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ORIGINAL ARTICLE

Association between neuropeptide Y receptor 2 polymorphism and the smoking behavior of elderly Japanese

Naomi Sato[1,2], Shinji Kageyama[1], Renyin Chen[1,4], Masaya Suzuki[1], Hiroki Mori[1], Fumihiko Tanioka[3], Hidetaka Yamada[1], Takaharu Kamo[1], Hong Tao[1], Kazuya Shinmura[1], Akiko Nozawa[2] and Haruhiko Sugimura[1]

Molecular heterogeneity of neuropeptide Y (NPY) and its three receptors (1, 2 and 5) has recently been discovered. *NPY2R* polymorphisms have been shown to be related to cocaine and alcohol dependence in European Americans. To test our hypothesis that these polymorphisms influence the smoking behavior of Japanese population, we investigated the prevalence of the rs4425326 and rs6857715 polymorphisms, which have been suggested to be related to alcohol dependence in European Americans, in 2517 Japanese elderly subjects for whom information on smoking behaviors was available. The prevalence of current smokers was greater among Japanese men having the rs4425326 C allele than ex-smokers. Among the ever-smokers, the Fagerström Test for Nicotine Dependence scores were higher in men having the rs4425326 homozygous T allelotype, and the numbers of cigarettes smoked per day were also significantly higher in the male smokers having the TT genotype. No correlations between the Tobacco Dependence Screener scores and any genotypes were detected. These results suggest that rs4425326 polymorphism may be related to smoking behavior in the Japanese elderly population. This study for the first time suggests NPY2R genotype as a possible genetic factor in nicotine dependence.
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Keywords: addiction; Fagerström Test for Nicotine Dependence (FTND); neuropeptide Y (NPY); *NPY2R*; single nucleotide polymorphism; smoking behavior; Tobacco Dependence Screener (TDS); nicotine dependence

INTRODUCTION

Neuropeptide Y (NPY) is a neuromodulator in the leptin–melanocortin axis. There are three types of receptors of NPY receptors (NPYRs) in humans: NPY receptor type 1 (NPY1R), NPY receptor type 2 (NPY2R) and NPY receptor type 5 (NPY5R). They are all G-protein-coupled receptors having a 7-transmembrane domain,[1] and the genes that encode them are located on chromosome 4.

The physiological and pathological functions of NPY and NPYRs have been widely investigated in relation to the pathogenesis of obesity (food-seeking behavior),[2] hypertension[3] and other neurovascular disorders.[4] Some psychological conditions have recently been claimed to be related to the NPY–NPYR system,[5] and the claim has generated controversy.[6] Abuse of several substances, including methamphetamine, phencyclidine, cocaine, marijuana and alcohol, is thought to be associated with the NPY–NPYR axis.[7] Polymorphisms in the *NPY* gene locus (chromosome 7) and *NPY* receptor loci are known, and they are currently being investigated for possible associations with individual differences in addictive behaviors. The *NPY* locus polymorphism has been extensively studied in regard to various aspects of many physiological and psychological disorders. For example, the Leu7Pro (rs16139) single nucleotide polymorphism (SNP) has been reported to be associated with alcohol consumption and the alcohol withdrawal syndrome in a European-American population.[8–10] However, it has a different prevalence in several other populations besides a European-American population,[11] and the association was not reproducible in Swedes and Finns.[12] Other polymorphisms in the *NPY* gene have been also reported to be related to alcohol dependence/preference.[13–15]

The roles of variants of the NPYRs, however, have not been thoroughly investigated. Wetherill *et al.*[16] carried out an extensive investigation of polymorphisms of *NPY* and *NPY* receptors: 7 SNPs in *NPY*, 15 SNPs in *NPY2R* including the 5′ end, and 17 SNPs in *NPY1R* and *NPY5R* in alcohol-dependent population; in populations with various degrees of the alcohol withdrawal syndrome and in populations with cocaine dependence. They found that several polymorphisms, including rs4425326 and rs6857715 located in the upstream of

[1]Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan; [2]Department of Clinical Nursing, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan and [3]Division of Pathology and Laboratory Medicine, Iwata City Hospital, Iwata, Shizuoka, Japan
[4]Current address: Department of Pathology, Zhengzhou University, Zhengzhou, Henan, China.
Correspondence: Dr H Sugimura, Department of Pathology, Hamamatsu University School of Medicine, 1-20-1, Handayama, Higashi-ward, Hamamatsu, Shizuoka 431-3192, Japan.
E-mail: hsugimur@hama-med.ac.jp
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NPY2R, were associated with these pathological conditions in non-Hispanic, European-Caucasian families.

In this study, we investigated the relationships between the polymorphisms rs4425326 (C/T) and rs6857715 (T/C), both of which were claimed to be associated with alcohol and cocaine dependence in the report by Wetherill *et al.*,[16] and nicotine dependence in an elderly Japanese population.

MATERIALS AND METHODS

Questionnaire

Blood was collected from 2517 subjects in the clinical laboratory of Iwata City Hospital during the 5-year period from 2003 to 2008. The criteria for recruitment as subjects of this study were being ambulant, able to communicate orally and 60 years of age or older. All subjects provided written informed consent regarding participation in this study. A leaflet containing a questionnaire about life style, including alcohol consumption, smoking, diet and cancer history, was handed to each subject, and professional interviewers assisted them in filling them out and confirmed their answers. Some of the questions on smoking behavior were the same as those in the revised Fagerström Tolerance Questionnaire,[17] that is, the Fagerström Test for Nicotine Dependence (FTND),[18] which contains six of the original eight questions in the Fagerström Tolerance Questionnaire, and the Tobacco Dependence Screener (TDS) (a screening questionnaire for tobacco/nicotine dependence according to the *International Statistical Classification of Diseases and Related Health Problems* (ICD)-10, *Diagnostic and Statistical Manual of Mental Disorders* (DSM)-III-R and DSM-IV),[19] which consists of 10 questions. The questionnaire also included questions about the numbers of cigarettes smoked per day (CPD), age when the subject started smoking, how many times current smokers had tried to quit and how many times ex-smokers had tried to quit smoking before they succeeded. FTND scores were available for 1296 subjects (1220 men, 76 women (90.4 and 91.6%, respectively, of the ever-smokers)), and TDS scores were available for 1252 subjects (1183 men, 69 women (87.6 and 83.1%, respectively, of the ever smokers)).

The study design was approved by the institutional review board of Hamamatsu University School of Medicine (19-87 and 21-8).

Genotype analysis

DNA was extracted from whole blood by using a QIAamp DNA Blood Maxi kit according to the manufacturer's instructions (Qiagen, Hamburg, Germany). A 50 ng sample of each subject's DNA was used for PCR amplification with the primer sets for NPY2R polymorphisms rs4425326 and rs6857715 by using the Start One (Applied BioSystems, Carlsbad, CA, USA), and assayed by using the Custom TaqMan SNP Genotyping Assay C__26159385_10 and C__29013142_10, respectively. The locations of the *NPY2R* polymorphisms and the exons are shown in Figure 1. The distribution of both genotypes distributions differs among populations. The minor alleles of rs4425326 and rs6857715 are Ts in the European populations, but C in rs4425326 and T in rs6857715 are listed as the ancestral alleles in the SNP database. The allele frequency of the C allele at the rs4425326 in Japanese is 0.341 and at the rs6857715 is 0.557. The background information regarding rs4425326 and rs6857715 is available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4425326 and at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=6857715, respectively.

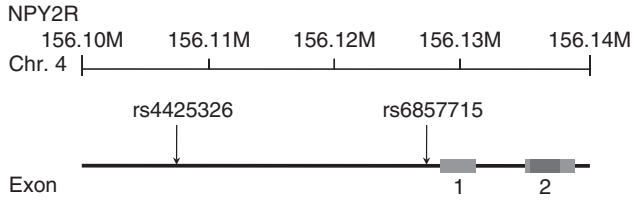

Figure 1 The locations of the *NPY2R* polymorphisms and the exons.

Statistical analysis

Genotype distributions were tested for Hardy–Weinberg equilibrium by using SPSS statistics 17.0. software (SPSS Japan, Tokyo, Japan). χ^2-Tests of each genotype or dominant model were performed for smoking status. The odds ratios were estimated by using a logistic model. The CPD values, FTND scores and TDS scores were evaluated according to smoking status and a dominant model of the polymorphisms by the Kruskal–Wallis test or Mann–Whitney *U*-test (SPSS Japan).

RESULTS

The age, sex and smoking status of the subjects are shown in Table 1. The subjects ranged in age from 60 to 94 years, and they consisted of generations born between 1910 and 1948. There were 1350 ever-smokers (current smokers and ex-smokers) (83.6%) among the 1615 male subjects and 83 (9.2%) among the 902 female subjects, and 21.5% of the men and 3.4% of the women were current smokers. These values are slightly lower than in a recent report[20] stating that the prevalence of male and female Japanese current smokers 60 years of age or more is 27.8 and 6.2%, respectively. The mean CPD values, FTND scores and TDS scores in this study were lower than those previously reported in Japanese[19,21] or American smokers[22] (Table 1). The CPD values of male ex-smokers were higher than those of male current smokers, and TDS scores of the current smokers of both sexes were higher than the TDS scores of ex-smokers of both sexes.

The drinking status and lung cancer history of the subjects are also shown in Table 1. Current drinkers were overrepresented among current smokers, and never-smokers had tendencies to be never-drinkers in both sexes (Supplementary Table 1).

Figure 2, a (male) and b (female), shows the distributions of the FTND scores, and Figure 3, a (male) and b (female), shows the distributions of the TDS scores. The FTND scores of the male smokers ranged from 0 to 10, and the mode value and the mean value were 2 and 3.59, respectively. The FTND scores of the female smokers ranged from 0 to 7, and the mode value and mean value were 1 and 2.41, respectively. The TDS scores of the male smokers ranged from 0 to 10, and the mode value and mean value were 2 and 3.06, respectively. The TDS scores of the female smokers ranged from 0 to 9, and the mode value and mean value were 0 and 2.87, respectively (Figures 2 and 3).

Spearman's rank correlation coefficient for the correlation between the FTND scores and the TDS scores of the male ever-smokers and the female ever-smokers was 0.307 and 0.347, respectively. The *P*-values of both rank correlation coefficients indicated that they were significant at the 1% level. The frequency distribution of both rs4425326 (men: χ^2=1.155, P=0.566; women: χ^2=1.595, P=0.451) and rs6857715 (men: χ^2=0.020, P=0.991; women: χ^2=1.035, P=0.593) obeyed the Hardy–Weinberg law in both sexes.

The distribution of rs4425326 of the smokers revealed that the subjects having the C allele (genotypes CC and CT) were over-represented among male current smokers (Table 2 and Supplementary Table 2), or expressed another way, men having the C allele in this SNP tended to continue to smoke (current smokers). This relation between the rs4425326 C allele and smoking status category was not found in female smokers. There were no differences in the prevalence of the rs6857715 polymorphism in any smoking category in either sex.

Male, but not female ever-smokers who had the rs4425326 TT genotype had significantly higher FTND scores and greater CPD than those with other genotypes (Table 3). In addition, male ever-smokers having the rs6857715 TT genotype had greater CPD than those having the C allele, but not significantly. Any other relations between the

Table 1 Subject profile

Variables	*Male*	P-*value*	*Female*	P-*value*
Number of subjects	1615		902	
Mean age, years (± s.d.)	73.1 (± 6.2)		73.0 (± 6.4)	
Age distribution, n *(%)*				
60–64	81 (5.0)		51 (5.7)	
65–69	426 (26.4)		251 (27.8)	
70–74	455 (28.2)		240 (26.6)	
75–79	418 (25.9)		197 (21.8)	
80–84	170 (10.5)		134 (14.9)	
85–89	51 (3.2)		25 (2.8)	
90–	14 (0.9)		4 (0.4)	
Smoking status, n *(%)*				
Current smokers	348 (21.5)		31 (3.4)	
Ex-smokers	1002 (62.1)		52 (5.8)	
Never-smokers	265 (16.4)		819 (90.8)	
Mean age according to smoking status, years (± s.d.)				
Current smokers	72.1 (± 6.0)	0.002[a]	71.1 (± 5.2)	0.078[a]
Ex-smokers	73.4 (± 6.0)		71.7 (± 6.4)	
Never-smokers	73.4 (± 7.0)		73.2 (± 6.4)	
Mean age at start of smoking, years (± s.d.)				
Ever smokers	19.7 (± 3.7)		34.5 (± 13.3)	
Current smokers	19.9 (± 4.3)	0.282[b]	37.5 (± 15.0)	0.136[b]
Ex-smokers	19.6 (± 3.5)		32.7 (± 11.9)	
Mean numbers of CPD (± s.d.)				
Ever smokers	21.1 (± 13.0)		13.3 (± 8.1)	
Current smokers	16.6 (± 9.1)	<0.001[b]	12.4 (± 6.1)	0.776[b]
Ex-smokers	22.7 (± 13.7)		13.8 (± 9.1)	
Mean FTND score (± s.d.)				
Ever smokers	3.59 (± 2.21)		2.41 (± 2.00)	
Current smokers	3.63 (± 2.10)	0.495[b]	2.26 (± 1.81)	0.777[b]
Ex-smokers	3.57 (± 2.25)		2.51 (± 2.13)	
Mean TDS score (± s.d.)				
Ever smokers	3.06 (± 2.49)		2.87 (± 2.47)	
Current smokers	3.69 (± 2.41)	<0.001[b]	3.78 (± 2.50)	0.018[b]
Ex-smokers	2.84 (± 2.48)		2.41 (± 2.35)	
Drinking status, n *(%)*[c]				
Current drinkers	852 (52.8)		177 (19.6)	
Ex-drinkers	319 (19.8)		50 (5.5)	
Never-drinkers	442 (27.4)		674 (74.8)	
Lung cancer history, n *(%)*				
Yes	47 (2.9)		12 (1.3)	
No	1568 (97.1)		890 (98.7)	

Abbreviations: CPD, cigarettes smoked per day; FTND, the Fagerström Test for Nicotine Dependence; s.d., standard deviation; TDS, the Tobacco Dependence Screener.
Ever smokers: current smokers and ex-smokers.
[a]Kruskal–Wallis test comparing three statuses.
[b]Mann–Whitney *U*-test comparing current smokers and ex-smokers.
[c]Information about alcohol drinking status were obtained from 1613 male subjects and 901 female subjects.

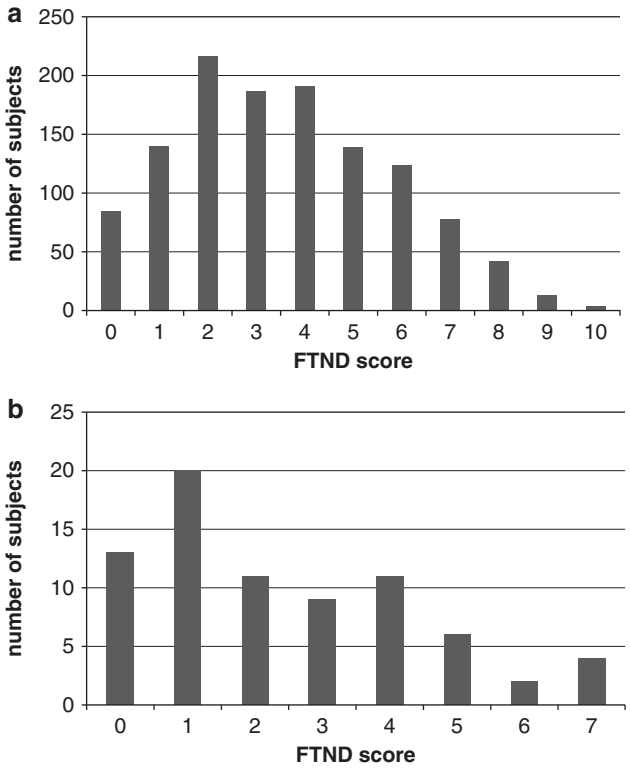

Figure 2 (**a**) Distribution of the Fagerström Test for Nicotine Dependence (male). (**b**) Distribution of the Fagerström Test for Nicotine Dependence (female).

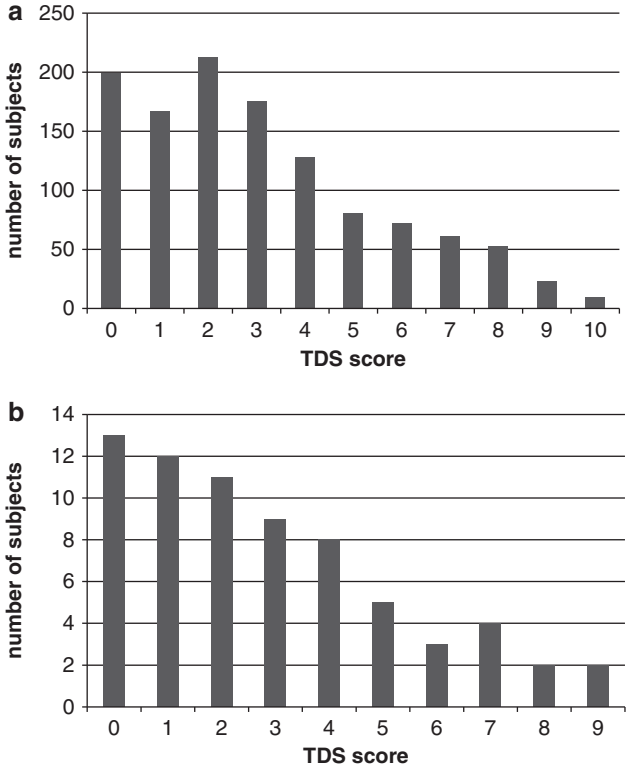

Figure 3 (**a**) Distribution of the Tobacco Dependence Screener (male). (**b**) Distribution of the Tobacco Dependence Screener (female).

Table 2 Comparison of subjects distribution of current smokers and ex-smokers according to two polymorphisms of *NPY2R*

	Smoking status				*Dominant model (CC+CT vs TT)*		*Dominant model (CC+CT vs TT)*	
	Total n *(%)*	*Current smokers* n *(%)*	*Ex-smokers* n *(%)*	P-*value*[a]	*OR*[b] *(95% CI)*	P-*value*	*OR*[c] *(95% CI)*	P-*value*
Males								
rs4425326								
TT	680 (50.4)	158 (45.4)	522 (52.1)		1		1	
CT	566 (41.9)	156 (44.8)	410 (40.9)	0.055	1.308 (1.024–1.670)	0.034	1.300 (1.017–1.662)	0.036
CC	104 (7.7)	34 (9.8)	70 (7.0)					
rs6857715								
TT	319 (23.6)	82 (23.6)	237 (23.7)		1		1	
CT	679 (50.3)	164 (47.1)	515 (51.4)	0.244	1.005 (0.754–1.339)	1.000	1.013 (0.759–1.351)	0.931
CC	352 (26.1)	102 (29.3)	250 (25.0)					
Females								
rs4425326								
TT	37 (44.6)	17 (54.8)	20 (38.5)		1		1	
CT	44 (53.0)	14 (45.2)	30 (57.7)	0.281	0.515 (0.209–1.268)	0.175	0.512 (0.208–1.264)	0.147
CC	2 (2.4)	0 (0)	2 (3.8)					
rs6857715								
TT	19 (22.9)	10 (32.3)	9 (17.3)		1		1	
CT	47 (56.6)	14 (45.2)	33 (63.5)	0.205	0.440 (0.155–1.245)	0.176	0.446 (0.157–1.268)	0.130
CC	17 (20.5)	7 (22.6)	10 (19.2)					

Abbreviations: CI, confidence interval; OR, odds ratio.
Allele frequency of C in rs4425326: 0.278. Allele frequency of T in rs6857715: 0.489. The risk alleles of these two polymorphisms are designated as C.
[a]The χ^2-tests were performed based on 3×2 tables.
[b]The odds ratios were calculated for the genotypes concerned in the current smoking status.
[c]The odds ratios were adjusted for age and calculated for the genotypes concerned in the current smoking status.

Table 3 Comparison of the numbers of CPD, FTND scores and TDS scores of ever smokers according to the dominant model (CC+CT vs TT) of two polymorphisms of *NPY2R*

	CPD			*FTND*			*TDS*		
	N	*Mean* ± *s.d.*	P-*value*[a]	n	*Mean* ± *s.d.*	P-*value*[a]	n	*Mean* ± *s.d.*	P-*value*[a]
Males (CPD, n=*1348; FTND,* n=*1220; TDS,* n=*1183)*									
rs4425326									
TT	680	22.3 ± 13.3	<0.001	614	3.80 ± 2.31	0.003	598	2.95 ± 2.49	0.085
CT+CC	668	19.9 ± 12.5		606	3.37 ± 2.08		585	3.18 ± 2.51	
rs6857715									
TT	319	21.8 ± 12.6	0.054	291	3.76 ± 2.35	0.197	281	2.88 ± 2.32	0.314
CT+CC	1029	20.9 ± 13.1		929	3.53 ± 2.16		902	3.12 ± 2.55	
Females (CPD, n=*83; FTND,* n=*76; TDS,* n=*69)*									
rs4425326									
TT	37	14.0 ± 8.0	0.417	34	2.56 ± 1.99	0.480	29	3.31 ± 2.99	0.501
CT+CC	46	12.7 ± 8.2		42	2.29 ± 2.02		40	2.55 ± 2.00	
rs6857715									
TT	19	11.2 ± 6.1	0.218	17	1.88 ± 1.50	0.334	14	2.21 ± 2.29	0.264
CT+CC	64	13.9 ± 8.5		59	2.56 ± 2.10		55	3.04 ± 2.51	

Abbreviations: CPD, cigarettes smoked per day; FTND, the Fagerström Test for Nicotine Dependence; s.d., standard deviation; TDS, the Tobacco Dependence Screener.
[a]Mann–Whitney *U*-test.

rs6857715 polymorphism and the FTND scores or CPD value were not found in either sex (Table 3). Neither the rs4425326 nor the rs6857715 polymorphism was associated with the TDS scores in either sex (Table 3).

DISCUSSION

A long list of genes is considered candidates for a relation to smoking behavior or nicotine dependence. Li and Burmeister,[23] recently conducted an extensive reviewed of research on genes related to addictions, and in that seminal review, they listed genes that are considered candidates for an association with at least one drug addiction (62 genes) in two large tables and genes having one or more of whose variants have been associated with addiction to at least one substance (41 genes) in supplementary tables. *NPY*, but not *NPYR*, was included in the tables, and thus far there have been few studies on associations between nicotine dependence and the NPY–NPYR axis.[23]

We attempted to determine whether the rs4425326 and rs6857715 polymorphisms of the *NPY2R* gene are related to another addictive behavior in humans, nicotine dependence, because Wetherill *et al.*[16] have recently shown an association between these polymorphisms and both alcohol dependence and cocaine addiction in humans. Dependence on nicotine is one of the addictions that has been extensively studied, and the genetic aspects of smoking behavior are being extensively investigated now.[23]

Our study on smoking behavior is the first to report a correlation between *NPY2R* gene polymorphism and nicotine dependence.

We found a significant correlation between rs4425326 C-containing allelotypes, which have been hypothesized to be high-risk allelotypes for addiction and current smoking status in male smokers. This is the first report to show that the *NPY2R* polymorphism is related to human addictive behaviors in a non-Caucasian population. This is also the first time an association between *NPY2R* and nicotine dependence has been shown. However, no relation was found between TDS scores and any of the *NPY2R* allelotypes, perhaps because the traits detected by the scores on two questionnaires (FTND and TDS) are different, and how the genetic components control these traits in establishing individual nicotine dependence is not elucidated.

The mechanistic significance of the rs4425326 locus, 0.2 Mb upstream from the first exon of *NPY2R* (Figure 1), is unknown.

Interestingly, 'addiction'-allele rs4425326 C holders (according to Wetherill *et al.*[16]) were more prevalent in the current smokers in this study than among the ex-smokers in spite of the lower FTND scores of the allele C holders, meaning that the rs4425326 C allele holders have milder nicotine dependence based on FTND scores but they do not quit smoking. Like the FTND scores, the CPD values of the rs4425326 C allele holders were lower, too. The analysis of male current smokers also showed lower CPD values and FTND scores in the rs4425326 C holders (P=0.100 and 0.098, respectively), but not significantly (Supplementary Table 3). Thus, Japanese male rs4425326 C allele holders are modest but very persistent nicotine-seekers. This paradoxical result may reflect the complex decision-making process regarding smoking in the elderly Japanese. No genetic tendencies were detected in any of the nicotine dependence scores among the female smokers in our study. The failure to find any genetic tendencies among them was mainly because of the relatively small numbers of female smokers in our study.

Smoking is a complicated personal behavior that is influenced by multiple factors, including the social, cultural and sometimes even the political environment. The role of a single genetic polymorphism must not be overestimated in explaining individual smoking behavior. The interpretations of this study have several limitations. We recruited the elderly people in a rural city, and this population has demographical and occupational characteristics different from those in urban cities or agricultural villages. We expected our study subjects have established smoking behavior. This might imply the dependence scores collected here have some biases, causing the limitation of our study. In a different point of view, the information on the established smoking behavior as a life-long habit in individuals may make a unique contribution to understand the genetic effect on whole life of humans. Anyway, it will be necessary to validate our observations by replication studies in the future. However, we think our data provide a major clue to understanding human smoking behavior.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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