9 (8%)	
Histology			
Ad	27 (79%)	57 (50%)	0.0028*
Non-Ad	7 (21%)	58 (50%)	
Stage			
≤IIIb	11 (32%)	44 (38%)	0.6862
IV	23 (68%)	71 (62%)	
Smoking history			
Current/former	21 (62%)	98 (85%)	0.0061*
Never	13 (38%)	17 (15%)	

Ad, adenocarcinoma.

Table 5 The number of patients receiving multiple chemotherapies ($n = 223$).

		Group A ('92–'99)	Group B ('00–'03)
2nd-line	yes	4 (5%)	62[a] (42%)
	no	70 (95%)	87 (58%)
3rd-line	yes	0 (0%)	22[b] (15%)
	no	74 (100%)	127 (85%)
4th-line	yes	0 (0%)	9 (6%)
	no	74 (100%)	140 (94%)

[a] docetaxel 27; EGFR-TKI 12; platinum-based 12; others 11.
[b] EGFR-TKI 14; docetaxel 1; others 7.

As we anticipated, the proportion of patients who received non-platinum-based chemotherapy, with either 1 or 2 agents, was higher in group B; however, more than 50% of patients in group B still received platinum-based chemotherapy. We further investigated group B to determine what clinical factors were associated with the selection of platinum-based or non-platinum-based chemotherapy. The results were unsurprising: patients with a PS of 2 or aged ≥75 tended to receive non-platinum-based chemotherapy. Several sub-group analyses from phase III trials indicated that, when PS was not impaired, platinum-based chemotherapy was equally safe and effective in patients aged over and under 70 years.[14–17] The first elderly-specific trial comparing platinum-based and non-platinum-based chemotherapy regimens also indicated that patients aged 70–74 years might derive more benefit from platinum-based chemotherapy than from non-platinum-based chemotherapy.[18] However, it is unclear whether platinum-based chemotherapy is safe and effective for patients aged ≥75 years.

In the present study, the proportion of patients who received salvage chemotherapy was also higher in group B. While only 5% of patients received second-line chemotherapy in group A, 42% received second-line chemotherapy in group B. In 15% of patients in group B, third-line chemotherapy was also administered.

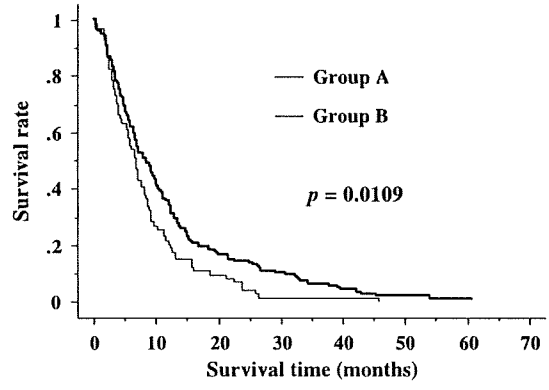

Figure 1 Kaplan-Meier curve of overall survival in all patients ($n = 232$). Median survival time, 1-year survival rate, and 2-year survival rate were 6.7 months, 14%, and 7%, respectively, in group A, and 8.1 months, 35%, and 20% in group B.

Table 6 Multivariate analysis for survival.

Variables	Category	MST (months)	Univariate	Multivariate		
			p	Risk ratio	95%CI	*p*
Age	<70	7.6	0.6	1.0930	0.815–1.465	0.5529
	≥70	7.0				
Sex	Female	9.7	0.0012*	1.4060	0.798–2.476	0.2382
	Male	7.0				
PS	0-1	7.5	0.15	1.3030	0.783–2.169	0.3084
	2	4.0				
Histology	Ad	7.5	0.06	0.8300	0.623–1.106	0.2034
	non-Ad	6.7				
Stage	≤IIIB	9.3	0.0094*	0.6120	0.524–0.949	0.0213*
	IV	6.8				
Smoking history	(−)	9.2	0.0062*	0.8510	0.634–2.044	0.6630
	(+)	7.0				
Platinum-doublet	(−)	7.3	0.86	1.1260	0.829–1.529	0.4466
	(+)	7.1				
3rd-generation agent	(−)	6.7	0.27	0.8640	0.596–1.252	0.4392
	(+)	7.3				
EGFR-TKI	(−)	6.7	<0.0001*	0.7900	0.453–1.379	0.4077
	(+)	15.0				
Salvage chemotherapy	(−)	5.8	<0.0001*	0.5100	0.335–0.776	0.0017*
	(+)	12.6				

EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; Ad, adenocarcinoma.

Overall survival time was significantly longer in group B, as compared with group A, and multivariate analysis revealed that clinical stage and the administration of salvage chemotherapy were independent prognostic factors. Between group A and B, the difference of clinical stage was not significant. Therefore, these results seem to be the effect of salvage chemotherapy and suggest that even elderly patients should be offered salvage chemotherapy regardless of age, if possible. To date, several agents, such as docetaxel, gefitinib, erlotinib, and pemetrexed, have been approved as salvage chemotherapy worldwide, including Japan.[19] Physicians should not miss a chance to offer these effective agents to elderly patients.

In conclusion, in our institute, chemotherapy regimens changed considerably after the year 2000. In addition, survival time significantly improved after 2000, and this improvement appears to be attributable mostly to the effect of salvage chemotherapy.

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work.

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Individuals susceptible to lung adenocarcinoma defined by combined *HLA-DQA1* and *TERT* genotypes

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Adenocarcinoma (ADC) is the commonest histological type of lung cancer, and its weak association with smoking indicates the necessity to identify high risk individuals for targeted screening and/or prevention. By a genome-wide association study (GWAS), we identified an association of polymorphisms in the 6p21.31 locus containing four HLA (human leukocyte antigen)-class II genes with lung ADC risk. DQA1*03 of the *HLA-DQA1* gene was defined as a risk allele with odds ratio (OR) of 1.36 (95%CI=1.21–1.54, P=5.3x10^{-7}) by analysis of 1,656 ADC cases and 1,173 controls. DQA1*03 and the minor allele for a polymorphism, rs2736100, in *TERT*, another lung cancer susceptibility locus identified in recent GWASs on Europeans and Americans, were indicated to independently contribute to ADC risk with per allele OR of 1.43 (95%CI=1.31–1.56, P=7.8x10^{-16}). Individuals homozygous both for the *DQA1*03* and minor *TERT* alleles were defined as high-risk individuals with an OR of 4.76 (95%CI=2.53–9.47, P=4.2x10^{-7}). The present results indicated that individuals susceptible to lung ADC can be defined by combined genotypes of *HLA-DQA1* and *TERT*.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the world. Adenocarcinoma (ADC) is the commonest histological type comprising ~40% of lung cancer cases among European, North American and Asian countries, and is increasing in incidence [1]. Development of ADC is more weakly associated with smoking than those of two other major histological types of lung cancer, squamous (SQC) and small (SCC) cell lung carcinomas [1-3]. Therefore, identification of high-risk individuals for lung ADC and targeted screening and/or prevention for these individuals will be a powerful way to reduce the number of lung cancer deaths in the world.

Recent GWASs with single nucleotide polymorphism (SNP) array methodology have led to the identification of three loci associated with lung cancer risk, *CHRNA3/5* at chromosome 15q25.1, *TERT* and *CLPTM1L* at 5p15.33, and *BAT3-MSH5* at 6p21.33 [4-10]. Among these loci, 5p15.33 was revealed as being a locus specifically associated with risk of ADC among major histological types of lung cancer [11]. However, loci associated with lung ADC risk in Asians remain obscure. Here, we performed a GWAS on the risk of lung ADC in a Japanese population for 23,010 polymorphic microsatellite loci and identified *HLA-DQA1* at 6p21.31 as a novel locus associated with lung ADC risk. We further examined whether or not individuals susceptible to ADC can be defined by combined genotypes of *HLA-DQA1* and other lung cancer susceptibility loci described above.

Subjects and Methods

Subjects. All the case and control subjects were Japanese, and were enrolled in institutions in the Kanto area of Japan, an approximately 200-km diameter region containing Tokyo. This region is located in the middle of the main island in Japan, where homogeneity of the genetic background of individuals with several common diseases, including lung cancer, has been shown by a recent GWAS on population structure of Japanese [12].

The NCCH set consisted of 2,343 lung cancer cases and 1,173 controls (**Table I**). The cases were 1,656 ADC, 390 SQC and 297 SCC cases. All ADC, SQC and SCC cases were enrolled in the National Cancer Center Hospitals (NCCH) from 1999 to 2008. All ADC, SQC and SCC cases, from whom informed consent as well as blood samples were obtained, were consecutively included in this study without any particular exclusion criteria. The participation rate was nearly 80%. All the cases were diagnosed by cytological and/or histological examinations according to WHO classification. The controls were 328 inpatients/outpatients of the NCCH; and 645 and 200 volunteers enrolled in Keio and Tokai Universities, respectively. The control NCCH subjects were selected with a criterion of no history of cancer from 1999 to 2007, while the 645 volunteers were the individuals with no known malignancies who offered blood on the occasion of a health check examination at Keio University in 2002 and 2003 [13]. The 200 volunteers in Tokai University were healthy individuals enrolled from 2001 to 2003 as control subjects in a previous case-control study [14].

The NNGH set were 84 ADC and 52 SQC cases and 145 controls who were enrolled in the National Nishi-Gunma Hospital (NNGH) from 1999 to

2003 (**Table I**). All ADC and SQC cases, from whom informed consent as well as blood samples were obtained, were consecutively included in this study without any particular exclusion criteria. The participation rate was nearly 80%. Controls were randomly selected from inpatients and outpatients with no history of cancer. Most of the controls had diseases other than lung cancer such as chronic obstructive pulmonary disease (COPD), pulmonary tuberculosis, bronchitis/pneumonia. Their characteristics were described in our previous studies [14-18].

Smoking histories of the subjects were obtained via interview using a questionnaire. Smokers were defined as those who had smoked at least one cigarette per day for 12 months or longer at any time in their life, while non-smokers were defined as those who had not. There were no individuals who had smoked less than one cigarette per day and/or for less than 12 months. Smoking exposure was represented by pack-years, which was defined as the number of cigarette packs smoked daily multiplied by years of smoking.

Genomic DNA was extracted from whole-blood cells using a Blood Maxi Kit (Qiagen, Tokyo, Japan) according to the supplier's instructions. Genomic DNAs for 645 and 200 volunteers enrolled in Keio and Tokai Universities, respectively, were extracted from EBV-transformed B-lymphocytes derived from the collected whole-blood cells [14,16].

GWAS. The method of GWAS on microsatellite loci was previously described [14]. Equal amounts of DNAs from 200 lung ADC cases and from 200 controls enrolled in Tokai University were mixed for the first set of case and control DNA pools, respectively. The second set of DNA pools was also

prepared from another 200 ADC cases and 200 controls enrolled in Keio University. Fifty ng of pooled-DNA was amplified by 40 cycles of PCR in 96-well plates using a pair of PCR primers designed for amplifying fragments that include polymorphic microsatellite sequences. Allele frequencies in pooled-DNA were estimated from the height of peaks: the frequency of each allele was determined by dividing the height of each allele by the summed height of all alleles. The significance for difference in allelic distribution was evaluated by Fisher's exact test, with the use of 2×m (where m is the number of alleles).

The first set of case and control DNA pools was examined for differences in allelic distribution for 23,010 microsatellite markers, and the distribution for 1,328 (5.8%) markers were judged as being significantly different by the criteria of $p<0.05$ (1st stage of GWAS in **Table II**). The inflation factor calculated by dividing the mean of the lower 90% of $-\log_{10}(P)$ values by the mean of the lower 90% of the expected values [19] for this screening was 0.639, indicating a deflation in the statistical tests (**Supplementary Table I**). However, in this screening, deduction of allele frequencies was affected by an inevitable experimental bias of the pooled DNA typing, i.e., "shadow bands" in electrophoregrams due to slippages in the PCR reaction particularly for microsatellite markers containing repeat units of 2-bp, as previously reported [20]. In fact, inflation factors for microsatellite markers containing repeat units of 3~6-bp were 0.919~1.022 (0.955 in total), i.e., deviations were within ± 10% as have been observed in previous GWASs in which adequacy of the case-control matching (i.e., lack of a significant hidden population substructure) was indicated [4,8,9,19]. Thus, the adequacy of the case-control matching was also indicated in the present screening with microsatellite markers containing repeat

units of 3~6-bp. On the other hand, inflation factor for microsatellite markers containing repeat units of 2-bp was 0.520, therefore, the deflation described above was considered to be caused by mis-estimation in allele frequency in the screening with microsatellite markers containing repeat units of 2-bp. Therefore, among 1,328 markers selected in the 1st stage of GWAS, 431 microsatellite markers with 3~6-bp units were further subjected to the 2nd stage of GWAS.

The second set of DNA pools was examined for differences in allelic distribution for 431 microsatellite markers containing repeat units of 3~6-bp which passed the criteria of $p<0.05$ in the 1st stage of GWAS. The distribution for 17 (3.9%) markers were significantly different by the criteria of $p<0.05$ (2nd stage of GWAS in **Table II**). The inflation factor for the 2nd stage screening was 1.010, indicating the adequacy of the case-control matching as well as the lack of differential genotyping of cases and controls (**Supplementary Table I**).

Next, individual typing was done on the 17 markers, which passed the criteria for the 3rd stage, for 576 cases and 576 controls, consisting of 384 cases and 384 controls used in the first and second pooled-DNA screening and an additional 192 cases and 192 controls from NCCH (3rd stage of GWAS in **Table II**). These 384 cases and 384 controls were consisted of two sets of 192 subjects which were chosen from two sets of 200 subjects examined in the 1st and 2nd GWAS stages, respectively, by simple random sampling. These analyses led to the identification of six loci, including D6S0067i, with differences in allelic distributions between the cases and controls with P values less than 0.05 by the $\chi 2$ test. The D6S0067i locus showed a P value of 2.4×10^{-7}, while the other five showed P values of 0.012~0.0011. A level of $P<2.2 \times 10^{-6}$ was judged as significant

by applying Bonferroni correction for multiple test (i.e., $P<2.2\times10^{-6}=0.05/23,010$).

Genotyping of SNPs in the 6p21.31 locus. Five-hundred-twenty-five cases and 525 controls, which were respectively chosen from the 576 cases and 576 controls examined in the 3rd GWAS stage by simple random sampling, were subjected to SNP analysis. Twenty-nine SNPs were selected from the 450-kb region surrounding the D6S0067i locus based on the following criteria; 1) SNPs whose minor allele frequency in the Japanese population was > 0.01 in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), and 2) SNPs for which pre-designed or validated Taqman probes were available from Applied Biosystems (Foster City, CA). Three other SNPs, rs1794282, rs3129763 and rs2187668, which showed significant associations with lung cancer risk in Europeans [8], were also examined. Thirty-two SNPs, in total, were genotyped using the TaqMan method according to the protocol for the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Twenty-four SNPs located in exon 2 of the *DRB1, DQA1* and *DQB1* genes, which enable allele discrimination for *DRB1, DQA1* and *DQB1* at high, low and high resolution levels, respectively, were genotyped by sequence-based typing methods recommended by the International Histocompatibility Working Group (Chapters 10-D, 11 and 12-A at http://www.ihwg.org/tmanual/TMcontents.htm). In brief, exon 2 of the *DRB1* and *DQB1* genes was amplified by PCR with mixtures of allele specific primers, while exon 2 of the *DQA1* gene was amplified with a set of common primers, and PCR products were directly sequenced using the ABI3700 DNA analyzer (Applied Biosystems). The location and alleles of the SNPs are described

according to the dbMHC database (http://www.ncbi.nlm.nih.gov/gv/mhc/). Based on the genotypes of 24 SNPs, alleles for *DRB1*, *DQA1*, *DQB1*, and *DR-DQ* were determined, and alleles with frequencies > 0.02 were subjected to the association study.

Statistical analyses. A Hardy-Weinberg equilibrium (HWE) test was performed using the SNPAlyze version 3 software (DYNACOM Co., Ltd), and SNPs with a *P* value for deviation > 0.01 were considered to be in HWE as described [7]. Calculation of the D′ and R^2 values between SNPs and allele/haplotype estimation was performed by the EM algorithm using the SNPAlyze version 3 software. The D6S0067i locus showed 19 polymorphic alleles in the same sets of cases and controls, and among them, alleles of 367-bp and 404-bp in sizes were significantly associated with an elevated risk for lung ADC (OR=1.60, $P= 9.9x10^{-3}$ and OR=1.42, 4.9×10^{-5}, respectively). Therefore, for the calculation of the D′ and R^2 values, genotypes for the D6S0067i polymorphism was expressed by presence or absence of these two alleles (**Supplementary Table II**).

Associations of SNPs/alleles with risks were digitized as crude ORs and ORs adjusted for gender, age and smoking with 95 % CIs by unconditional logistic regression analysis using the JMP version 6.0 software (SAS Institute Inc., NC, USA). Variables used for adjustment in each test are described in the footnotes to Supplementary Tables. A level of $P<0.05$ for an OR was judged as significant and that of $0.05 \leq P<0.1$ was judged as marginal in association studies other than GWAS.

Genotyping of SNPs in the lung cancer susceptibility loci identified by previous GWASs. SNPs in the lung cancer susceptibility loci identified by previous GWASs were genotyped by the TaqMan method. Two intronic SNPs, rs2736100 and rs401681, in the *TERT* and *CLPTM1L* genes [4,21] were genotyped for the 5p15.33 locus against 2,343 cases and 1,173 controls (subjects of the NCCH set in **Table I**). Association results of the rs1051730 SNP in the *CHRNA3* gene for the 15q25.1 locus in a subset of the present study population were previously reported [22]. Therefore, in this study, 1,094 ADC cases and 236 controls which had not been examined in our previous study were genotyped [22]. Eight SNPs in the 6p21.33 locus, consisting of rs3117582 and seven SNPs in LD with this SNP in Europeans (D'=1 in the HapMap database), were genotyped for 525 ADC cases and 525 controls used for the mapping stage (**Table II**).

Results and Discussion

We performed a GWAS on the risk of lung ADC in a Japanese population for 23,010 polymorphic microsatellite loci. After a three stage screening against 576 ADC cases and 576 controls from the NCCH set (**Table I**), a locus, D6S0067i, at 6p21.31 was identified as being significantly different in allelic distribution after Bonferroni correction (i.e., $P=2.4\times10^{-7}$, which is less than $0.05/23{,}010=2.2\times10^{-6}$) (details in **Subjects and Methods** and **Table II**).

The D6S0067i locus was mapped between two linkage disequilibrium (LD) blocks previously defined [23], one containing four HLA-class II genes, *HLA-DRA*, *-DRB1*, *-DQA1*, and *-DQB1*, and the other containing two pseudogenes, *HLA-DQA2* and *–DQB2* (**Figure 1**). Therefore, the locus of the strongest association was searched for in the 450-kb region containing these two LD blocks by analyzing 32 SNPs. Five-hundred-twenty-five cases and 525 controls, randomly selected from the GWAS subjects, were genotyped by the Taqman method (**Table III**). The rs1794282 SNP was monomorphic in the study subjects, while the other 31 were polymorphic. A SNP in the *DRA* gene, rs16822586, significantly deviated from the HWE in cases (P=0.001), while other SNPs did not deviate in either the cases or the controls, suggesting that SNPs in the regions examined in the present study normally segregated in the Japanese irrespective of lung cancer susceptibility. The 31 SNPs, which were polymorphic in our study population, comprised three LD blocks. The largest difference in allelic distribution between the cases and controls was observed at an intronic SNP in the *DQA1* gene, rs17426593 (OR=1.51, P=4.2x10^{-6}) (**Figure 1**) in the block containing four HLA-class II genes (LD block 1 in **Table III**). The D6S0067i polymorphism was in LD (D'=0.516 in controls and D'=0.603 in cases) and showed a considerably high correlation coefficient (R^2=0.225 in controls and R^2=0.349 in cases) with the rs17426593 SNP (**Subjects and Methods** and **Supplementary Table II**). Therefore, we further examined associations of SNPs in this LD block with lung ADC risk.