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Contribution of nicotine acetylcholine receptor polymorphisms to lung cancer risk in a smoking-independent manner in the Japanese

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Recent genome wide association (GWA) studies on European and American populations revealed association with lung cancer risk of single-nucleotide polymorphisms (SNPs) in the locus containing two nicotine acetylcholine receptor (*CHRNA*) genes, whose involvement in tobacco addiction had been indicated. Association with lung cancer risk in smokers was consistently, but that in non-smokers as well as that with smoking behavior was inconsistently, observed in these studies. To obtain further information on the significance of *CHRNA* SNPs in lung cancer risk, association of seven SNPs in this locus with lung cancer risk as well as smoking status was examined in a Japanese population by a case–control study of 1250 cases (562 adenocarcinoma, 391 squamous cell carcinoma and 297 small cell carcinoma) and 936 controls. The frequency of the haplotype consisting of minor alleles for three SNPs, rs8034190, rs16969968 and rs1051730, which had been defined as a susceptible haplotype in the GWA studies, was much lower in the Japanese population (0.013) than in European and American populations (0.3–0.4). However, this haplotype was significantly associated with lung cancer risk also in Japanese (odds ratio = 2.3, 95% confidence interval = 1.5–3.7, $P = 0.00028$, respectively). The association was observed both in smokers and non-smokers and in all histological types of lung cancers. Individuals with this haplotype showed higher smoking doses than those without; however, the difference was not statistically significant. These results strongly indicate that *CHRNA* SNPs confer lung cancer susceptibility in a small subset of Japanese in a smoking-independent manner.

Introduction

Tobacco smoking is the major cause of lung cancer in most human populations (1). Recently, the locus containing two genes encoding nicotine acetylcholine receptor subunits, *CHRNA3* and *CHRNA5*, was shown to be associated with lung cancer risk in European and American populations by three genome wide association (GWA) studies (2–4). CHRNA proteins are expressed in lung epithelial cells and bind nicotine, an addictive compound in cigarette smoke, and nitrosamines, potential lung carcinogens in cigarette smoke and foods (5–7). Signal transduction through CHRNA proteins was suggested to cause cell proliferation and also to facilitate neoplastic transformation (8,9). Therefore, if we assume that CHRNA proteins generally transduces a signal of nicotinic substrates in cigarette smoke, a single-nucleotide polymorphism (SNP) in the *CHRNA* genes will be associated with lung cancer risk especially in smokers. Alternatively, if CHRNA proteins transduces a signal of nitrosamines in food as well, a SNP in the *CHRNA* genes could be associated in both smokers and non-smokers. On the other hand, involvement of the same locus in tobacco addiction has been also indicated (4,10,11). Particularly, the rs16969968 SNP causes a change of amino acid conserved across species in CHRNA5 protein, thus, is thought to be a responsible SNP for tobacco addiction (10). Therefore, it is also possible that *CHRNA* SNPs confer lung cancer risk in tobacco addiction-dependent manner. Association of *CHRNA* SNPs with lung cancer risk in smokers was consistently observed among three GWA studies (2–4). However, association in non-smokers was observed only in individuals of European countries and Canada (3) and was not in those of the USA and UK (2). Association of *CHRNA* SNPs with tobacco addiction was also observed inconsistently among the three studies. Association was observed in individuals of Iceland, Spain and The Netherlands (4); however, such an association was observed only in former but not in current smokers of the USA and UK (2) and was not observed in individuals of European countries and Canada (3). Therefore, the mode of association of *CHRNA* SNPs with lung cancer risk is still unclear. Thus, more information on the association in a variety of populations is necessary to elucidate the significance of *CHRNA* SNPs in lung cancer risk. In addition, in the GWA studies above, the association of *CHRNA* SNPs was not examined in Asians due to the low frequency of risk alleles, therefore, it remains also unknown whether or not and how *CHRNA* polymorphisms confer susceptibility to lung cancer in Asians. We conducted here a case–control study to examine the association of *CHRNA* polymorphisms with risks for three major histological types of lung cancer, adenocarcinoma (ADC), squamous cell carcinoma (SQC) and small cell carcinoma (SCC), as well as smoking status in a Japanese population, and the results were compared with those from European and American populations.

Abbreviations: ADC, adenocarcinoma; GWA, genome wide association; LD, linkage disequilibrium; SCC, small cell carcinoma; SNP, single-nucleotide polymorphism; SQC, squamous cell carcinoma.

Subjects and methods

Case–control study

All cases and controls were Japanese. The cases consisted of 562 ADC, 391 SQC and 216 SCC patients of the National Cancer Center Hospital located in Tokyo and 81 SCC patients of the National Cancer Center Hospital East located in Chiba, a prefecture neighboring Tokyo, from 1999 to 2007. The controls were 936 volunteers of National Cancer Center Hospital and Keio University in Tokyo. All the lung cancer cases, from whom informed consents as well as blood samples were obtained, were consecutively included in this study without any particular exclusion criteria. All the lung cancer cases were diagnosed as ADC, SQC or SCC by histological examinations according to World Health Organization classification (12,13). All the control subjects were selected with a criterion of no history of any cancer. Characteristics of a subset of cases and controls were described previously (14,15). This study was approved by the institutional review boards of the National Cancer Center.

Smoking history of cases and controls was obtained via interview using a questionnaire. Smoking dose of each subject was expressed by 'cigarettes per day', i.e. the number of cigarettes smoked per day on average on most days, whereas smoking exposure of each subject was expressed by 'pack-years', which was defined as the number of pack per day (i.e. cigarettes per day divided by 20) multiplied by years of smoking as in previous studies (2–4). Smokers were defined as those who had smoked regularly for 12 months or longer at any time in their life, whereas non-smokers were defined as those who had not. There were no individuals who had smoked regularly for <12 months. From each individual, a 10 or 20 ml whole-blood sample was obtained. Genomic DNA was extracted from whole-blood samples as described previously (14) and 10 ng of genomic DNA was subjected to genotyping using TaqMan assays and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster city, CA).

Statistical analysis

A Hardy–Weinberg equilibrium test was performed using the SNPAlyze version 3.1 software (DYNACOM Co., Ltd, Chiba, Japan). Calculation of the

D' and R^2 values as well as haplotype estimation was undertaken using the expectation-maximization algorithm of the same software. The strength of association of alleles and genotypes with ADC, SQC and SCC risks was measured as crude odds ratios (ORs). The strength of association of genotypes was also measured as ORs adjusted for gender, age and smoking using an unconditional logistic regression analysis (16). These statistical analyses were performed using the JMP version 6.0 software (SAS Institute, Cary, NC). A level of $P < 0.05$ for an OR was considered significant, whereas a level of $0.05 \leq P < 0.10$ for an OR was considered marginal.

Results

We conducted a case–control study consisting of 1250 cases and 936 controls (Table I). All the subjects were Japanese. Most of the SQC and SCC cases were male and smokers, whereas approximately half of ADC cases were smokers, as has been reported (1,17). Seven SNPs were selected from the locus containing the *CHRNA* genes (Figure 1). Two of them, rs8034191 and rs1051730, were the SNPs whose contribution to lung cancer susceptibility as well as smoking behavior was shown in previous GWA studies (2–4). Another one was rs16969968 in *CHRNA5*, whose association with nicotine dependence was previously reported (10). The remaining four were selected based on the fact that minor allele frequencies in the Japanese are reported to be >10% in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

All the cases and controls were genotyped for the seven SNPs (Table II). All these SNPs were in Hardy–Weinberg equilibrium both in cases and controls ($P > 0.05$). Similar and significant differences in the minor allele frequencies between controls and cases were observed in three SNPs, rs8034191, rs16969968 and rs1051730, all of which had minor allele frequencies of <0.02 in the controls (Table II). Two SNPs, rs16969968 and rs1051730 located in the *CHRNA5* and *CHRNA3* genes, respectively, showed significant allelic differentiations irrespective of histological types and smoking status, whereas the minor allele for the rs8034191 SNP located in the *LOC123688* gene showed significantly increased ORs for all histological types of lung cancer and for lung cancer in non-smokers but not for lung cancer in smokers. Four other SNPs, whose minor allele frequencies were >0.1 in the controls, did not show significant allelic differentiations (Table II). Thus, it was indicated that three SNPs, rs8034191, rs16969968 and rs1051730, were associated with lung cancer risk in this population. Crude ORs and ORs adjusted for age, sex and smoking for genotypes for these three SNPs were further calculated (Table III). Heterozygotes and carriers of the minor alleles showed similarly increased ORs (Table III and supplementary Table I is available at *Carcinogenesis* Online). On the other hand, ORs of homozygotes for the minor alleles were not consistently increased among populations probably due to the small number of homozygotes.

We next examined linkage disequilibrium (LD) among these seven SNPs, and haplotypes were estimated. Five of the seven SNPs examined, rs8034191, rs6495306, rs621849, rs16969968 and rs1051730, were in LD with one another ($D' > 0.8$) (Figure 1), and the size of the region with LD was 88–172 kb. The rs8034191, rs16969968 and rs1051730 SNPs, which showed significant association with lung cancer risk, showed high correlation coefficients ($R^2 > 0.6$) with

Table I. Lung cancer cases and controls used for case–control study

Variable	Control	Case			
		All	ADC	SQC	SCC
Total	936	1250	562	391	297
Age (mean ± SD; years)	50 ± 13	60 ± 8	58 ± 8	62 ± 7	62 ± 9
Sex					
Male (%)	560 (60)	910 (73)	316 (56)	355 (91)	239 (80)
Female (%)	376 (40)	340 (27)	246 (44)	37 (9)	58 (20)
Smoking habit					
Non-smoker (%)	575 (61)	264 (21)	238 (44)	13 (3)	14 (5)
Smoker (%)	361 (39)	986 (79)	324 (56)	379 (97)	283 (95)
Pack-years[a] (mean ± SD)	26 (26)	56 (31)	44 (30)	61 (29)	62 (32)

[a]Values for smokers.

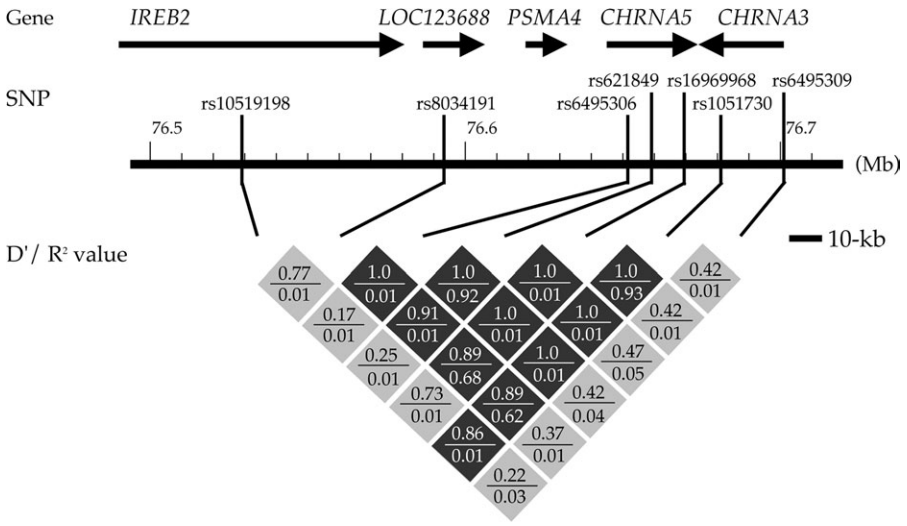

Fig. 1. SNPs in the locus containing nicotine acetylcholine receptor genes. Location of SNPs and genes are shown on the top. D' and R^2 values between two SNPs in the control subjects are shown below. Cells with D' values >0.8 are in black.

Table II. Allele and haplotype differentiation between controls and cases

SNP	Allele	Gene	Category	Frequency		OR (95% CI, *P*)[a]
				Control	Case	
rs10519198	A	*IREB2*	All	0.377	0.367	1.0 (0.9–1.1, 0.50)
			ADC		0.356	0.9 (0.8–1.1, 0.36)
			SQC		0.354	0.9 (0.8–1.1, 0.27)
			SCC		0.397	1.1 (0.9–1.3, 0.38)
			Non-smoker	0.380	0.362	0.9 (0.8–1.2, 0.93)
			Smoker	0.373	0.369	1.0 (0.8–1.2, 0.85)
rs8034191	C	*LOC123688*	All	0.018	0.035	2.0 (1.3–2.9, 0.00091)
			ADC		0.035	1.9 (1.2–3.1, 0.0045)
			SQC		0.035	1.9 (1.2–3.2, 0.010)
			SCC		0.035	2.0 (1.1–3.4, 0.013)
			Non-smoker	0.015	0.036	2.5 (1.3–4.8, 0.0056)
			Smoker	0.024	0.035	1.5 (0.9–2.5, 0.15)
rs6495306	G	*CHRNA5*	All	0.169	0.176	1.1 (0.9–1.2, 0.54)
			ADC		0.176	1.1 (0.9–1.3, 0.63)
			SQC		0.183	1.1 (0.9–1.4, 0.40)
			SCC		0.168	1.0 (0.8–1.3, 0.96)
			Non-smoker	0.175	0.181	1.0 (0.8–1.4, 0.75)
			Smoker	0.161	0.175	1.1 (0.9–1.4, 0.38)
rs621849	G	*CHRNA5*	All	0.179	0.177	1.0 (0.8–1.2, 0.82)
			ADC		0.177	1.0 (0.8–1.2, 0.87)
			SQC		0.183	1.0 (0.8–1.3, 0.84)
			SCC		0.168	0.9 (0.7–1.2, 0.54)
			Non-smoker	0.184	0.181	1.0 (0.8–1.3, 0.87)
			Smoker	0.172	0.176	1.0 (0.8–1.3, 0.81)
rs16969968	A	*CHRNA5*	All	0.015	0.034	2.2 (1.5–3.4, 0.00015)
			ADC		0.032	2.1 (1.3–3.5, 0.0026)
			SQC		0.033	2.2 (1.3–3.7, 0.0034)
			SCC		0.168	2.6 (1.5–4.5, 0.00060)
			Non-smoker	0.014	0.032	2.4 (1.2–4.7, 0.013)
			Smoker	0.015	0.033	2.2 (1.1–4.1, 0.016)
rs1051730	A	*CHRNA3*	All	0.014	0.033	2.4 (1.5–3.6, 0.000088)
			ADC		0.031	2.2 (1.3–3.7, 0.0019)
			SQC		0.033	2.4 (1.4–4.1, 0.0016)
			SCC		0.037	2.6 (1.5–4.7, 0.00058)
			Non-smoker	0.013	0.034	2.7 (1.3–5.3, 0.0041)
			Smoker	0.017	0.033	2.0 (1.1–3.8, 0.024)
rs6495309	T	*CHRNA3*	All	0.497	0.482	0.9 (0.8–1.0, 0.19)
			ADC		0.477	0.9 (0.8–1.1, 0.18)
			SQC		0.465	0.9 (0.7–1.0, 0.081)
			SCC		0.485	1.1 (0.9–1.3, 0.60)
			Non-smoker	0.493	0.455	0.9 (0.7–1.1, 0.14)
			Smoker	0.482	0.490	0.9 (0.8–1.1, 0.20)
Haplotype[b]	CAA	*LOC123688*	All	0.013	0.029	2.3 (1.5–3.7, 0.00028)
		CHRNA5	ADC		0.028	2.3 (1.3–3.9, 0.0022)
		CHRNA3	SQC		0.028	2.2 (1.2–4.0, 0.0059)
			SCC		0.032	2.5 (1.4–4.7, 0.0019)
			Non-smoker	0.011	0.026	2.4 (1.1–5.1, 0.022)
			Smoker	0.015	0.030	2.0 (1.0–3.8, 0.034)

[a]CI, confidence interval.
[b]rs8034191-rs16969968-rs1051730 = CAA.

one another, however, showed low coefficients ($R^2 = 0.01$) with two other SNPs, rs6495306 and rs621849, due to large differences in allele frequency between the former three and the latter two SNPs (Figure 1). The result of LD in this study population was consistent with the results of LD in Japanese subjects deposited in the HapMap database (http://www.hapmap.org) (supplementary Figure 1 is available at *Carcinogenesis* Online). Therefore, the lack of association of the rs6495306 and rs621849 SNPs with lung cancer risk were considered to be due to their low correlation coefficients with rs8034191, rs16969968 and rs1051730. The rs8034191, rs16969968 and rs1051730 SNPs were also in LD in European and American populations (supplementary Figure 1 is available at *Carcinogenesis* Online), and minor alleles for these three SNPs were reported to comprise a single haplotype in European and American populations with frequencies of 0.3–0.4 (2,3). Consistently, minor alleles for these three SNPs were also deduced to comprise a single haplotype in the Japanese population (supplementary Table II is available at *Carcinogenesis* Online). However, the frequency of this haplotype was much lower in the Japanese population (0.013) than in European and American populations.

ORs for the haplotype consisting of minor alleles for the three SNPs were then calculated (haplotype CAA in Table II). This haplotype showed significantly increased ORs for lung cancer risk, and the association was observed in all histological types of lung cancers and in both smokers and non-smokers. Crude ORs and ORs adjusted for age, sex and smoking for genotypes with the CAA haplotype were further calculated (haplotype CAA in Table III). Heterozygotes and carriers (1 and 1 + 2 in Table III) for the CAA haplotype showed similarly increased crude and adjusted ORs in all three histological types of lung cancer and in both smokers and non-smokers. Crude

Table III. Genotype differentiation for the rs8034191, rs1051730 SNPs and the haplotype CAA between controls and cases

SNP/haplotype	Category	Genotype	No. of controls (%)	No. of cases (%)	Crude OR (95% CI, *P*)	Adjusted OR (95% CI, *P*)
rs8034191	All	T/T	905 (96.7)	1166 (93.3)	Reference	Reference
		T/C	28 (3.0)	81 (6.4)	2.3 (1.5–3.5, 0.00021)	1.9 (1.2–3.2, 0.0058)[a]
		C/C	3 (0.3)	3 (0.2)	0.8 (0.2–3.9, 0.76)	0.6 (0.1–3.3, 0.52)[a]
		T/C + C/C	31 (3.3)	84 (6.7)	2.1 (1.4–3.2, 0.00041)	1.8 (1.1–2.9, 0.013)[a]
	ADC	T/T		526 (93.6)	Reference	Reference
		T/C		33 (5.9)	2.0 (1.2–3.4, 0.0061)	1.8 (1.1–3.1, 0.031)[a]
		C/C		3 (0.5)	1.7 (0.4–8.6, 0.50)	1.1 (0.2–6.1, 0.90)[a]
		T/C + C/C		36 (6.4)	2.0 (1.2–3.3, 0.0050)	1.7 (1.0–2.9, 0.037)[a]
	SQC	T/T		364 (93.1)	Reference	Reference
		T/C		27 (6.9)	2.4 (1.4–4.1, 0.0012)	1.9 (0.9–3.8, 0.077)[a]
		C/C		0 (0)	0 (—, 0.27)	0 (—, 0.38)[a]
		T/C + C/C		27 (6.9)	2.2 (1.3–3.7, 0.0035)	1.7 (0.9–3.5, 0.11)[a]
	SCC	T/T		276 (92.9)	Reference	Reference
		T/C		21 (7.1)	2.5 (1.4–4.4, 0.0018)	1.7 (0.8–3.5, 0.14)[a]
		C/C		0 (0)	0 (—, 0.34)	0 (—, 0.28)[a]
		T/C + C/C		21 (7.0)	2.2 (1.3–3.9, 0.0050)	1.6 (0.8–3.1, 0.21)[a]
	Non-smoker	T/T	559 (97.2)	241 (93.2)	Reference	Reference
		T/C	15 (2.6)	17 (6.4)	2.6 (1.3–5.2, 0.0073)	2.3 (1.1–4.9, 0.032)[b]
		C/C	1 (0.2)	1 (0.4)	2.3 (0.1–36, 0.55)	1.0 (0–25, 0.98)[b]
		T/C + C/C	16 (2.8)	18 (6.8)	2.6 (1.3–5.1, 0.0061)	2.2 (1.0–4.5, 0.039)[b]
	Smoker	T/T	346 (95.8)	919 (93.3)	Reference	Reference
		T/C	13 (3.6)	64 (6.5)	1.9 (1.0–3.4, 0.044)	1.7 (1.0–3.4, 0.068)[b]
		C/C	2 (0.6)	2 (0.2)	0.4 (0.1–2.7, 0.31)	0.5 (0.1–5.0, 0.54)[b]
		T/C + C/C	15 (4.2)	66 (6.7)	1.7 (0.9–2.9, 0.082)	1.6 (0.9–3.0, 0.11)[b]
rs1051730	All	G/G	910 (97.2)	1170 (93.6)	Reference	Reference
		G/A	25 (2.7)	77 (6.2)	2.4 (1.5–3.8, 0.00013)	2.2 (1.4–3.8, 0.0014)[a]
		A/A	1 (0.1)	3 (0.2)	2.3 (0.2–22, 0.45)	1.2 (0.1–25, 0.87)[a]
		G/A + A/A	26 (2.8)	80 (6.4)	2.4 (1.5–3.8, 0.000096)	2.2 (1.3–3.7, 0.0016)[a]
	ADC	G/G		530 (94.3)	Reference	Reference
		G/A		29 (5.2)	2.0 (1.2–3.4, 0.012)	1.9 (1.1–3.4, 0.029)[a]
		A/A		3 (0.5)	5.2 (0.5–50, 0.11)	2.8 (0.3–56, 0.35)[a]
		G/A + A/A		32 (5.7)	2.1 (1.3–3.6, 0.0046)	1.9 (1.1–3.4, 0.019)[a]
	SQC	G/G		365 (93.4)	Reference	Reference
		G/A		26 (6.6)	2.6 (1.5–4.6, 0.00060)	2.6 (1.2–5.5, 0.011)[a]
		A/A		0 (0)	0 (—, 0.53)	0 (—, 0.56)[a]
		G/A + A/A		26 (6.6)	2.5 (1.4–4.4, 0.00092)	2.5 (1.2–5.2, 0.014)[a]
	SCC	G/G		275 (92.6)	Reference	Reference
		G/A		22 (7.4)	2.9 (1.6–5.3, 0.00021)	2.6 (1.2–5.6, 0.012)[a]
		A/A		0 (0)	0 (—, 0.58)	0 (—, 0.46)[a]
		G/A + A/A		22 (7.4)	2.8 (1.6–5.0, 0.00033)	2.4 (1.2–5.2, 0.017)[a]
	Non-smoker	G/G	560 (97.4)	248 (93.6)	Reference	Reference
		G/A	15 (2.6)	16 (6.0)	2.4 (1.2–5.0, 0.014)	2.2 (1.0–4.7, 0.051)[b]
		A/A	0 (0)	1 (0.4)	0 (—, 0.13)	0 (—, 0.36)[b]
		G/A + A/A	15 (2.6)	17 (6.4)	2.6 (1.3–5.2, 0.0074)	2.2 (1.0–4.8, 0.040)[b]
	Smoker	G/G	350 (97.0)	922 (93.6)	Reference	Reference
		G/A	10 (2.8)	61 (6.2)	2.3 (1.2–4.6, 0.013)	2.3 (1.2–4.9, 0.012)[b]
		A/A	1 (0.2)	2 (0.2)	0.8 (0.1–8.4, 0.82)	0.8 (0.1–21, 0.90)[b]
		G/A + A/A	11 (3.0)	63 (6.4)	2.2 (1.1–4.2, 0.017)	2.2 (1.1–4.5, 0.016)[b]
Haplotype CAA[c]	All	0[d]	913 (97.5)	1179 (94.3)	Reference	Reference
		1[d]	22 (2.4)	69 (5.5)	2.4 (1.5–4.0, 0.00024)	2.2 (1.3–3.9, 0.0031)[a]
		2[d]	1 (0.1)	2 (0.2)	1.6 (0.1–17, 0.72)	0.7 (0.1–17, 0.81)[a]
		1 + 2[d]	23 (2.5)	71 (5.7)	2.4 (1.5–4.0, 0.00024)	2.1 (1.3–3.7, 0.0042)[a]
	ADC	0[d]		532 (94.7)	Reference	Reference
		1[d]		28 (5.0)	2.2 (1.2–3.9, 0.0059)	2.0 (1.1–3.6, 0.027)[a]
		2[d]		2 (0.4)	3.4 (0.3–38, 028)	1.9 (0.2–42, 0.58)[a]
		1 + 2[d]		30 (5.3)	2.2 (1.3–3.9, 0.0035)	2.0 (1.1–3.5, 0.022)[a]
	SQC	0[d]		369 (94.4)	Reference	Reference
		1[d]		22 (5.6)	2.5 (1.4–4.5, 0.0024)	2.4 (1.1–5.4, 0.029)[a]
		2[d]		0 (0)	0 (—, 0)	0 (—, 0.55)[a]
		1 + 2[d]		22 (5.6)	2.4 (1.3–4.3, 0.0036)	2.3 (1.1–5.1, 0.040)[a]
	SCC	0[d]		278 (93.6)	Reference	Reference
		1[d]		19 (6.4)	2.8 (1.5–5.3, 0.00071)	2.2 (1.0–4.9, 0.047)[a]
		2[d]		0 (0)	0 (—, 0.58)	0 (—, 0.46)[a]
		1 + 2[d]		19 (6.4)	2.7 (1.5–5.1, 0.0011)	2.1 (0.9–4.5, 0.063)[a]
	Non-smoker	0[d]	562 (97.7)	251 (94.7)	Reference	Reference
		1[d]	13 (2.3)	14 (5.3)	2.4 (1.1–5.2, 0.021)	2.0 (0.9–4.7, 0.089)[b]
		2[d]	0 (0)	0 (0)	0 (—, 0)	0 (—, 0)[b]
		1 + 2[d]	13 (2.3)	14 (5.3)	2.4 (1.1–5.2, 0.021)	2.0 (0.9–4.7, 0.089)[b]
	Smoker	0[d]	351 (97.2)	928 (94.2)	Reference	Reference
		1[d]	9 (2.5)	55 (5.6)	2.3 (1.1–4.7, 0.018)	2.3 (1.2–5.1, 0.017)[b]
		2[d]	1 (0.3)	2 (0.2)	0.8 (0.1–8.4, 0.82)	0.8 (0–20, 0.89)[b]
		1 + 2[d]	10 (2.8)	57 (5.8)	2.2 (1.1–4.3, 0.024)	2.2 (1.1–4.6, 0.022)[b]

[a]Adjusted for sex, age and smoking.
[b]Adjusted for sex and age.
[c]rs8034191-rs16969968-rs1051730 = CAA.
[d]Expressed by the number of CAA haplotype carried.

ORs were significantly increased and adjusted ORs were significantly or marginally increased. On the other hand, ORs of homozygotes for the CAA haplotype and those for the minor allele for each SNP were not consistently increased among populations probably due to the small number of homozygotes.

We next examined the association of the rs8034191, rs16969968 and rs1051730 SNPs and the CAA haplotype with smoking status. The value of cigarettes per day was used as a measure of smoking doses of subjects for this analysis since this value was commonly used in previous three GWA studies (2–4). Values of cigarettes per day of individuals with the minor alleles for these SNPs or with the CAA haplotype were higher than those without in both controls and cases; however, the difference did not reach a statistical significance (Table IV).

Discussion

In this study, minor alleles for three *CHRNA* SNPs, rs8034191, rs16969968 and rs1051730, which were defined as risky alleles for lung cancer in Europeans and Americans, also showed significantly increased ORs in Japanese. Results of recent association studies (2–4) on *CHRNA* SNPs, including this study, are summarized in Table V. The frequency of risky alleles in the Japanese population was $<2\%$ and was considerably lower than those in European and American populations as previously indicated (3). Therefore, homozygotes for risky alleles were rare ($<1\%$) in Japanese. Accordingly, an increase in ORs of the homozygotes was not observed in the present study, although an increase in ORs of minor allele carriers (i.e. OR in dominant model) was observed. Therefore, it was suggested that *CHRNA* risky alleles makes a smaller subset of individuals more susceptible to lung cancer in Japanese than in European and American populations.

Association of *CHRNA* SNPs with lung cancer risk by dividing subjects into smokers and non-smokers was examined in two GWA studies (2,3). Association with risk in smokers was commonly observed in these two studies, whereas association in non-smoker was not [Table V, (2,3)]. In the presents study, the minor alleles for three SNPs as well as the haplotype carrying them showed similarly increased ORs in both smokers and non-smokers (Table II), indicating that *CHRNA* SNPs are associated with lung cancer risk irrespective of smoking. This result was consistent with the study by Hung *et al.* (3). The reason for the lack of association in non-smokers in the other study (2) might be a low statistical power due to a small number of non-smoking lung cancer cases (i.e. 125) as discussed (18). Alternatively, other factors that have not been taken into account, such as food intake and passive smoking, might differentiate the mode of contribution of the *CHRNA* SNPs in non-smokers.

Effects of *CHRNA* SNPs according to histological types of lung cancers were examined in a previous study [Table V, (3)] and were similar among ADC, SQC and SCC. In the present study, the minor alleles for rs8034191, rs16969968 and rs1051730 SNPs as well as the haplotype with these minor alleles also showed similarly and significantly increased ORs among them (Table II). Therefore, it was strongly indicated that *CHRNA* SNPs are associated with lung cancer risk irrespective of histological types of cancer. Recent studies suggested the presence of several types of lung ADCs according to accumulated genetic alterations, and ADCs with mutations in the epidermal growth factor gene are predominantly developed in female non-smokers (19). Therefore, we calculated ORs of genotypes for *CHRNA* SNPs for ADC risk after dividing subjects according to sex and smoking. It was found that ORs of heterozygote and minor allele carriers were significantly or marginally significantly increased in female non-smokers (supplementary Table III is available at *Carcinogenesis* Online). Similar but insignificant increases in ORs were also observed in male non-smokers, male smokers and female smokers. These results indicated that *CHRNA* SNPs confer risk for several types of lung ADCs including those developed in female non-smokers, and further validated that *CHRNA* SNPs are associated with lung cancer risk in non-smokers.

In the present study, carriers of the minor alleles for *CHRNA* SNPs as well as the CAA haplotype showed larger values of cigarettes per day than non-carriers by four to six and by one to two in the control and case populations, respectively, although the difference did not reach a statistical significance (Table IV). Associations of *CHRNA*

Table IV. Cigarettes per day values according to genotypes for *CHRNA* SNPs

Control/ case	SNP/ haplotype	Cigarettes per day (mean ± SD)		*P* by Wilcoxon test
		Non-carrier	Carrier	
Control	rs8034191	20.0 (12.2)	25.9 (15.7)	0.20
	rs1051730	20.2 (12.3)	24.2 (15.6)	0.52
	rs16969968	20.0 (12.2)	26.4 (16.6)	0.27
	CAA[a]	20.1 (12.3)	25.7 (15.7)	0.30
Case	rs8034191	28.6 (14.0)	30.6 (12.2)	0.096
	rs1051730	28.7 (14.0)	29.5 (11.6)	0.32
	rs16969968	28.7 (14.0)	29.5 (11.7)	0.32
	CAA[a]	28.6 (14.0)	30.1 (11.8)	0.19

[a]rs8034191-rs16969968-rs1051730 = CAA.

Table V. Association of *CHRNA* SNPs with lung cancer risk and smoking behavior

	SNP	Category	Minor allele frequency	OR (*P*)[a]		Association with lung cancer risk by smoking	Association with smoking status	Reference
				Homozygotes for the minor allele	Minor allele carriers			
USA and UK	rs8034191 (T/C)	NSCLC[b]	0.33	1.8 (2.5×10^{-12})		Smoker only	Yes	Amos *et al.* (2)
	rs1051730 (G/A)		0.33	1.8 (4.6×10^{-12})				
France, 15 other European countries and Canada	rs8034191 (T/C)	ADC	0.34		1.4 (2.0×10^{-10})	Smoker, non-smoker	No	Hung *et al.* (3)
		SQC			1.2 (6.0×10^{-6})			
		SCC			1.3 (2.0×10^{-4})			
Iceland, Spain and The Netherlands	rs1051730 (G/A)	NSCLC[b] + SCC	0.35	1.7 (1.1×10^{-7})		Not examined	Yes	Thorgeirsson *et al.* (4)
Japan	rs8034191 (T/C)	ADC	0.018	1.1 (0.9)	1.7 (3.7×10^{-2})	Smoker, non-smoker	No	This study
		SQC		0 (0.4)	1.7 (0.1)			
		SCC		0 (0.3)	1.6 (0.2)			
	rs1051730 (G/A)	ADC	0.014	2.8 (0.4)	1.9 (1.9×10^{-2})			
		SQC		0 (0.6)	2.5 (1.4×10^{-2})			
		SCC		0 (0.5)	2.4 (1.7×10^{-2})			

[a]OR against homozygotes for the major allele.
[b]NSCLC, non-small cell lung cancer.

SNPs with values of cigarettes per day were also investigated in control populations of the three GWA studies (2–4). In the Iceland, Spain and The Netherland study, the individuals with one and two copies of the minor allele for the rs1051730 SNP were estimated to smoke approximately one and two more cigarette per day than those without, respectively, and the association was statistically significant (4). In the USA and UK study, significant differences in the cigarettes per day values were observed only in former but not in current smokers, and the reason for the inconsistency was unclear (2). In another GWA study, difference in the values of cigarette per day according to genotypes for *CHRNA* SNPs was not observed (3). Thus, minor alleles for *CHRNA* SNPs might have an effect to make individuals more addictive to smoking and to make them consume a few more cigarettes a day. However, since the results of four studies are inconsistent, further studies are still needed to draw a conclusion on this issue.

The present study suggested that *CHRNA* SNPs contribute to lung cancer susceptibility in multiple ethnic populations, including Asians, and the contribution is irrespective of histological types of cancers. Since the association was observed in non-smokers in several populations including Japanese, *CHRNA* SNPs is probably to contribute to lung cancer risk in a smoking-independent manner. Recently, it was reported that *CHRNA* SNPs confer risk of familial lung cancer in Americans, whereas association of these SNPs with smoking status was not significant (20). Therefore, contribution of *CHRNA* SNPs to lung cancer risk through tobacco addiction remains inconclusive. Further studies on a cohort of subjects, for whom data on lung cancer development, smoking exposure, nicotine dependence and duration and intensity of smoking are available, should be done to elucidate this issue as previously discussed (18). CHRNA proteins transduce signals of not only nicotinic substrates but also non-nicotinic substrates, as described in the Introduction (5–7). Therefore, identification of SNPs in the *CHRNA* genes causing differences in the signal transduction by non-nicotinic substrates will be a way to further elucidate the involvement of *CHRNA* in lung cancer susceptibility. rs16969968 is a candidate since it causes a change of a conserved amino acid in *CHRNA5* protein (10) and other non-synonymous or regulatory polymorphisms that show high correlation coefficients with the rs8034191, rs16969968 and rs1051730 SNPs have not been found to date. Alternatively, polymorphisms in two other genes, *LOC123688* and *PSMA4*, which are in LD with these three SNPs (Figure 1), might be responsible. Further genetic and functional studies on the *CHRNA* genes are needed to elucidate the significance of the *CHRNA* locus in lung cancer susceptibility.

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