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Evaluation of *CYP2A6* genetic polymorphisms as determinants of smoking behavior and tobacco-related lung cancer risk in male Japanese smokers

Masaki Fujieda[1,7], Hiroshi Yamazaki[1], Tetsuya Saito[1], Kazuma Kiyotani[1], Maxwell Afari Gyamfi[1], Masaharu Sakurai[2], Hirotoshi Dosaka-Akita[3], Yuichi Sawamura[4], Jun Yokota[5], Hideo Kunitoh[6] and Tetsuya Kamataki[1]

[1]Laboratory of Drug Metabolism, Division of Pharmacobio-dynamics, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, [2]Division of Pharmacology and Therapeutics, Graduate School of Clinical Pharmacy, Kumamoto University, Kumamoto 862-0973, Japan, [3]Department of Medical Oncology, Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan, [4]Maruyama Clinic, Sapporo 064-0820, Japan, [5]Biology Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan and [6]Department of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo 104-0045, Japan

[7]To whom correspondence should be addressed
Email: fujieda@pharm.hokudai.ac.jp

We reported previously that subjects homozygous for the *cytochrome P450 2A6* (*CYP2A6*) **4* have a lower risk of lung cancer. The purpose of this study was to clarify whether or not the alterations of smoking behavior and risk for lung cancer could be found in subjects possessing novel *CYP2A6* variants discovered recently. An epidemiological study was performed with 1094 cases and 611 controls in male Japanese smokers. It was found that the amounts of daily cigarette consumption in subjects who harbored *CYP2A6*4/*7*, **4/*10*, **7/*7*, **7/*9* and **4/*4* genotypes were significantly less than those in subjects carrying the **1/*1* genotype ($P < 0.01$). Even after adjustment with cigarette consumption, the adjusted odds ratios (ORs) for lung cancer were significantly lower in subjects who harbored *CYP2A6*1/*4*, **1/*7*, **1/*9*, **1/*10*, **4/*4*, **4/*7*, **4/*9*, **7/*7* and **7/*9* genotypes than those who possessed the **1/*1* genotype ($P < 0.05$). When participants were classified into four groups according to the *CYP2A6* genotypes, group 1 (1/*1*), group 2 (heterozygotes for the **1* and a variant allele), group 3 (heterozygotes and homozygotes for variant alleles except for **4/*4*) and group 4 (**4/*4*), lung cancer risk was found to be less in subjects with the variant of *CYP2A6* alleles (group 2, OR of 0.59 [95% confidence interval (CI), 0.44–0.79]; group 3, OR of 0.52 (95% CI, 0.37–0.72); group 4, OR of 0.30 (95% CI, 0.16–0.57)). The reduced risk for lung cancer was seen more clearly in heavy smokers than in light smokers. Additional stratification analysis showed that the ORs for squamous cell carcinoma (OR of 0.07) and small cell carcinoma (OR of 0.10) were lower than that of adenocarcinoma (OR of 0.39) in group 4. These results suggest that the CYP2A6 is one of the principal determinants affecting not only smoking behavior but also susceptibility to tobacco-related lung cancer.**

Abbreviations: Ad, adenocarcinoma; CI, confidence interval; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; OR, odds ratio; SCC, small cell carcinoma; SqCC, squamous cell carcinoma.

Introduction

Cytochrome P450 2A6 (CYP2A6) is known as an enzyme responsible for the metabolism of chemicals and drugs such as coumarin (1), nicotine (2), (+)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride (SM-12502) (3), tegafur (4), fadrozole (5), methoxyflurane (6) and valproic acid (7). The enzyme can also metabolically activate a number of carcinogens including tobacco-specific *N*-nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (8,9).

The existence of a genetic polymorphism of *CYP2A6* was first suggested by evidence that there was a large inter-individual difference in the capacity of coumarin 7-hydroxylation (10,11). In fact, various variants of the *CYP2A6* gene have been found in recent years (1,12–18). Analyzing the genes of subjects who showed a poor metabolizer phenotype toward SM-12502, we found two novel deletion-type variants of the *CYP2A6* gene, *CYP2A6*4B* and *CYP2A6*4C* (19,20); the *CYP2A6*4C* was one of the major variants in Japanese. Following the discovery of *CYP2A6*4C*, we discovered two additional alleles, *CYP2A6*7* and *CYP2A6*11*, showing a decrease in enzymatic activity (21,22). A novel variant, the *CYP2A6*9*, has a −48T to G nucleotide substitution in the TATA box of the 5′-flanking region of the *CYP2A6*, which reduced the expression levels of CYP2A6 mRNA and protein in human livers (23). The *CYP2A6*10* allele possessing two simultaneous amino acid substitutions seen in the *CYP2A6*7* and *CYP2A6*8* also shows decreased enzymatic activity (24).

Most cancers are caused by chemical carcinogens present in our environment (25,26). These chemical carcinogens exert their genotoxicity after undergoing metabolic activation by enzymes present in our bodies. Thus, the capacity of enzymes to activate chemical carcinogens has been recognized as one of the factors determining the risk of cancer. Genetic polymorphism of the genes for such enzymes has been expected to be the most typical factor altering the activity and the amounts of the enzymes. Thus, it has been hypothesized that the genetic polymorphism alters the risk of chemical carcinogenesis. However, no conclusive evidence for the association between the genetic polymorphism of carcinogen-activating enzyme and the lung cancer risk has been reported as yet. Recently, several reports, including our group, have demonstrated the role of *CYP2A6* genetic polymorphisms in lung cancer risk with some conflicting results in several populations from different ethnicities (27–32). In our previous paper (31), we reported a clear relationship between *CYP2A6* genetic polymorphisms and lung cancer risk in smokers. To our knowledge, our results were not supported by other investigators, who reported that no clear association between *CYP2A6* genetic polymorphisms and lung cancer risk could be seen (27,29,30,32). The reason for this discrepancy is not known as yet. However, the most possible explanation for this discrepancy is that they analyzed the genes from smokers and non-smokers. Our epidemiology has indicated that the

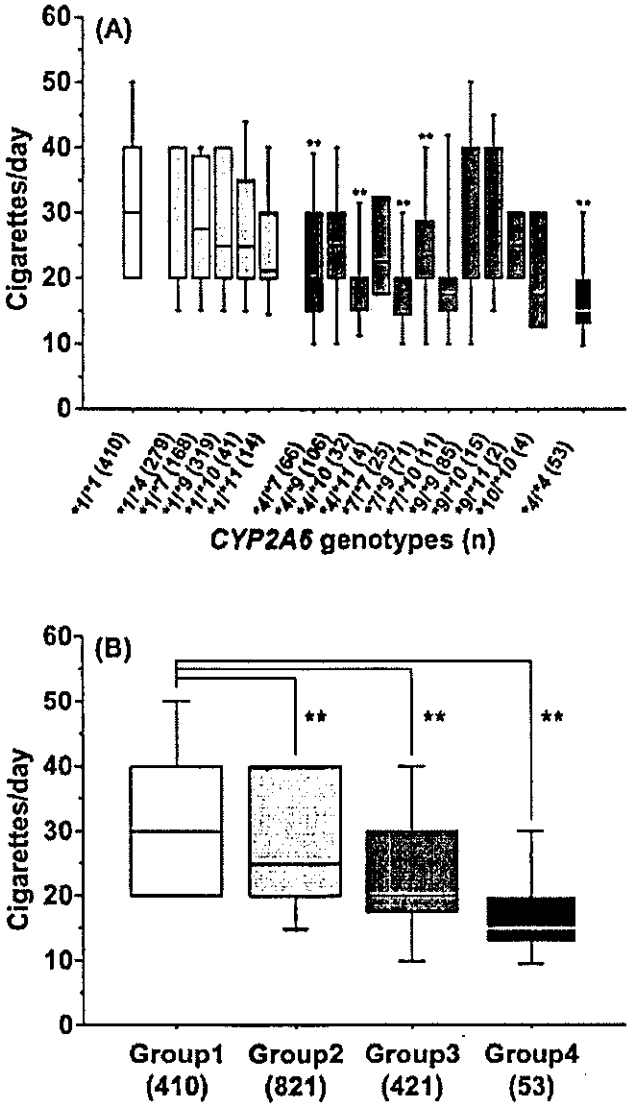

Fig. 1. Relationship between the *CYP2A6* genotypes and the amounts of daily cigarette consumption (A), and between CYP2A6 groups classified by *CYP2A6* genotypes and daily cigarette consumption (B). Cigarette consumption was investigated with all subjects employed in this study. *CYP2A6*1* consists of *CYP2A6*1A* and **1B* alleles. *CYP2A6*4* consists of *CYP2A6*4B* and **4C* alleles. The number of subjects is shown under each genotypic group in brackets. Subjects were classified into four groups, according to the *CYP2A6* genotypes. Group 1 includes the subjects carrying *CYP2A6*1/*1* (wild-type). Group 2 contains subjects heterozygous for wild-type allele (*CYP2A6*1/*4*, *CYP2A6*1/*7*, *CYP2A6*1/*9*, *CYP2A6*1/*10*, *CYP2A6*1/*11*). Group 3 consists of subjects carrying *CYP2A6*4/*7*, *CYP2A6*4/*9*, *CYP2A6*4/*10*, *CYP2A6*4/*11*, *CYP2A6*7/*7*, *CYP2A6*7/*9*, *CYP2A6*7/*10*, *CYP2A6*9/*9*, *CYP2A6*9/*10*, *CYP2A6*9/*11* or *CYP2A6*10/*10*. Group 4 contains subjects homozygous for *CYP2A6* deletion allele (*CYP2A6*4/*4*). Genotypes are shown in numerical order. Horizontal lines mean medians. Boxes show 25th and 75th percentile of the observed values. Bars mean 10th and 90th percentiles. The amount of daily cigarette consumption was significantly less than those carrying the *CYP2A6*1/*1* genotype (**$P < 0.01$).

study and the present one may be explained by several possibilities. First, the sample size was small in the study reported by Yoshida *et al.* (40). They analyzed only 92 Japanese individuals, whereas we analyzed 611 controls. Secondly, the

Table II. Distribution of *CYP2A6* genotypes in lung cancer patients

CYP2A6[a]	Cases (%) (n = 1094)	Controls (%) (n = 611)	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
**1/*1*	300 (27.4)	110 (18.0)	1.00[c]	1.00[c]
**1/*4*	185 (16.9)	94 (15.4)	0.72 (0.52–1.00)	0.68 (0.47–0.98)[d]
**1/*7*	106 (9.7)	62 (10.1)	0.63 (0.43–0.92)[d]	0.55 (0.36–0.84)[d]
**1/*9*	207 (18.9)	112 (18.3)	0.68 (0.49–0.93)[d]	0.59 (0.41–0.84)[d]
**1/*10*	21 (1.9)	20 (3.3)	0.39 (0.20–0.74)[d]	0.30 (0.14–0.61)[d]
**1/*11*	9 (0.8)	5 (0.8)	0.66 (0.22–2.01)	1.05 (0.29–3.78)
**4/*4*	25 (2.3)	28 (4.6)	0.33 (0.18–0.59)[d]	0.29 (0.15–0.56)[d]
**4/*7*	36 (3.3)	30 (4.9)	0.44 (0.26–0.75)[d]	0.45 (0.25–0.82)[d]
**4/*9*	62 (5.7)	44 (7.2)	0.52 (0.33–0.81)[d]	0.51 (0.31–0.83)[d]
**4/*10*	16 (1.5)	16 (2.6)	0.37 (0.18–0.76)[d]	0.55 (0.24–1.28)
**4/*11*	2 (0.2)	2 (0.3)	0.37 (0.05–2.64)	0.52 (0.05–5.62)
**7/*7*	12 (1.1)	13 (2.1)	0.34 (0.15–0.76)[d]	0.28 (0.11–0.71)[d]
**7/*9*	39 (3.6)	32 (5.2)	0.45 (0.27–0.75)[d]	0.44 (0.25–0.79)[d]
**7/*10*	7 (0.6)	4 (0.7)	0.64 (0.18–2.24)	0.98 (0.25–3.92)
**9/*9*	55 (5.0)	30 (4.9)	0.67 (0.41–1.10)[d]	0.71 (0.41–1.23)
**9/*10*	11 (1.0)	4 (0.7)	1.01 (0.31–3.23)	0.98 (0.28–3.41)
**9/*11*	1 (0.1)	1 (0.2)	0.37 (0.02–5.91)	0.36 (0.02–5.81)
**10/*10*	0 (0.0)	4 (0.7)	NA[e]	NA[e]

Significant difference in the distribution of *CYP2A6* genotypes was found between lung cancer cases and control subjects (χ^2 value 42.6, $P = 0.0005$).
[a]*CYP2A6*1* consists of *CYP2A6*1A* and **1B* alleles. *CYP2A6*4* consists of *CYP2A6*4B* and **4C* alleles.
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.
[e]Not applicable.

ethnic difference of the *CYP2A6*4* allele frequency might exist between Japanese and Chinese populations. In fact, the frequencies of *CYP2A6*4* in controls in their two different studies were almost the same (30,32), although the frequencies in lung cancer cases were largely different.

We assessed the impact of *CYP2A6* genetic polymorphisms on the number of cigarettes smoked per day and the risk of lung cancer. Regarding the basis for the classification of the *CYP2A6* genotypes into groups 1–4, we recently analyzed that the relationship between the *in vivo* catalytic activity of CYP2A6 towards nicotine and the polymorphism of the *CYP2A6* gene in healthy Thai volunteers (unpublished data). The levels of plasma cotinine concentration in subjects genotyped as *CYP2A6*1/*4*, *CYP2A6*1/*7*, *CYP2A6*1/*9*, *CYP2A6*1/*10*, *CYP2A6*4/*7*, *CYP2A6*4/*9*, *CYP2A6*7/*7* and *CYP2A6*9/*9* showed 53.9, 61.4, 72.2, 63.4, 11.7, 35.8, 20.4 and 58.9% of the plasma cotinine concentration of subjects carrying *CYP2A6*1/*1*, respectively, suggesting that the catalytic activity of CYP2A6 is lower in the subjects homozygous for either *CYP2A6*7* or *CYP2A6*9*, or heterozygous within the *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9* and *CYP2A6*10* variants. Additionally, we also found the *CYP2A6*4/*11* genotype from a patient who also showed a poor metabolic phenotype in the metabolism of tegafur to yield 5-fluorouracil (22), suggesting that the enzyme encoded by *CYP2A6*11* had a lower metabolic capacity. In fact, we clarified that the recombinant CYP2A6.11 had a lower capacity to metabolize tegafur (41% of CYP2A6.1) and coumarin (59%) (22). Furthermore, analyzing the plasma concentration of nicotine, Xu and colleagues (24) have reported that individuals who possessed the *CYP2A6*7/*7*, *CYP2A6*4/*7* and *CYP2A6*4/*10* genotypes showed apparently intermediate and poor metabolic phenotype, probably indicating that the *CYP2A6*7* and *CYP2A6*10* are among the causative alleles

Table III. Relationship between the CYP2A6 groups and lung cancer risk

	Group[a]	1	2	3	4
All cases	Cases (%)/controls (%)	300 (27.4)/110 (18.0)	528 (48.3)/293 (47.9)	241 (22.0)/180 (29.5)	25 (2.3)/28 (4.6)
	OR (95% CI)[b]	1.00[c]	0.59 (0.44–0.79)[d]	0.52 (0.37–0.72)[d]	0.30 (0.16–0.57)[d]
<38.3 pack-years	Cases (%)/controls (%)	66 (19.0)/63 (18.1)	161 (46.4)/146 (42.0)	102 (29.4)/117 (33.6)	18 (5.2)/22 (6.3)
	OR (95% CI)[b]	1.00[c]	0.95 (0.61–1.49)	0.73 (0.46–1.18)	0.48 (0.22–1.04)
≧38.3 pack-years	Cases (%)/controls (%)	234 (31.3)/47 (17.9)	367 (49.1)/147 (55.9)	139 (18.6)/63 (24.0)	7 (1.0)/6 (2.2)
	OR (95% CI)[b]	1.00[c]	0.42 (0.28–0.62)[d]	0.39 (0.25–0.63)[d]	0.19 (0.05–0.65)[d]

Significant difference in the distribution of *CYP2A6* genotypic groups was found between lung cancer cases and control subjects (χ^2 value 24.2, $P < 0.0001$).
[a]Groups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. Group 1 includes the subjects carrying *CYP2A6*1/*1* (wild-type). Group 2 contains subjects heterozygous for wild-type allele (*CYP2A6*1/*4*, *CYP2A6*1/*7*, *CYP2A6*1/*9*, *CYP2A6*1/*10*, *CYP2A6*1/*11*). Group 3 consists of subjects carrying *CYP2A6*4/*7*, *CYP2A6*4/*9*, *CYP2A6*4/*10*, *CYP2A6*4/*11*, *CYP2A6*7/*7*, *CYP2A6*7/*9*, *CYP2A6*7/*10*, *CYP2A6*9/*9*, *CYP2A6*9/*10*, *CYP2A6*9/*11* or *CYP2A6*10/*10*. Group 4 contains subjects homozygous for *CYP2A6* deletion allele (*CYP2A6*4/*4*).
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.

Table IV. Allele frequency of *CYP2A6* in lung cancer patients

Allele[a]	Cases (%) ($n = 2188$)	Controls (%) ($n = 1222$)	OR (95% CI)[b]
*CYP2A6*1*	1128 (51.6)	513 (42.0)	1.00[c]
*CYP2A6*4*	351 (16.0)	242 (19.8)	0.66 (0.54–0.80)[d]
*CYP2A6*7*	212 (9.7)	154 (12.6)	0.63 (0.50–0.79)[d]
*CYP2A6*9*	430 (19.7)	253 (20.7)	0.77 (0.64–0.93)[d]
*CYP2A6*10*	55 (2.5)	52 (4.3)	0.48 (0.32–0.71)[d]
*CYP2A6*11*	12 (0.5)	8 (0.7)	0.68 (0.28–1.68)

Significant difference in the distribution of the six alleles between lung cancer cases and control subjects was found (χ^2 value 35.7, $P < 0.0001$).
[a]*CYP2A6*1* consists of *CYP2A6*1A* and **1B* alleles. *CYP2A6*4* consists of *CYP2A6*4B* and **4C* allele.
[b]Crude OR.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.

reducing enzymatic activity of CYP2A6. These lines of evidence support our idea that not only the amounts of daily cigarette consumption but also the risk for tobacco-related lung cancer decrease in association with the impaired function of CYP2A6 (Figure 1B and Table III). Results reported by Tyndale and coworkers (41–44) on the association between the *CYP2A6* genetic polymorphism with smoking behavior are in agreement with our results. The present study also clearly indicates that the predicted capacity of CYP2A6 correlates well with the tobacco-related lung cancer risk, suggesting that the inhibition of this enzyme by some inhibitors of this enzyme results in the prevention of the occurrence of tobacco-related lung cancer. Supporting this idea, our recent study showed that treatment of A/J mice with NNK together with 8-methoxypsoralen, a specific inhibitor of CYP2As, completely abolished the occurrence of NNK-induced adenoma (33).

SqCC and SCC have been recognized as major types of lung cancer caused by smoking, whereas Ad has not been regarded as a common histological type of lung cancer caused by smoking until recent years, when it was demonstrated that Ad could be increased by smoking (37,38). Thus, it is of interest to note that in the present study the decreased ORs are seen in SqCC and SCC rather than in Ad, which was in agreement with a previous concept that SqCC and SCC appeared highly related to tobacco smoking.

Conflicting results have been reported on the association of *CYP2A6* genetic polymorphisms and lung cancer risk (27–32). These contradictory results seem to be caused by several factors. First, the original genotyping method (45), which was employed in the previous two reports (27,46) is rather non-specific, which caused a misclassification of *CYP2A6* genotypes. Secondly, the frequencies of the inactive alleles such as *CYP2A6*2* and *CYP2A6*4* in their studies were too small to detect a potential relationship with sufficient statistical power (29,30). A larger population is needed to confirm their findings. Thirdly, they analyzed the genes of combined groups of smokers and non-smokers (30,32) as pointed out in the Introduction section. As reported in this and a previous paper (31), we found that the association between the genotype of *CYP2A6* and the lung cancer risk could be seen only in smokers. In our preliminary results, ORs of subjects heterozygous for the *CYP2A6*1* and *CYP2A6*4* allele and homozygous for the *CYP2A6*4* allele were 0.79 (95% CI of 0.59–1.07) and 1.48 (95% CI of 0.80–2.76) among 331 healthy controls and 743 cases in Japanese non-smokers, respectively (data not shown). In contrast, Tan *et al.* (30) recently reported that Chinese individuals carrying at least one *CYP2A6*4* allele were at a 2-fold increased risk of lung cancer compared with those without a *CYP2A6*4* allele. However, this effect was limited mainly to non-smokers in their study (30). In their more recent report, they reported again that no association was observed between the *CYP2A6* genotype and the risk of lung cancer (32). In this study, they analyzed the gene from subjects of smokers and non-smokers (OR = 0.97, 95% CI of 0.72–1.31). Careful analyses using only smokers will be needed to elucidate the impact of *CYP2A6*4* for lung cancer risk in their studies (30,32), since CYP2A6 is one of the key enzymes in the metabolic activation of NNK and other *N*-nitrosamines in tobacco smoke (9) and in the metabolism of nicotine (2). The importance of categorization by the number of cigarettes smoked was first proposed by analyzing data between the genetic polymorphisms of *CYP2D6* and lung cancer risk (47). In our study, it is of interest to note that a reduced risk for lung cancer associated with the *CYP2A6* genetic polymorphisms was seen more clearly in heavy smokers (≧38.3 pack-years). The 50th percentile pack-years value (38.3 pack-years) in the Japanese population was higher than in the Chinese population (30,32). The reason for this is not known at present. It is also of interest to perform another subanalysis separating subjects by current and ex-smokers. However, we could not analyze according to this classification, because of the small number of ex-smokers in the present study.

Table V. Relationship between the *CYP2A6* groups and tobacco-related lung cancer risk according to the histological types of lung cancer

	Group[a]	1	2	3	4
SqCC	Cases (%)/controls (%)	80 (27.0)/110 (18.0)	152 (51.4)/293 (47.9)	60 (20.9)/180 (29.5)	2 (0.7)/28 (4.6)
	OR (95% CI)[b]	1.00[c]	0.62 (0.41–0.93)[d]	0.52 (0.32–0.84)[d]	0.07 (0.01–0.33)[d]
<38.3 pack-years	Cases (%)/controls (%)	13 (19.4)/63 (18.1)	33 (49.3)/146 (42.0)	20 (29.8)/117 (33.6)	1 (1.5)/22 (6.3)
	OR (95% CI)[b]	1.00[c]	0.93 (0.44–1.98)	0.75 (0.34–1.70)	0.12 (0.01–1.02)
≧38.3 pack-years	Cases (%)/controls (%)	67 (29.3)/47 (17.9)	119 (52.0)/147 (55.9)	42 (18.3)/63 (24.0)	1 (0.4)/6 (2.2)
	OR (95% CI)[b]	1.00[c]	0.46 (0.28–0.74)[d]	0.41 (0.23–0.73)[d]	0.09 (0.01–0.84)[d]
SCC	Cases (%)/controls (%)	45 (33.6)/110 (18.0)	65 (48.5)/293 (47.9)	23 (17.2)/180 (29.5)	1 (0.7)/28 (4.6)
	OR (95% CI)[b]	1.00[c]	0.46 (0.28–0.77)[d]	0.39 (0.21–0.72)[d]	0.10 (0.01–0.78)[d]
<38.3 pack-years	Cases (%)/controls (%)	5 (23.8)/63 (18.1)	11 (52.4)/146 (42.0)	5 (23.8)/117 (33.6)	0 (0.0)/22 (6.3)
	OR (95% CI)[b]	1.00[c]	0.81 (0.26–2.51)	0.51 (0.14–1.90)	NA[e]
≧38.3 pack-years	Cases (%)/controls (%)	40 (35.4)/47 (17.9)	54 (47.8)/147 (55.9)	18 (15.9)/63 (24.0)	1 (0.9)/6 (2.2)
	OR (95% CI)[b]	1.00[c]	0.36 (0.21–0.63)[d]	0.31 (0.15–0.62)[d]	0.17 (0.02–1.60)
Ad	Cases (%)/controls (%)	143 (25.7)/110 (18.0)	256 (46.0)/293 (47.9)	138 (24.8)/180 (29.5)	20 (3.6)/28 (4.6)
	OR (95% CI)[b]	1.00[c]	0.59 (0.42–0.81)[d]	0.54 (0.37–0.78)[d]	0.39 (0.20–0.77)[d]
<38.3 pack-years	Cases (%)/controls (%)	41 (18.0)/63 (18.1)	100 (43.9)/146 (42.0)	71 (31.1)/117 (33.6)	16 (7.0)/22 (6.3)
	OR (95% CI)[b]	1.00[c]	0.95 (0.58–1.57)	0.82 (0.48–1.38)	0.67 (0.30–1.51)
≧38.3 pack-years	Cases (%)/controls (%)	102 (31.0)/47 (17.9)	156 (47.4)/147 (55.9)	67 (20.4)/63 (24.0)	4 (1.2)/6 (2.2)
	OR (95% CI)[b]	1.00[c]	0.42 (0.27–0.64)[d]	0.43 (0.26–0.72)[d]	0.23 (0.06–0.92)[d]

Significant association between the CYP2A6 groups and lung cancer risk with SqCC, SCC and Ad seen as χ^2 value 20.8 ($P = 0.0001$), χ^2 value 15.8 ($P < 0.01$) and χ^2 value 15.6 ($P < 0.01$), respectively.
[a]Groups 1, 2, 3 and 4 were classified according to the *CYP2A6* genotypes. See Table III for details.
[b]To adjust age and smoking habit, OR and 95% CI were calculated by logistic regression.
[c]Reference category.
[d]Significant decrease of OR is indicated by 95% CI.
[e]Not applicable.

Tobacco smoke contains a number of tobacco-specific *N*-nitrosamines, such as *N*-nitrosodiethylamine, NNK and *N'*-nitrosonornicotine (48). In addition to CYP2A6, CYP1A1 and CYP2A13 are able to activate NNK (8,9,49,50). Thus, it can be expected that the genetic polymorphism of the *CYP1A1* and *CYP2A13* genes affect the tobacco-related cancer risk. Recently, we found 14 novel *CYP2A13* haplotypes including the Arg257Cys variant, which was named as *CYP2A13*2* (51). Wang *et al.* (32) have reported recently that the frequency of the *CYP2A13* variant associated with the reduced risk of lung Ad in light smokers. However, analyzing our data using the same subjects employed in the present study, we found no clear association between the lung cancer risk and the *CYP2A13*2* allele (data not shown). The reason for this discrepancy is unknown at present. Furthermore, the contribution to cancer risk of other carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines in tobacco smoke could not be ruled out. In fact, the enzymes belonging to the *CYP1* gene family play central roles in the metabolic activation of these compounds present in tobacco smoke (52,53). However, we were unable to find out any clear relationships between genetic polymorphism of *CYP1A1* and tobacco-related lung cancer risk with the same population employed in the previous epidemiological study (31), probably suggesting that the metabolic activation by CYP2A6 of nitrosamines or carcinogens other than polycyclic aromatic hydrocarbons is the key step determining the tobacco-related lung cancer risk.

In conclusion, our results suggest strongly that the genetic polymorphism of *CYP2A6* is one of the principal determinants affecting not only smoking behavior but also tobacco-related lung cancer risk in the Japanese population.

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Fusion of HIV-1 Tat protein transduction domain to poly-lysine as a new DNA delivery tool

H Hashida[1,3], M Miyamoto*[,1,3], Y Cho[1], Y Hida[1], K Kato[1], T Kurokawa[1], S Okushiba[1], S Kondo[1], H Dosaka-Akita[2] and H Katoh[1]

[1]Department of Surgical Oncology, Division of Cancer Medicine, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan; [2]Department of Medical Oncology, Division of Cancer Medicine, Hokkaido University Graduate School of Medicine, Hokkaido, Japan

Effective gene therapy depends on the efficient transfer of therapeutic genes to target cells. None of the current technologies, however, satisfy all of the requirements necessary for gene therapy, because the plasma and nuclear membranes of mammalian cells are tight barriers against gene transfer using synthetic delivery systems. The protein transduction domain (PTD) of human immunodeficiency virus type 1 (HIV-1) Tat protein greatly facilitates protein transfer via membrane destabilisation. We synthesised polylysine peptides containing Tat PTD (TAT-pK), or other sequences, and investigated their potential as agents for gene transfer. The synthesised polypeptide TAT-pK retains DNA binding function and mediates delivery of a reporter gene to cultured cells. RGD motif binds with low affinity to alpha integrins which induce cell activation. Two control polypeptides, GGG-pK and RGD-pK, were synthesised and tested, but their gene transfer abilities were weaker than those of TAT-pK. TAT-pK-mediated gene transfer was enhanced in the presence of chloroquine or ammonium chloride, to a greater extent than that of cationic lipid-mediated gene transfer in most cancer cell lines tested. These data suggest that TAT-pK may be a potent candidate delivery vehicle that promotes gene transfer, dependent on the endocytic pathway. We conclude that the TAT-pK/DNA complex is useful as a minimal unit to package therapeutic genes and to transduce them into mammalian cells.
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Gene therapy for cancer has been developed and a number of clinical therapeutic protocols are now being investigated. Vectors based on various viruses are useful for delivering therapeutic genes into primary cells *in vitro* and have also been applied in a number of gene therapy trials with humans. Viruses have some disadvantages as tools for medical application, however, with many elements of their biology yet to be elucidated. The utility of viral vectors for gene therapy is limited by DNA carrying capacity, difficulty in reliable and cost-effective manufacturing, and by immunogenicity and other safety concerns. One goal of cancer gene therapy is the development of gene delivery tools with lowered immunogenicity. While the construction of some viral vectors with reduced immunogenicity have been reported (Fisher *et al*, 1996; Haecker *et al*, 1996; Kochanek *et al*, 1996; Kumar-Singh and Chamberlain, 1996; Morral *et al*, 1999), preparation of these vectors is difficult because the virus is composed of several kinds of large molecules.

Two elements are necessary to efficiently express foreign genes in cells: passage of DNA across the cell membrane and transport into the nucleus (Colin *et al*, 2000). From this point of view, recombinant viral vectors have a great advantage by depending on their intrinsic machinery for infection. Basic peptides derived from human immunodeficiency virus type 1 (HIV-1) Tat protein and *Drosophila* Antennapedia protein have been reported to translocate through the cell membrane and to carry exogenous molecules into the cytoplasm and nucleus (Derossi *et al*, 1994, 1996; Vivès *et al*, 1997; Nagahara *et al*, 1998; Schwartz *et al*, 1999; Dostman *et al*, 2000). HIV-1 Tat is an 86 amino-acid protein, and aa 47–57 of Tat (YGRKKRRQRRR) possess a high net positive charge at physiological pH, with nine of its 11 amino acids being either arginine or lysine. Fusion of several proteins and this 11 aa region of Tat protein enables the delivery of proteins into cells. Thus, this 11 aa region is considered a protein transduction domain (PTD). A 119-kDa protein, β-galactosidase, genetically fused to HIV-1 Tat PTD, was successfully carried into various mouse tissues, including the brain, following intraperitoneal injection (Nagahara *et al*, 1998).

Molecular conjugates of poly-lysine with natural or artificial ligands utilise the DNA-binding and -condensing properties of poly-lysine to mediate interaction with DNA (Wagner *et al*, 1991; Perales *et al*, 1994). Upon formation of a DNA-poly-lysine-ligand complex (polyplex (Felgner *et al*, 1997)), gene transfer is facilitated via receptor-mediated endocytosis.

In this study, we investigated the potential of a poly-lysine fused Tat PTD (TAT-pK) as a gene delivery agent. We demonstrate that TAT-pK combines with DNAs and efficiently transports them into several human cell lines.

*Correspondence: Dr M Miyamoto;
E-mail: m-miyamo@med.hokudai.ac.jp
[3]These authors have contributed equally to this work.
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MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: chloroquine and ammonium chloride from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan); 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N'N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/dioleoyl phosphatidylethanolamine (DOPE) (LipofectAMINE™) from Life Technologies, a division of Invitrogen (Rockville, MD, USA).

Cell lines

The human embryonic kidney cell line HEK 293 was obtained from CLONTECH Laboratories. Human pancreatic carcinoma cell lines PCI10, PCI19, PCI35, and PCI43 were generously provided by Dr Yoshiki (Hokkaido University, Japan). Human oesophageal squamous cell carcinoma cell lines TE2, TE5, TE8, and TE13 were provided by Dr Nishihira (University of Tohoku, Japan). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Rockville, MD, USA) with 2 mM L-glutamine, supplemented with 10% heat-inactivated foetal calf serum (FCS), at 5% CO2. Human lung carcinoma cell lines A549, RERF-LC-MS, and PC3 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and NCI-H226 was obtained from American Type Culture Collection (Manassas, VA, USA). They were maintained in RPMI medium (GIBCO) with 10% FCS.

Polypeptides synthesis

Three pKs (TAT-pK, RGD-pK, GGG-pK) and TAT were supplied by Hokkaido System Science Co.,. Ltd. (Sapporo, Japan). They were chemically synthesised by solid-phase methods, using Fmoc (9-fluorenylmethyloxycarbonyl) with a Pioneer™ Peptide Synthesis System (Applied Biosystems, CA, USA). Primary structures of the polypeptides were shown in Table 1.

Plasmid DNAs

The expression plasmid for enhanced green fluorescent protein (EGFP) (CLONTECH Laboratories, CA, USA) was constructed by inserting the cDNA into pcDNA3.1+ to produce pcDNA-EGFP. The expression vector for firefly luciferase pGL3 was obtained from Promega Corp (Madison, WI, USA).

Agarose gel electrophoresis of DNA-peptides complex

For the agarose gel electrophoresis assay, 0.5 μg of DNA (lambda DNA/*Hind*III digest) and peptides were mixed and incubated for 10 min at room temperature. The samples were loaded on a 1% agarose gel containing 0.5 $\mu g\,ml^{-1}$ of ethidium bromide and run for 30 min at 100 V in 1 × TBE buffer.

Transfection conditions

A standard protocol for gene transfer into cultured cells was followed. Cells were seeded at 2×10^4–1×10^5 cells $well^{-1}$ in tissue culture plates, and cultured for 6 h. The cells were washed once with serum-free medium and incubated with medium containing DNA, DNA-peptides complex, or DNA complexed with cationic lipids (DOSPA/DOPE) for 8 h at 37°C. The cells were cultured for 48 h in medium with 10% FCS before assaying for the expression of reporter genes. DNAs were complexed with cationic lipids, according to the procedures recommended by the suppliers.

Table 1 Primary structures of synthesised polypeptides

Peptides	Sequences
TAT-pK	NH_2-YGRKKRRQRRR-GGG-KKKKKKKKKKKKK-COOH
RGD-pK	NH_2-AIRGDTFATGAS-GGG-KKKKKKKKKKKKK-COOH
GGG-pK	NH_2-GGG-KKKKKKKKKKKKK-COOH
TAT	NH_2-YGRKKRRQRRR-GGG-COOH

Luciferase assay and UV microscopy

Luciferase activity was evaluated using the Luciferase Assay System (Promega) and relative light units (RLU) were measured with Mini Lumat LB 9506 (BERTHOLD, Germany). RLUs are shown as averages with standard deviations. GFP and FITC were detected with fluorescence microscopy (Olympus Optical Co. Ltd., Japan) using a GFPA cube. The cell nucleus was localised with fluorescence microscopy, using the fluorescent DNA binding dye, Hoechst 33342, and a WU cube (Olympus Optical Co. Ltd.). FITC labelled DNA fragments were prepared by phosphoramidite synthesis and purified by RP-HPLC purification.

WST-8 assay

Cytotoxicity of peptides was investigated using WST-8 assay. HEK293 cells were seeded in 96-well tissue culture plate at a density of 2×10^4 cells per well and incubated at 37°C for 72 h in fresh medium containing various peptides at a concentration of 10–320 $\mu g\,ml^{-1}$. After incubation, 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-8; Wako Pure Chemical Industries, Ltd, Japan) were added to each well. After 4 h of incubation, the optical density was read on a microplate autoreader (SPECTRAmax® 190; Molecular Devices Corp, Sunnyvale, CA, USA) using a test wavelength of 490 nm and a reference wavelength of 620 nm.

FACS analysis

Where indicated, 10 000 events were counted on a Becton Dickinson FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ, USA) using a 15 mW air-cooled argon laser set at 488 nm, and recorded with a 530 nm emission filter in the FL1 emission channel. Cell populations are represented on a FACS histogram plotting FITC intensity on a logarithmic scale against cell number. Fluorescence intensity of cell populations is indicated by a shift to the right of the histogram plots of treated cells. Fluorescence enhancement was determined by obtaining the number of gated fluorescent events for untreated and treated cells.

RESULTS

DNA mobility shift analyses of pKs using agarose gel electrophorase

The pK tracts should impart DNA binding function to the fusion protein, by interacting with the negatively charged phosphate backbone of nucleic acids. To determine DNA binding ability, increasing concentrations of peptides were incubated with constant amounts of DNA marker (*λ*/*Hind*III), and the resulting effects on DNA mobility were analysed on agarose gels (Figure 1). At concentrations where peptides completely bind and thus neutralise the DNA, it appears immobilised on the gel. The DNA was immobilised with peptides at a protein-to-DNA ($w\,w^{-1}$) ratio of 1. These results show that peptides can bind DNA. Furthermore, excess amounts of peptides did not induce the DNA to migrate in the opposite direction from the positive electrode.

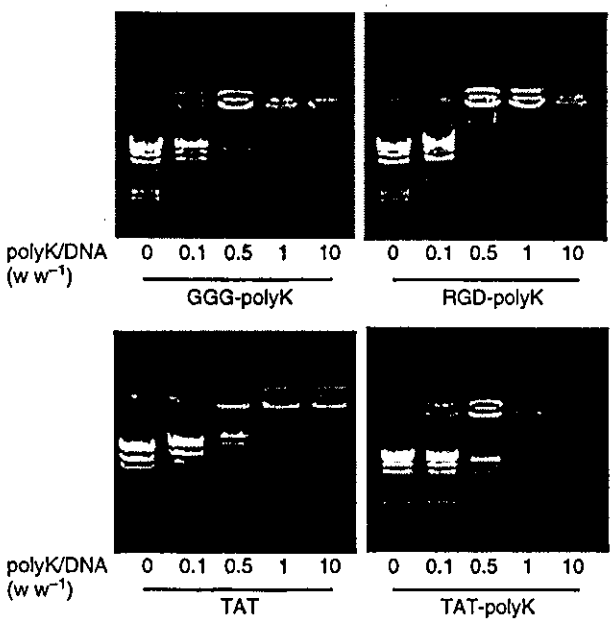

Figure 1 DNA mobility shift analyses of pKs. Synthesised pKs bind to plasmid DNA electrically. Three pKs were preincubated with 0.5 μg of λ/*Hind*III DNA marker. The plasmid DNA was electrophoresed alone or after preincubation with a given concentration (0.05–5 μg) of polypeptides. All plasmids were immobilised with pKs at a protein-to-DNA ($w w^{-1}$) ratio of 1.

Induction of EGFP and luciferase gene expressions by various peptides

We examined the efficiency of peptide-mediated gene transfer by evaluating the expression of the complexed EGFP gene. Under standard transfection conditions, we detected strong EGFP expression in approximately 5% of HEK 293 cells treated with the TAT-pK/pcDNA-EGFP complex. In cells treated with the GGG-pK/ or RGD-pK/pcDNA-EGFP complex, however, EGFP expression was detected in less than 1% of cells (Figure 2A). The number of cells expressing EGFP increased in proportion to the dose of pKs/DNA complex (figure not shown). In this study, the RGD-pK/DNA complex was also introduced into cells in a dose-dependent manner, but there was no difference in the transduction efficiency of RGD-pK and of GGG-pK. On the other hand, DNA complexed with TAT-pK was efficiently introduced, even at lower doses. The transduction efficiency of TAT peptide without a sequence of poly-lysine was lower than those of pKs (Figure 2B).

Cell-binding activities evaluated by FACScan

To investigate the reason for the high efficiency of TAT-pK-mediated gene transfer, cell-binding activity of pK/DNA complexes using FITC-labelled DNA was assessed (Figure 3A-H). There was scarcely any complex bound to cells with GGG-pK (Figure 3A) and RGD-pK (Figure 3B), and the amount of complex bound to cells did not increase, even 6 h after incubation (data not shown). TAT-pK/DNA complex, however, bound to almost all cells 60 min after incubation (Figure 3C). The difference in binding activity between TAT-pK and other pKs should be reflected in the DNA transduction efficiency. Interestingly, treatment with a small quantity of TAT-pK/DNA complex rarely led to cell binding (Figure 3D), and less than 0.1% of cells expressed EGFP when exposed to the equivalent quantity of TAT-pK/pcDNA-EGFP (data not shown). Most of the TAT-pK/DNA complex binds to the cells

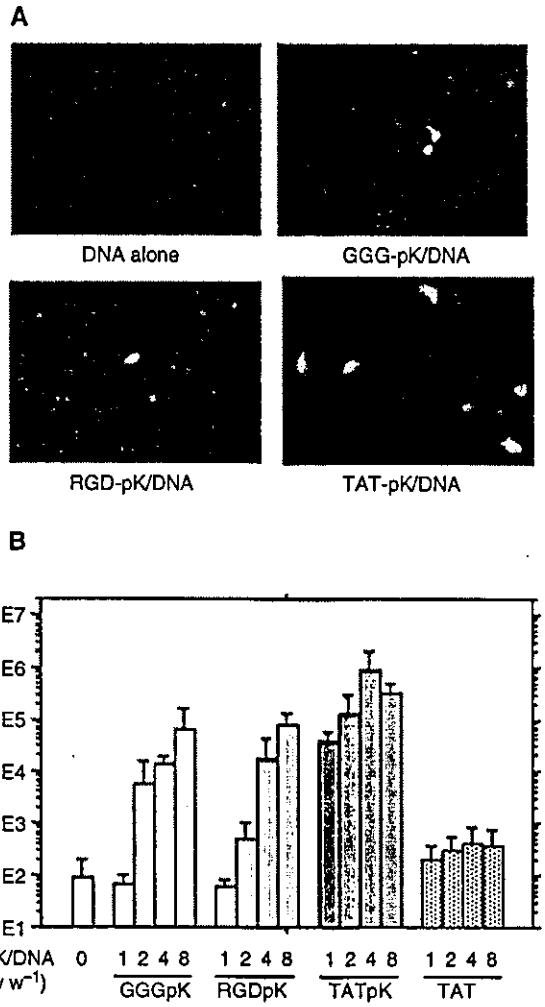

Figure 2 Induction of marker gene expression by various pKs. (**A**) *In situ* detection of pK-mediated GFP gene expression in HEK 293 cells. Cells were seeded in 12-well tissue culture plates at a density of 5×10^4 cells well^{-1}. The cells were treated with pK/pcDNA-EGFP complex (12 μg of pKs and 3 μg of DNA) as described under Materials and Methods. After 8 h, medium with 10% FCS was added, and the cells were grown for another 48 h before fluorescent microscopic observation. (**B**) Induction of peptide-mediated luciferase gene expression. Cells were seeded in six-well tissue culture plates at a density of 1×10^5 cells well^{-1}. The cells were treated with peptide/pGL3-promoter complex (5 μg of DNA). After 8 h, medium with 10% FCS was added, and the cells were grown for another 48 h before they were harvested for analysis. Luciferase activity was evaluated using the Luciferase Assay System (Promega) and estimated in average relative light units (RLU) with standard deviations.

10–30 min after incubation (Figure 3E and F). Tat-(48–60) has been reported to enter cells extremely rapidly, reaching the nucleus within 5 min (Futaki *et al*, 2001). Similarly, in our analysis, binding activity was observed immediately after incubation, and 1 h after incubation, FITC emission was detected in the nucleus (Figure 3I). No FITC emission was observed in cells exposed to the other pK/DNA complexes (data not shown). On the other hand, a four-fold excess of peptides, for neutralisation of the electrical charge, was required for high affinity (Figure 3G and H). A large number of peptides appear to require the complex to remain stably on the cell membrane or in the cytoplasm.

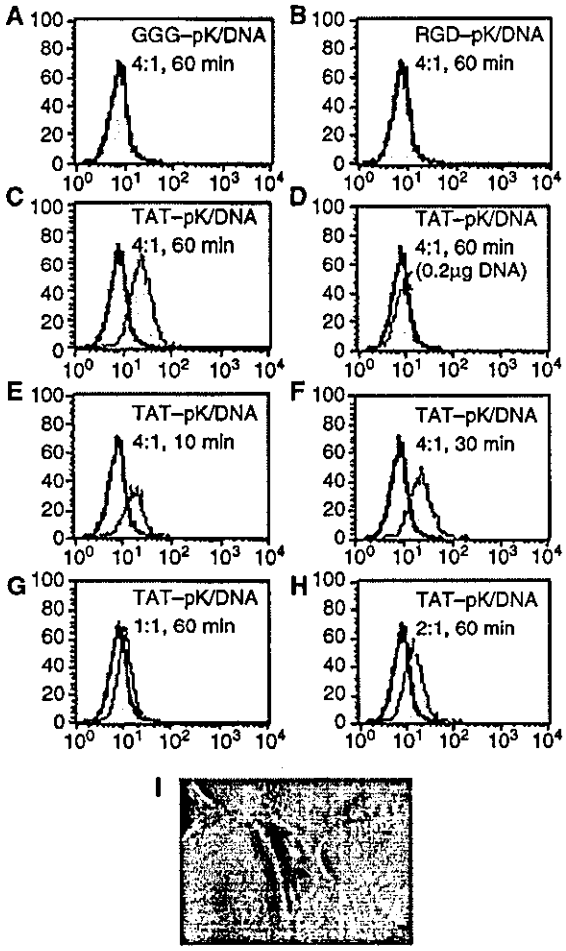

Figure 3 Cell binding activity of pK/DNA complex. HEK 293 cells were grown on 12-well dishes. Peptides and FITC-labelled-DNA were incubated together at the indicated ratios, at room temperature for 5 min in 1 ml of serum-free DMEM, and then added to cell monolayers. Cells were exposed to mixtures (2 μg of DNA) for 60 min at 37°C, 5% CO_2 (**A–C**). Cells exposed to mixtures containing 0.8 μg of TAT-pK and 0.2 μg of DNA for 60 min (**D**), 8 μg of TAT-pK and 2 μg of DNA for 10 min (**E**) and 30 min (**F**), indicated ratios of TAT-pK and 2 μg of DNA for 60 min (**G**, **H**). *In situ* detection of TAT-pK/FITC-labelled-DNA complexes (**I**). Cells were exposed to mixtures containing 8 μg of TAT-pK and 2 μg of DNA for 2 h and FITC was detected with fluorescent microscopy as described under Materials and Methods.

Cytotoxicity of pKs evaluated by WST-8 assay

The cytotoxicity of the three pKs was investigated after incubating 293 cells with peptide concentrations up to 320 μg ml^{-1} for 72 h. The cytotoxicity of TAT-pK and of RGD-pK were almost equal, while that of GGG-pK was slight (Figure 4).

Characterisation of TAT-pK-mediated gene transfer in HEK 293 cells

TAT-pK-mediated EGFP expression was dramatically improved in the presence of 100 μM chloroquine (Figure 5A). Efficiency of TAT-pK-mediated luciferase expression was also elevated dose-dependently, but exposure to excess amounts of agent reduced the luciferase activity (Figure 5B). Moreover, as TAT-pK contains a GRKKR nuclear localisation signal within its sequence, we thought it would be advantageous for cellular gene expression. These

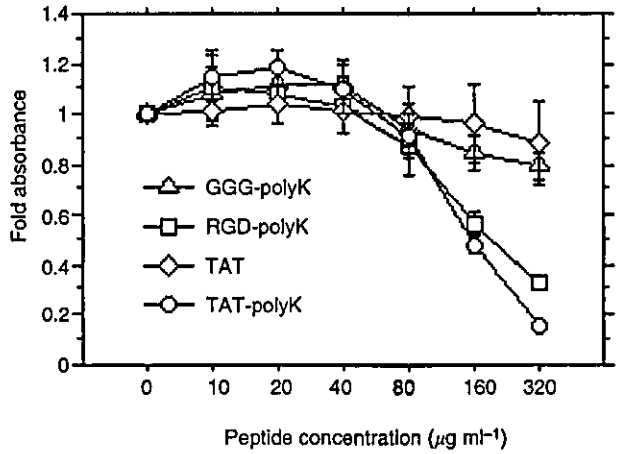

Figure 4 Cytotoxicity of polypeptides on HEK 293 cells. Cells were seeded into 96-well plates and incubated at 37°C for 72 h in fresh medium containing a given concentration of various polypeptides. Absorbance was measured by the WST-8 assay, as described under Materials and Methods. Each end point represents the mean ± s.d.

functions are similar to those of viral vectors that actively bind to the cell surface via high-affinity ligands, and transport their DNA into the nucleus by endocytosis. Thus, TAT-pK/DNA complexes possess the necessary elements for transfection as a small particle, although the efficiency is lower than that of adenoviral vectors. When we compared the efficiency of TAT-pK-mediated gene transfer with that mediated by cationic lipids (DOSPA/DOPA), we found that TAT-pK can induce higher levels of luciferase activity in the presence of chloroquine, but not in its absence (Figure 5B). The cytotoxicity of ammonium chloride or chloroquine was investigated (Figure 5C). Both of ammonium chloride and chloroquine showed cytotoxicity at a higher concentration. There were no cytotoxic effects with TAT-pK.

TAT-pK-mediated transduction efficiency in various human cancer cell lines

To utilise TAT-pK for various purposes, we investigated the efficiency of TAT-pK-mediated luciferase gene transfer using several human cancer cell lines that tend to accept gene transfer with low efficiency (Figure 6). The TAT-pK complex was successfully introduced into almost all cell lines at superior levels to DOSPA/DOPA, in the presence of chloroquine.

DISCUSSION

In order to be utilised for gene therapy, gene delivery systems require convenience and safety. Even transporting a single DNA encoding a small protein needs a vector construct. Moreover, the vector has longer DNA and 'the shell' that wraps it. Because large vectors may have adverse effects, our aim was to construct the smallest possible unit permitting efficient and safe transfection of DNA into mammalian cells. Cationic polypeptides, such as poly-arginine and poly-lysine, have been reported to bind DNA, form complexes with DNA, and introduce themselves into cells (Wagner *et al*, 1991; Perales *et al*, 1994; Felgner *et al*, 1997; Futaki *et al*, 2001; Suzuki *et al*, 2002). Their efficiency for practical applications to gene therapy, however, remains untested. In the present study, we fused the Tat PTD to poly-lysine in order to improve the efficiency of DNA delivery.

As the pancreatic cancer cell line PCI35 has poor DNA transfection efficiency with cationic liposomes, we investigated

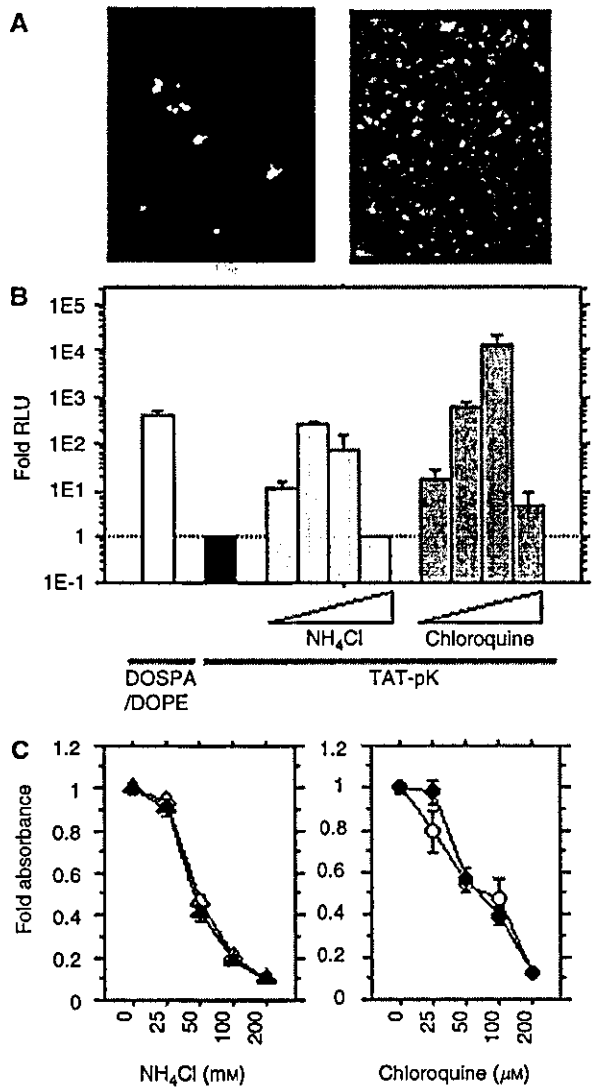

Figure 5 Characterisation of TAT-pK-mediated gene transfer in HEK 293 cells. (**A**) Enhancement of TAT-pK-mediated GFP gene expression in HEK 293 cells. Cells were seeded in 24-well tissue culture plates at a density of 2×10^4 cells well^{-1}. The cells were treated with 1 ml fresh medium containing TAT-pK/pcDNA-EGFP complex (4 μg of peptide and 1 μg of DNA) in the absence (left) or the presence of 100 μM chloroquine (right). EGFP expression was detected with fluorescent microscopy as described under Materials and Methods. (**B**) Comparison of transfection activity of DOSPA/DOPE/DNA complex with TAT-pK/DNA complex and effects of ammonium chloride or chloroquine on TAT-pK-mediated gene transfer. HEK 293 cells (5×10^4 well^{-1}) were seeded into 12-well tissue culture plates. The cells were treated with DOSPA/DOPE/DNA complex (2 μl of DOSPA/DOPA and 1 μg of DNA, open bar), according to the procedures recommended by the suppliers, or with TAT-pK/DNA complex (4 μg of peptide and 1 μg of DNA) as described under Materials and Methods. The cells with TAT-pK/DNA complex were incubated for 48 h in the absence (filled bar) or presence (grey bars) of ammonium chloride (25, 50, 100, and 200 mM) or chloroquine (25, 50, 100, and 200 μM). After incubation, cells were harvested and luciferase activity was evaluated. The luciferase activities were averaged from the results of duplicate experiments and are presented relative to the control value, indicated with the filled bar. (**C**) Cytotoxicity of ammonium chloride or chloroquine on HEK293 cells. Cells were seeded into 96-well plates and incubated at 37°C for 48 h in fresh medium containing a given concentration of ammonium chloride (left) or chloroquine (right) with (filled) or without (open) 20 μg ml^{-1} TAT-pK. After incubation, absorbance was measured by the WST-8 assay, as described under Materials and Methods. Each end point represents the mean $\pm$ s.d.

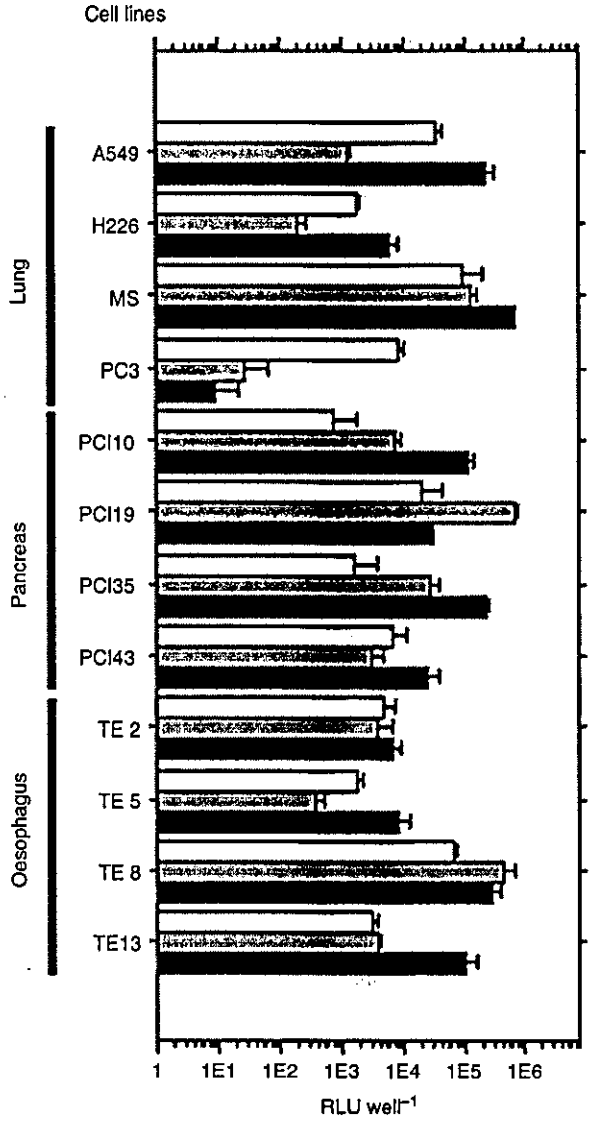

Figure 6 Transduction efficiency of DOSPA/DOPE or TAT-pK with chloroquine in various human cancer cell lines. Cells (5×10^4) were incubated with 1 ml medium containing pGL3-promoter DNA (1 μg) complexed with DOSPA/DOPA (open bars) or with TAT-pK in the presence of 50 μM chloroquine (grey bars) or 100 μM chloroquine (filled bars) and grown as described under Materials and Methods. After 48 h incubation, cells were harvested and luciferase activity was measured. Each end point represents the mean $\pm$ s.d. RLU, relative light units.

modalities for efficiently transfecting pancreatic cancer cell lines. Initially, a recombinant Histidine6-tagged TAT-pK (H6-TAT-pK) construct was produced in *E. coli* strain BL21(DE3), carrying the pLysS plasmid (One Shot™; Invitrogen Corp., Carlsbad, CA, USA) to control leak-through expression and to allow subsequent cell lysis by freeze thawing. This construct was purified using a HiTrap™ column (Amersham Pharmacia Biotech., Buckinghamshire, UK) and the ÄKTA prime™ system (Amersham Pharmacia Biotech), and a standard protocol for protein production was followed. The H6-TAT-pK/DNA complex was successfully introduced into PCI35 cells and EGFP expression was detected. The efficiency of transfection, however, was not better than with lipofection (data not shown). Subsequently, in order to elucidate the mechanism for TAT-pK-mediated gene transfer and to

improve the efficiency, we synthesised TAT-pK without the His6 tag, as well as two control polypeptides GGG-pK and RGD-pK. The adenoviral RGD (Arg-Gly-Asp) motif AIRGDTFATGAS was fused to pK to compare the transfection efficiency with that of TAT-pK, because interaction of the RGD motif in the adenoviral penton base with cell membrane integrins is required to induce or trigger endocytosis (Wickham *et al*, 1993).

We anticipated that the cytotoxicity of TAT-pK would be stronger than that of RGD-pK, but interestingly, this was not the case. Actually, as cells would not be exposed to high concentrations for a prolonged time, toxicity is not likely to be a serious concern. No toxicity was observed, even at the highest doses examined for DNA transduction.

Most nonviral vehicles deliver their genes passively, relying on uptake into vesicular compartments by endocytosis, thus we examined the effects of ammonium chloride or chloroquine on transduction. Ammonium chloride is a weak acidotropic base. Chloroquine elevates the pH of vesicular compartments (Cotten *et al*, 1990), and either stimulates or inhibits the efficiency of endocytosis-mediated gene transfer, depending on the delivery vehicle. We found that TAT-pK-mediated gene transfer is affected by either of these agents, suggesting that transduction relies on the endocytic pathway. These results are similar to past reports showing that gene transfer via receptor-mediated endocytosis (Cotten *et al*, 1990) or mediated by DEAE-dextran (Luthman and Magnusson, 1983) is markedly enhanced with endosomotrophic agents, such as chloroquine. However, our data contradict other studies on TAT-peptide-mediated protein transduction (Mann and Frankel, 1991; Derossi *et al*, 1996; Vivès *et al*, 1997; Elliott and O'Hare, 1997) and TAT-phage-mediated gene transfer (Eguchi *et al*, 2001) that do not depend on endosomotrophic reagents. The mechanism of action of Tat-(48–60) peptide and the full-length Tat protein may not be the same (Liu *et al*, 2000). Rather, TAT-pK-mediated gene transfer seems to share features of both systems, operating by both an energy-dependent endocytic pathway and an independent pathway. These dual mechanisms may account for the high efficiency of DNA transduction. In short, the Tat PTD anchors the TAT-pK/DNA complex to the cell surface within a few minutes by membrane destabilisation, and then the complex crosses the cell membrane by endocytosis.

The conditions suitable for gene transfer differed for each cell line. As PBS or RPMI medium reduce the efficiency of TAT-pK-mediated gene transfer (data not shown), we used sterilised water for diluting pKs and only used DMEM in transfection. Transduction efficiency of TAT-pK was easily influenced by several factors, such as pH or temperature of medium, preincubation period, and quantity of DNA (data not shown). Moreover, the fold absorptions were decreased at high concentrations of chloroquine because of its cytotoxicity.

Several Tat PTD fused proteins have been reported as potential therapeutic strategies for cancer (Mann and Frankel, 1991; Derossi *et al*, 1996; Elliott and O'Hare, 1997; Vivès *et al*, 1997), but the quantity of protein transduced into tissues would be lower than that from administration of vector DNA. Moreover, selectivity and transduction efficiency are very important factors in order to apply gene therapy for cancer patients. Although we have not achieved targeting transduction for cancer cells by using TAT-pK, it may be easily modified to target cancerous, but not normal cells, since TAT-pK is much smaller than the capsid proteins of viral vectors. Also immunogenicity by TAT-pK should be investigated, but we do not think it higher than that of viral vectors because of its size. We have started *in vivo* experiments to assess these factors and to improve them.

In conclusion, although there is need for further improvement, TAT-pK is a candidate for a new DNA transfection system. Many problems still exist in clinical trials using viral vector-mediated gene therapy, therefore the development of artificial viral vector systems is urgently needed. TAT-pK is likely a minimal unit to efficiently package therapeutic genes and transduce them into mammalian cells.

ACKNOWLEDGEMENTS

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Phase I Trial of Carboplatin and Weekly Paclitaxel in Patients With Advanced Non-small-cell Lung Cancer

Junko Kikuchi[1], Koichi Yamazaki[1], Ichiro Kinoshita[2], Hajime Asahina[1], Mikado Imura[1], Eiki Kikuchi[1], Jun Konishi[1], Naofumi Shinagawa[1], Hiromitsu Oki[3], Hirotoshi Dosaka-Akita[2] and Masaharu Nishimura[1]

[1]First Department of Medicine, Hokkaido University School of Medicine, Sapporo, [2]Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo and [3]Department of Pharmacy, Hokkaido University Hospital, Sapporo, Japan

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Objective: This study was designed to determine the maximum tolerated dose of paclitaxel administered weekly in combination with carboplatin and to assess its dose limiting toxicity and preliminary activity in patients with previously untreated, advanced non-small-cell lung cancer.
Methods: Carboplatin was administered at a fixed dose that maintained an area under the curve of 6. Paclitaxel was given over 1 h once a week for 3 weeks starting at 60 mg/m^2 and escalated in 10 mg/m^2 increments.
Results: Twenty-one patients were treated with six dose levels (60, 70, 80, 90, 100, 110 mg/m^2) of paclitaxel. The dose limiting toxicity was infection and the maximum tolerated dose was 110 mg/m^2. Nine of 21 (42.9%) patients demonstrated a therapeutic response.
Conclusion: Weekly paclitaxel and carboplatin were well tolerated. Based on our results, 100 mg/m^2 of paclitaxel for 3 weeks of a 4-week cycle, in combination with carboplatin, was recommended for a phase II study.

Key words: carboplatin – non-small-cell lung cancer – paclitaxel – phase I study

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the leading cause of death from cancer in the world. Unfortunately, when NSCLC is diagnosed most patients have locally advanced or disseminated cancers. The median survival of patients with stage IIIB and IV NSCLC ranges from 6 to 8 months and only 20–30% survive for 1 year.

Paclitaxel (Taxol; Bristol-Myers Squibb) is a clinically active anticancer drug that inhibits cell division by promoting the assembly of microtubules and stabilizing the tubulin polymers in the G_2/M phase (1). Consequently, paclitaxel causes the formation of abnormal bundles of microtubules during the cell cycle, and it has antiangiogenic activity (2). Carboplatin (Paraplatin; Bristol-Myers Squibb) is a less toxic analog of cisplatin, which is thought to inhibit DNA synthesis by forming interstrand and intrastrand cross-linking of DNA molecules. Carboplatin is as efficacious as cisplatin in treating NSCLC.

A recent Eastern Cooperative Oncology Group (ECOG) study compared third-generation chemotherapy regimens, which included cisplatin with paclitaxel, cisplatin with gemcitabine, cisplatin with docetaxel and carboplatin with paclitaxel. The results showed that there were no differences in survival, and carboplatin with paclitaxel had the lowest degree of toxicity. Therefore, ECOG selected carboplatin with paclitaxel as its reference regimen (3). In addition, the combination of carboplatin [area under the curve (AUC) = 6] and paclitaxel (225 mg/m^2) administered every 3 weeks is the most commonly used regimen in the USA. The response rate with this regimen ranges from 17% to 25%, with median survival times averaging 8 months (3–5). While the regimen is well tolerated, it is associated with a 10% to 17% incidence of grade 3 neuropathy (3–5).

Weekly regimens of paclitaxel and carboplatin were developed in an effort to increase efficacy and reduce toxicity. Belani et al. (6) studied various regimens and found that paclitaxel (paclitaxel 100 mg/m^2 weekly for three of 4 weeks) plus carboplatin (AUC = 6 on day 1) was the most effective and least toxic. For example, this regimen had a response rate of 32%, a median survival time of 49 weeks, and a 1-year survival rate of 47%. Comparison with the previous studies using the standard every-3-week schedule of paclitaxel and carboplatin indicated that the weekly regimens achieved favorable efficacy with a highly tolerable toxicity profile.

In Japan, a phase I trial of carboplatin plus weekly paclitaxel was conducted in advanced NSCLC, and the recommended dose level of paclitaxel was 70 mg/m^2 on days 1, 8 and 15 in

For reprints and all correspondence: Koichi Yamazaki, First Department of Medicine, Hokkaido University School of Medicine, North 15, West 7, Kitaku, Sapporo 060-8638, Japan. E-mail: kyamazak@med.hokudai.ac.jp

combination with carboplatin (AUC = 6) on day 1 of a 4-week cycle (7). The dose level of paclitaxel was much lower than in Belani's study. In order to reconfirm the dose level of paclitaxel, we conducted a phase I trial of weekly paclitaxel (on days 1, 8 and 15) with carboplatin (on day 1) of a 4-week cycle for advanced NSCLC.

SUBJECTS AND METHODS

Patient Eligibility

This phase I trial was designed to determine the maximum tolerated dose (MTD) and toxicity of paclitaxel administered on a weekly schedule to patients with advanced NSCLC. A secondary objective was the determination of efficacy. The ethics committee at Hokkaido University School of Medicine approved this study. Inclusion criteria were: (i) histological or cytological evidence of NSCLC with no prior chemotherapy; (ii) stage IIIB or IV disease that was not curable with chemo-radiation as the first choice; (iii) measurable or assessable disease; (iv) ECOG performance status (PS) ≤1; (v) between 20 and 75 years of age with white blood cell (WBC) counts $>4 \times 10^9$/l, hemoglobin (Hb) >9.5 g/dl, platelet (PLT) counts $>100 \times 10^9$/l, bilirubin <1.5 mg/dl, GOT and GPT less than twice upper limits of normal, and creatinine <1.5 mg/dl, P_aO_2 >70 torr; (vi) anticipated survival at least 3 months and (vii) provided written informed consent. Exclusion criteria were: (i) serious concomitant systemic disorders; (ii) severe heart failure within 3 months, uncontrollable angina or hypertension; (iii) diabetes mellitus; (iv) interstitial pneumonia; (v) active infection, ulcer or second primary malignancy; (vi) history of severe hypersensitivity or hypersensitivity to the study drug or polioxyetilene and (vii) pregnancy. We also measured plasma paclitaxel concentrations during treatment to compare with those established for every-3-week regimens.

Treatment Schedule

The trial was designed as a dose-escalation study of paclitaxel and carboplatin used in combination therapy scheduled every 4 weeks. The dose of carboplatin was fixed to target AUC 6 on day 1. The starting dose of paclitaxel was 60 mg/m^2 given in 1-h i.v. infusions on days 1, 8 and 15 every 4 weeks. If treatment was well tolerated, then successive dose levels were increased in intervals of 10 mg/m^2 in groups of three patients to 70, 80, 90 mg/m^2, if there was no dose-limiting toxicity (DLT). DLT was defined as: (i) persistent (>4 days) leucopenia (<1000/μl); (ii) active infection or fever (>38°C) with grade 3/4 neutropenia; (iii) a PLT count <20 000/μl; (iv) any grade 3/4 non-hematological toxicities, except appetite loss, nausea and vomiting; (v) a delay of second cycle within 6 weeks. If no one encountered dose limiting toxicity, then subsequent patients entered the study at the next greater dose level. If one of the three patients encountered DLT, then subsequent patients entered at the same level, to a total of six patients. If more than one of three or more than two of six patients had DLT at a specific dose level, then that dose levels was defined as the maximum tolerated dose. Anaphylactic premedication included diphenhydramine (10 mg i.v.) and ranitidine (50 mg i.v.) and dexamethasone (20 mg i.v.) 1 h before paclitaxel infusion.

The National Cancer Institute (NCI) common toxicity scale (8) was used to grade side effects. The treatment plan was put on hold for any of the following reasons: (i) white blood cell (WBC) count <3000/μl or PLT count 75 000/μl within 24 h of the day of treatment; (ii) fever >38°C; (iii) PS 3 or (iv) grade 3/4 non-hematological toxicities. Treatment was discontinued when disease progressed, patient died, patient withdrew or experienced septic shock or grade 4 non-hematological toxicity, or decision of clinician. The dose modifications were made after the first 4 weeks of therapy as necessary. Repeated cycles were delivered as their assigned first-dose level unless modified for toxicity. If a patient developed hematologic DLT, each drug was reduced by 20% of previous cycle. The response was evaluated according to WHO criteria (9).

Phamacokinetic Analysis

The disposition of paclitaxel was determined in three patients who received 100 mg/m^2. Blood samples were collected at 0, 2.5, 3.0, 6.0, 10, 24 and 48 h after infusion in tubes that contained potassium edetic acid. Plasma was immediately separated by centrifugation at 3000 r.p.m. for 3 min and stored at −20°C until analysis. Plasma paclitaxel concentrations were determined by high-performance liquid chromatography (HPLC) at SBS, Inc. (Sagamihara, Japan). The pharmacokinetic parameters were calculated using MOMENT (EXCEL), which was developed using Microsoft Excel.

RESULTS

Twenty-one patients with NSCLC entered this trial through six dose levels (Table 1). There were 14 men and seven women with median age of 65 years (range, 44–75). Seven patients had stage IIIB disease and 14 patients had stage IV disease. Adenocarcinoma was the most common histology (n = 17) followed by squamous cell carcinoma (n = 4). Two patients were treated at relapse after surgical resection and 19 were treated during their initial presentation.

Three patients entered at 60, 70, 80, 90 and 110 mg/m^2 (Table 2). Six patients entered at 100 mg/m^2, because one of the first three patients had a delayed second cycle of 6 weeks because of decrease in WBC counts. This patient developed asymptomatic neutropenia. Paclitaxel on day 15 was postponed to day 30. Since five of six patients completed the first cycle at the 100-mg/m^2 dose level, the dose of paclitaxel was advanced to the next greater level. Two patients at the 110-mg/m^2 dose level developed cases of pneumonia that were associated with grade 3/4 neutropenia that cleared with antibiotics. Thus, criteria of DLT were met in two of three patients treated with 110 mg/m^2 (Table 2).

Table 1. Patient characteristics

Characteristic	No. of patients
Age, years	
Median	65
Range	44–75
Sex	
Male	14
Female	7
Performance status	
0	9
1	12
Histological type	
Adenocarcinoma	17
Squamous cell carcinoma	4
Stage	
IIIB	7
IV	14
Prior therapy	
Surgery	2
Radiotherapy	0
No therapy	19

Table 2. Dose level, emergence of DLT and response

Level	CBDCA (AUC)	Paclitaxel (mg/m^2)	No. of patient	DLT	Resonse
1	6	60	3	0	1 PR
2	6	70	3	0	2 PR
3	6	80	3	0	1 CR + 1 PR
4	6	90	3	0	1 PR
5	6	100	6	1*	1 PR
6	6	110	3	2†	2 PR

*Delay of second cycle within 6 weeks.
†Infection with grade 3/4 neutropenia. CBDCA, carboplatin; AUC, area under the curve (mg/ml/min); DLT, dose limiting toxicity; CR, complete response; PR, partial response.

Seven patients received one cycle of therapy, three completed two cycles, six completed three cycles, three completed four cycles, one completed five cycles and one completed seven cycles. Progression of disease was the most common reason for discontinuation. More than 148 doses of paclitaxel were administered to these 21 patients. The average cumulative dose of paclitaxel was 569 mg/m^2, and the maximum dose was 1278 mg/m^2. The longest duration of therapy was 13 months.

Toxicities of Therapy

Twenty-one patients and six dose levels were eligible for evaluation. The toxicities associated with this schedule were generally mild (Table 3). No significant (grade 3/4) red blood cell and PLT toxicities were noted. Although neuropathy is a complication of paclitaxel therapy, only one patient had grade 1 peripheral neuropathy. There was no anaphylaxis or hypersensitivity. Although no formal analysis of long-term toxicity was performed, no obvious cumulative hematologic, pulmonary or neurologic toxicity was noted.

Table 3. Toxicity by dose level in the first cycle (grade 3/4)

Toxicity*	Level					
	1	2	3	4	5	6
Hematological toxicity						
Leucopenia	1/0	0/0	0/0	0/0	0/0	1/0
Granulocytopenia	0/1	1/0	0/0	1/0	2/1	2/1
Anemia	0/0	0/0	0/0	0/0	0/0	0/0
Thrombocytopenia	0/0	0/0	0/0	0/0	0/0	0/0
Non-hematological toxicity						
Anorexia	0/0	0/0	0/0	0/0	1/0	1/0
Fatigue	0/0	0/0	0/0	0/0	1/0	0/0
Infection with grade 3/4 neutropenia	0/0	0/0	0/0	0/0	0/0	2/0

*NCI common toxicity scale

Response to Therapy

Of 21 assessable patients, one complete response (CR) and eight partial responses (PR) (42.9%) were observed. One occurred at level 1, two at level 2, two at level 3, one at level 4, one at level 5 and two at level 6 (Table 2). One patient attained a CR and he remained disease-free for 13 months. A minor response was also seen in five (23.8%) patients. Seven (33.3%) patients showed progression of their disease.

Pharmacokinetics

Pharmacokinetic studies were performed on three patients at 100 mg/m^2. Their mean peak concentration was 5.3 ± 0.72 (±SD) μmol/l. Their average interval peak plasma paclitaxel concentrations are depicted graphically in Figure 1. The mean 48-h concentration was 0.02 ± 0.00 μmol/l. Plasma paclitaxel concentrations at 48-h were >0.01 μmol/l, which is the minimum for a therapeutic response. Plasma paclitaxel levels remained >0.01 μmol/l for more than 144 h and >0.05 μmol/l for 27.9 ± 4.11 h. Key pharmacokinetic characteristics are shown in Table 4.

DISCUSSION

We conducted a phase I trial of weekly paclitaxel with carboplatin in advanced NSCLC. The recommended dose of paclitaxel in a phase II study was 100 mg/m^2, and the DLT was infection with grade 3/4 neutropenia. While the 100 mg/m^2 dose of paclitaxel was greater than doses previously reported in Japan (7), it was equivalent to the dose reported in Belani's

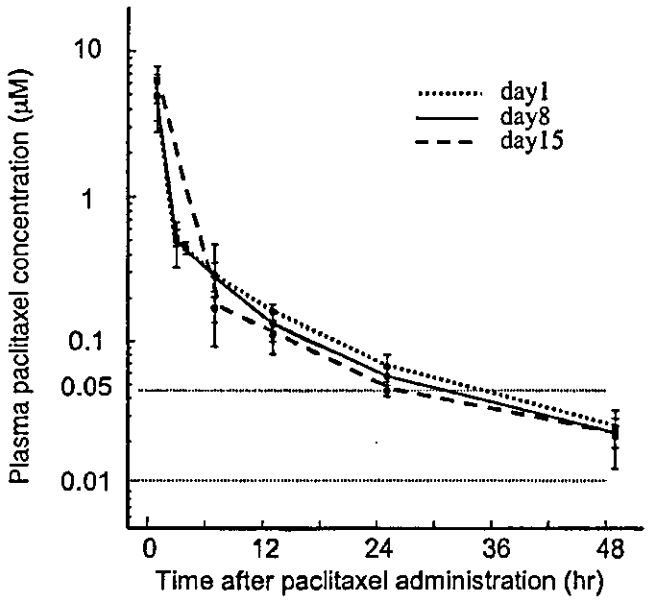

Figure 1. Time versus concentration curves of paclitaxel. Straight line, dotted line, and dashed line show mean ± SD concentration of three patients treated with 100 mg/m^2 on days 1, 8 and 15, respectively.

Table 4. Key pharmacokinetic parameters of paclitaxel

	Cmax (μM)	$t_{1/2}$ (h)	AUC (μM·h)	Time above 0.05 μM (h)
Day 1	6.13 ± 1.75	13.7	15.56	31.8
Day 8	4.93 ± 1.62	14.9	13.26	28.3
Day 15	4.84 ± 2.07	14.6	14.83	23.6

Cmax, maximum plasma concentration; $t_{1/2}$, half-life; AUC, area under the plasma concentration time curve.

study (6). Differences in the ratio of patients with PS 2 between another Japanese trial and the present may be a reason.

Since paclitaxel is a phase-specific agent, frequent or continuous schedules offer the greatest theoretical benefit (10). Depending on the duration of exposure, cellular cytotoxicity can be achieved at relatively low concentrations of this drug that are around 0.01 μmol/l (11,12). On the other hand, myelosuppression was related to the duration of exposure to plasma paclitaxel concentrations >0.05 μmol/l (13). In our study, the time that plasma paclitaxel concentrations remained >0.01 μmol/l was more than 144 h and the time it remained >0.05 μmol/l was 27.9 ± 4.11 h. Our results are similar to the findings in patients with metastatic breast cancer who received similar 1-h infusions of 100 mg/m^2 paclitaxel (14). Thus, a weekly schedule of paclitaxel extended the duration >0.01 μmol/l and >0.05 μmol/l of plasma paclitaxel concentration, as compared to an every-3-week regimen. The pharmacokinetic data of paclitaxel might explain the favorable response rate of a weekly schedule despite weakened intensity of carboplatin every 4 weeks in comparison with an every-3-week regimen. In addition to exposure duration issues, cellular cytokinetic considerations imply that frequent exposure to cytotoxic agents with brief intervals between exposures affords less opportunity for the emergence and regrowth of drug-resistant cell clones (15). On the other hand, severe myelosuppression was not seen in this study, which was not compatible with pharmacokinetic data.

Weekly administration of paclitaxel is dose-intense, but it also has a favorable toxicity profile (16,17). Non-hematologic toxicity was less common with weekly paclitaxel regimens. In our study, no patients developed grade 3 or 4 peripheral neuropathy. A peripheral neuropathy may begin as soon as 24–72 h after treatment with higher doses (>250 mg/m^2) but usually occurs only after multiple courses at conventional doses. Clinically, peripheral neurotoxicity occurs at cumulative doses of approximately 1500 mg/m^2 given at weekly doses of >110 mg/m^2 (18,19). Thus, weekly paclitaxel combination with carboplatin was a favorable regimen in the view of neuropathy, compared to a standard every-3-week regimen.

In conclusion, weekly paclitaxel and carboplatin were well tolerated and 100 mg/m^2 of paclitaxel for 3 weeks of a 4-week cycle in combination with carboplatin was recommended for a phase II study. A multi-institutional phase II study of this treatment is currently underway.

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Expression of RCAS1 in human gastric carcinoma: A potential mechanism of immune escape

Yuichi Nakamura,[1] Koichi Yamazaki,[1,4] Satoshi Oizumi,[1] Manabu Nakashima,[2] Takeshi Watanabe,[2] Hirotoshi Dosaka-Akita[3] and Masaharu Nishimura[1]

[1]First Department of Medicine, Hokkaido University School of Medicine and [3]Department of Medical Oncology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638; and [2]Department of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582

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RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) inhibits the *in vitro* growth of receptor-expressing cells and induces apoptosis, which may contribute to the ability of tumor cells to evade host immune surveillance. In this study, we investigated RCAS1 expression in gastric cancer and precancerous lesions by immunohistochemical means. We then analyzed the relationship between RCAS1 expression and clinicopathological variables, and examined whether RCAS1 expression is associated with infiltration of tumor-infiltrating lymphocytes (TILs) and apoptosis of TILs. Of 54 gastric cancers analyzed, RCAS1 expression was positive in 52 (96%) of them. The expression pattern of RCAS1 in gastric cancer cells could be classified as granular staining either enriched in the glandular side of the cytoplasm with polarity (P pattern) or scattered diffusely in the cytoplasm and on the cell membranes (D pattern). Nineteen of 39 intestinal-type carcinomas (49%) showed the P pattern, and all of 13 diffuse type carcinomas (100%) showed the D pattern. In contrast, all RCAS1-positive specimens of gastric adenoma and metaplastic mucosa were of the P pattern. The D pattern of gastric cancers was more frequently recognized in carcinomas with large size ($P<0.01$), in those with regional lymph node metastasis ($P<0.05$) and in those that had invaded beyond the submucosa ($P<0.01$), compared with the P pattern. On the same sections, significantly less TILs were identified in RCAS1-positive areas than RCAS1-negative areas. Furthermore, the rate of apoptosis of TILs was significantly higher in RCAS1-positive areas than in RCAS1-negative areas. The expression and distribution of RCAS1 may be involved in malignant transformation, tumor progression, histological type and tumor escape from host immune surveillance in gastric cancer. (Cancer Sci 2004; 95: 260–265)

Despite early diagnosis and improved outcome following curative resection, gastric cancer is still one of the most frequent causes of worldwide cancer death.[1] Chemotherapy and irradiation have little effect on the advanced stage of gastric cancer. Accordingly, novel effective therapeutic modalities such as immunotherapy must be developed. Tumor rejection antigens such as CEA and MAGE1-3 are considered to be potential targets for specific immunotherapy in patients with gastric cancer because they are recognized by cytotoxic T lymphocytes (CTLs), which results in tumor rejection.[2,3] However, regardless of the expression of these antigens, many neoplasms escape host immune surveillance. The precise mechanisms involved in this process remain undefined.

The novel tumor-associated antigen, receptor-binding cancer antigen expressed on SiSo cells (RCAS1), is recognized by the mouse monoclonal antibody, 22-1-1, which was raised against the human uterine adenocarcinoma cell line, SiSo.[4,5] A cDNA encoding the antigen recognized by the 22-1-1 antibody has been isolated and the Ag was named RCAS1.[6] RCAS1 is a type II membrane protein that forms homo-oligomers through its C-terminal coiled-coil structures. It is expressed on human cancer cells and acts as a ligand for a putative receptor present on various human cell lines and peripheral lymphocytes, such as T, B and NK cells. The receptor expression was enhanced by activation of the lymphocytes. RCAS1 inhibited the *in vitro* growth of receptor-expressing cells and induced apoptotic cell death. Given these results, tumor cells might evade immune surveillance by expressing RCAS1 and inducing the apoptosis of RCAS1 receptor-positive immune cells. In human tumor tissues, RCAS1 was originally detected at a high frequency on uterine and ovarian tumor cells, and subsequently identified in some other types of cancer.[5,7–13] In the case of gastric cancer, the altered intracellular distribution of RCAS1 is strictly associated with tumor progression.[12] However, thus far, few detailed studies have investigated the relationship between RCAS1 expression on tumor cells and the apoptotic depletion of tumor-infiltrating lymphocytes (TILs) *in vivo*.

In the present study, we investigated RCAS1 expression in gastric cancer and precancerous lesions, and its association with clinicopathological variables. Furthermore, we investigated the association between RCAS1 expression and the number of TILs or apoptosis of TILs to determine whether RCAS1 contributes to regulation of the immune system in gastric cancer.

Materials and Methods

Patients and tissue specimens. We evaluated tissue specimens from 54 patients with gastric adenocarcinoma, five with gastric adenoma, ten with intestinal metaplastic mucosa and five with normal gastric mucosa using immunohistochemical methods. All patients with gastric adenocarcinoma and gastric adenoma had undergone surgical resection or endoscopic mucosal resection at Hokkaido University Medical Hospital between 1997 and 2000. Samples of normal gastric mucosa and intestinal metaplasia were obtained from endoscopic biopsy. None of the patients with gastric adenocarcinoma had received chemotherapy or radiotherapy before resection. Histopathological features were evaluated according to the criteria of Laurèn.[14]

Immunohistochemical detection of RCAS1 expression. Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxide was blocked with 3% hydrogen peroxide in methanol for 20 min. Sections were then washed three times in phosphate-buffered saline (PBS). After blocking of non-specific binding with serum (Histofine SAB-PO kit; Nichirei, Tokyo) for 30 min, sections were incubated with the primary antibodies in a humid chamber at 4°C overnight. The primary antibody was anti-RCAS1 mouse monoclonal antibody (Medical & Biological Laboratories, Nagoya) diluted at 1:500. After three PBS washes, sections were incubated with biotinylated secondary antibody for 30 min, washed three times in PBS and incubated with streptavidin-conjugated peroxidase for 30 min. After three ad-

[4]To whom correspondence should be addressed.
E-mail: kyamazak@med.hokudai.ac.jp