厚生労働科学研究費補助金
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ゲノム・遺伝子解析に基づく、胃がん、肺腺がん高危険度群の捕捉、及び予防標的分子の同定に資する研究

平成２４年度　総括研究報告書

研究代表者　　椙村　春彦

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Ⅰ．総括研究報告

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研究要旨

胃がんや肺がんは、頻度からいっても、死亡数からいっても本邦でもっとも重要ながんと言っても良いが、その原因ついては、喫煙習慣、ヘリコバクター感染などの外的要因がよく知られているが、遺伝性瀰慢性胃がんを極型としてこれらの外的要因と独立あるいは相関して遺伝的素因の存在が従来から想定されてきた。本研究班は分担研究者の全ゲノム領域をカバーする胃がんや肺がんの相関研究、研究代表者の家族集積性胃がん例の解析をもとに、ゲノム・遺伝子解析により胃がんや肺がんとくに肺腺がんの高危険度群を抽出することを狙ってきた。これまで、胃がんにおいて、ときに de novo の形で生じる copy number 変異が若年発症の胃がんにみられたこと、epigenetic な変化を修飾する遺伝子の多型が肺腺がんのリスクに関与することなどを示したが、引き続き、濃厚な遺伝的要因が想定される若年あるいは家族性胃がんの症例を重ねるとともに、今回エクソーム解析により、未知の遺伝的変化を抽出した。その変化についても上記の症例で確認をしていきたい。

A. 研究目的

胃がんや肺がんの原因については、喫煙習慣、ヘリコバクター感染などの外的要因がよく知られているが、遺伝性瀰慢性胃がんを極型としてこれらの外的要因と独立あるいは相関して遺伝的素因の存在が従来から想定されてきた。本研究班は分担研究者の全ゲノム領域をカバーする胃がんや肺がんの相関研究、研究代表者の家族集積性胃がん例の解析をもとに、ゲノム・遺伝子解析により胃がんや肺がんとくに肺腺がんの高危険度群を抽出することを目的とする。

B. 研究方法

[既存の家族集積例、若年発症例からのアプローチ] 家族集積例、あるいは若年発症で組織像が signet ring cell type の症例を引き続き、内視鏡医などの協力を得て収集している。そのなかで、CDH1 の塩基配列や copy number を前年度と同様に Multiplex Ligation dependent Probe Amplification (MLPA) 法による スクリーニングを続けた。その際、いくつかの陽性例の経験から、

組織像の特徴つまり粘膜内に多発していること、signet ring cell in situ などの所見があることを伝え、現場の臨床医と広く連携をしながら症例を収集している。また、文献にあるコンソーシアムの基準に必ずしも合致しない例でも見つかることがあることを周知せしめて、たとえば家族歴のまったくない症例なども検索の協力を要請した。

[GWAS からのアプローチ] 500 例の肺腺がんについての網羅的解析により、クロマチン修飾遺伝子などの多型が新たに見つかっており、一部は Nature Genetics などに発表しているが、それらで見いだされつつある候補遺伝子について、後述の極めて稀な若年肺がんのエクソーム解析所見とあわせて、検討をはじめた。

[エクソーム解析からのアプローチ] アジレント SureSelect を用いエクソン DNA を濃縮し、イルミナの次世代シークエンサー HiSeq 2000 を用い、全エクソンの塩基配列解析(Whole exon sequence, WES)を行った。Bioinformatics の面からの分析として、WES の pair-end の read data を BWA で hg19+ decoy 5 にマップし、PCR duplicate 疑いを samtools で削除。既知の indel 周辺の realign, Q-value の付け替えを GATK で実施。 ゲノムコホート 192 人と当班の 53 人（家族性胃がんと若年性肺がん）をまとめ、GATK の UnifiedGenotyper を用いて multiple sample で bait ターゲット領域内の mutation call を実行した。また更に sensitivity をあげるために個別に single sample でも同様の call を行い、Cancer Gene Census に登録されている遺伝子領域において multiple sample では call されなかった変異を追加した。そのうち、PhyloP, SIFT, PolyPhen2, LRT, Mutation Taster などにより、deleterious と判定された変異(多型)を、さらに、胃がん例、肺がん例で有意に頻度の高いものを検出していった。

（倫理面への配慮）

ゲノム解析研究を含む研究であり、浜松医科大学医の倫理委員会、浜松医科大学遺伝子解析研究倫理委員会の審議を経てエクソーム解析も含め承認を得ている (23-91)。またコントロールとして、JHPC の検体を用いる承認も国立がん研究センターから得ている（課題 2011-044)。

C. 研究結果

[若年性家族性胃がん例] CDH1 の生殖細胞系列のうち機能差のありそうなものとして、ミスセンスバリアントが 4 つ、ナンセンスバリアントが 1 つ、フレームシフトバリアントが 1 つ、5' UTR のバリアントが 2 つ遺伝性びまん性胃がんおよびびまん性胃がんの若年孤発例で見つかった。MLPA 法では、遺伝性びまん性胃がんおよびびまん性胃がんの若年孤発例で 5 つのコピー数変化が見つかった。これらのコピー数変化については胃がんを発症していない検体について TaqMan コピー数解析法を用いて探索したが見つからなかった。つまり、対照群には存在する可能性の低い多型である。1 例については家族内の構成員の検索が可能であり、その結果、コピー数変異がある世代で新たに生じ、次の世代にうけつかれていくものであることを確認した。また、その組織像の詳細な観察から signet ring cell carcinoma in situ の多発が確認され、欧米で報告されている組織像と同様のものであった。

[肺がんの GWAS と若年肺がんのエクソーム] 肺腺がんの GWAS により、従来いわれていた locus 以外に、クロマチン修飾に関わる遺伝子座など新たなものも見いだし、さらにこれらの遺伝子群についてエクソームデータとあわせて注視しており、一般的な肺がん集団あるいは対象群での分布の解析の準備もすすめている。

[エクソーム解析] single sample の解析で call された変異および多型は 47134 種類であった。これらのうち胃がん例のみで見つかり、PhyloP, SIFT, PolyPhen2, LRT, Mutation Taster, GERP++すべてでdeleteriousと判定された変異および多型は187種類であり、その多くは、Cancer Gene Census に登録されていない新規なものであった。登録されていた遺伝子としてはCHEK2, ARID1A, APC などがあった。CHEK2 については同一家系内の胃がん発症者2例で同じ変異が見つかった。

D. 考察

胃がんのリスクについて、ヘリコバクターの感染などがよく知られており、その除菌が、胃がん予防の対策として有効である。また肺がんについても喫煙という大きな因子があり、両疾患とも環境要因の強い癌であるとされ、逆にいえば preventable な癌とされてきた。しかし、遺伝性弥慢性胃癌のような劣性遺伝病として認識されてきたいわば極型ともいえる phenotype の存在、また本班で収集してきた若年や家族性の胃癌・肺癌の本邦における実態を鑑みるとおそらくは遺伝的要因がつよい未知の entity が存在すると考えられた。また、遺伝性弥慢性胃癌と同一遺伝子が原因でも、比較的大きな遺伝子領域の欠落という現象がいままで同定されてこなかったことが明らかになり、しかも、家族歴がない症例で de novo で生じ得るということがわかった。胃癌については、従来から、一定の割合で 20 代、30 代、40 代といった発症が知られており、いわゆるスキラスといわれる病理学的な特徴や、発見が遅れた場合の予後の悪さなど、100 年近く前の山極勝三郎の胃癌発生論にもスケッチがある。本班のいままでの研究により、この日本人の胃がんのこの phenotype の少なくとも一部が遺伝的要因であるという認識をもたらした。また、エクソーム解析は非常に有用な方法で、多数の候補遺伝子の検証が必要であるにせよ、理論的に機能的障害のあるような遺伝子多型が見いだされている。ヒトは誰でも複数の有害な遺伝子の変異を持っているといわれているが、これらのうちあるものが、preventable な胃癌や肺癌といった疾患と関係するという期待は本班の趣旨そのものである。おりしも、別の臓器ではあるが遺伝性乳癌の感受性遺伝子のキャリアーが予防的乳房切除を受けたというニュースが、全国紙にもとりあげられた（朝日新聞、2013年5月20日朝刊）。ハイリスクグループがわかった場合、その確度によりどのような臨床的、倫理的、社会的対応が適切であるかという議論がますます進むと思われる。これまで、とくに本邦の場合、遺伝的感受性という言葉がしばしば、研究者の概念的な話題にとどまるところがあったが、ある程度の患者数がおり、surveillance や、治療が確立されている common cancer での実態を、海外の情報をそのまま適応するのではなく（現実的に人種差などがあり、種々

の遺伝子多型の分布も意義も大変異なる）本邦の基礎的 data をさらに臨床家の認識と協力を得てすすめたいと思う。
一方肺癌のリスクは組織像や喫煙者に発生したかどうか、喫煙量との用量反応関係などを考慮する必要があるが、本班の収集している若年肺癌は極めて貴重な例であり、そのエクソームデータがでたことは非常に意義がある。さらに、これまで多くの実績を積んできた班員の河野博士のグループの GWAS による data との比較、関連が非常に興味深い。

E. 結論

ゲノム解析によるデータは、胃癌や肺癌の高リスク群の同定に極めて有用であり、とくに塩基変異ばかりでなく、copy number の変異や、家族歴の有無に関わらない遺伝的リスクの存在を明らかにできる。そのような変化は、前もって検索あるいはサーベイランスが可能であり、胃癌や肺癌は早期に発見されると非常に予後が良い疾患になっている現状を考えると、若年や壮年におけるこの疾患による死亡をふくめた重篤な損失を防ぐことができる基礎的知見であると思われる。

F. 健康危険情報

ゲノム変化による胃癌のリスクについて、特に現在は広報していず、マスコミなどにみられる胃癌の予防については、保険収載という昨今の情況もあり、ヘリコバクターの除菌をすすめている。若年胃癌についての、現状（頻度、割合、検査の簡便さ）をさらに確度の高いものにする必要があるが、すくなくとも胃癌の臨床にたずさわっている医師にとっては、本邦の胃癌の遺伝的要因の存在が現実的に考え得るものとして明確になり（以前はほとんどないという報告があったため、検索意欲も現場では少なかった）、あらたな症例の発掘や、その症例の早期治療が行われだしたというのが現状である。

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

別紙4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ. 研究成果の刊行物・別冊

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ORIGINAL ARTICLE

Association between dopamine beta hydroxylase rs5320 polymorphism and smoking behaviour in elderly Japanese

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The dopaminergic brain pathway is involved in many addictive behaviours, hence represents a good candidate in the study of smoking behaviour and nicotine addiction. Dopamine beta hydroxylase (DBH) is an enzyme that catalyses the conversion of dopamine into noradrenaline. This study, the first of its kind, was done to investigate the role of *DBH* rs5320 polymorphism in smoking behaviour of elderly Japanese. This was done by collecting blood samples from 2521 subjects with various smoking habits to genotype the *DBH* rs5320 polymorphism. Participants also had to fill out a questionnaire containing questions regarding their lifestyles. Some of the questions were from the Fagerström Test for Nicotine Dependence (FTND) and the Tobacco Dependence Screener (TDS). It was found that male ever-smokers with AA genotype smoked less cigarettes per day than those with GG and AG genotypes. FTND scores were also lowest in male ever-smokers with AA genotype and in female ever-smokers with AG genotype. There was no correlation detected between the TDS scores and any of the genotypes. This study shows that *DBH* rs5320 polymorphism influences nicotine dependence.
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Keywords: addiction; dopamine beta hydroxylase (*DBH*); Fagerström Test for Nicotine Dependence (FTND); nicotine dependence; single-nucleotide polymorphism (SNP); smoking behaviour; Tobacco Dependence Screener (TDS)

INTRODUCTION

Dopamine beta hydroxylase (DBH) is an enzyme that catalyses the conversion of dopamine to noradrenaline in sympathetic nerves. DBH is expressed in noradrenaline-containing neurons, occurring in both membrane-bound and soluble forms.[1] Because of this, noradrenaline and DBH are released together during synaptic transmission,[2,3] hence they can be found in cerebrospinal fluid, and plasma or serum. The human gene encoding DBH is located on chromosome 9q34.[4] The *DBH* gene is composed of 12 exons and comprises a sequence of approximately 23 kb.[5] Serum DBH activity of human as well as gorilla have been known to be polymorphic for the last 30 years.[6–9] This inter-individual difference in serum DBH of European population has been stated as being mostly related to a promoter polymorphism at the −1021 promoter region (C to T, rs1611115).[10] Furthermore, there are probably a few pathogenetic germline mutations of *DBH* that explain rare congenital deficiency. The amino-acid substitutions from Asparaginate to Glutaminate at codon 100 in the exon 2 of DBH (300C-A transversion), from Valine to Methionine at codon 87 in the exon 1 of DBG (259 G-A transition) and from Asparaginate to Asparagine at codon 331 in the exon 6 (991 G-A transition) together with splice site mutation (IVS1DS, T-C, +2) are known.[11,12] Clinical phenotypes of these deficiencies are mainly severe orthostatic hypotension and other autonomic nerve symptoms. On the other hand, other polymorphisms also have been investigated in view of a possible modulator of human conditions including psychiatric ones.

In this study, we investigated various aspects of smoking behaviour in relation to single-nucleotide polymorphism in *DBH*. The particular polymorphism investigated was the rs5320, as it was found that the minor allele frequency of this polymorphism existed in substantial number in our Japanese sample. This particular polymorphism has been shown to be associated with Parkinson's disease among North Indians,[13] but our study is the first to investigate its involvement in the role of smoking behaviours.

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MATERIALS AND METHODS

Questionnaire

Blood was collected from 2521 subjects (1616 males and 905 females) between the ages of 60 and 94 years. This was done at the Iwata City Hospital during a 5-year period from 2003 to 2008. The participants involved in this experiment had various smoking habits (1349 male ever-smokers (current-smokers and ex-smokers; 83.5%) and 83 female ever-smokers (9.2%)). To be eligible for this experiment, the participants had to be ambulant and be able to communicate orally. All subjects provided written consent before participating in this study. The overall portraits of subjects have been described previously.[14] The participants were required to fill in a questionnaire leaflet containing various questions about lifestyle, including alcohol consumption, smoking, diet and cancer history. They were assisted in filling out the leaflet by professional interviewers. Some of the questions were from the Fagerström Test for Nicotine Dependence (FTND; a test that yields a continuous measure of nicotine dependence),[15] and 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), which consists of 10 questions.[16] The questionnaire also included questions about the numbers of cigarettes smoked per day (CPD), the participants' age when they started smoking, how many times current-smokers had tried to quit smoking and how many times ex-smokers had tried to quit smoking before succeeding.

The study design was approved by the Institutional Review Board of Hamamatsu University School of Medicine.

Genotype analysis

DNA was extracted from the blood samples given by the participants using a QIAamp DNA Blood Maxi kit according to the manufacturers' instructions (Qiagen, Hamburg, Germany). A 50 ng sample of each subjects DNA was amplified by PCR, with the primer set for *DBH* rs5320 polymorphism using the StepOne (Applied BioSystems, Carlsbad, CA, USA). The Assay ID is C_12020332_20. Successful genotyping of *DBH* was performed in 100% of the enrolled subjects. The rate of successful genotyping was almost the same as the other genotype.[17]

Statistical analysis

The genotype of the *DBH* rs5320 polymorphism was tested for Hardy–Weinberg equilibrium using the SPSS statistics software (SPSS Japan, Tokyo, Japan). χ^2 tests of each genotype were performed for smoking status and lung cancer history. The CPD values, FTND scores, TDS scores and trial times for quitting smoking were evaluated according to smoking status and each genotype by the Kruskal–Wallis test or Mann–Whitney *U* test (SPSS Japan).

RESULTS

The age, sex and smoking status of the participants have been reported previously[14] and are shown in Table 1. The age of participants whose DNA could be genotyped ranged from 60 to 94, with the mean age for males being 73.1 years and for females being 73.0 years. Most of the male participants (62%) were ex-smokers, whereas most of the female participants (90.8%) had never smoked. Current-smokers of both sexes had higher TDS than ex-smokers of both sexes. The average CPD for male current-smokers was 16.6 and for female current-smokers 12.2.

Most current-smokers were also current drinkers, and never-smokers of both sexes tended to be never-drinkers ($\chi^2 = 17.7$, $P = 0.001$ for males and $\chi^2 = 42.1$, $P < 0.001$ for females). Table 1 also shows that ex-smokers of both sexes went through more trials to quit smoking than current-smokers.

Table 2 shows that most of the male and female participants had the GG genotype of the *DBH* rs5320 polymorphism ($n = 1275$, 78.9% for males and $n = 693$, 76.6% for females), followed by the AG genotype and finally the AA phenotype (1.4% of males and 1.3% of

Table 1 Subjects profile

Variables	*Males*	P-*value*	*Females*	P-*value*
Number of subjects	1616		905	
Mean age, years (± s.d.)	73.1 (± 6.2)		73.0 (± 6.4)	
Age distribution, n (%)				
60–64	81 (5.0)		51 (5.6)	
65–69	426 (26.4)		253 (28.0)	
70–74	456 (28.2)		240 (26.5)	
75–79	418 (25.9)		198 (21.9)	
80–84	170 (10.5)		134 (14.8)	
85–89	51 (3.1)		25 (2.8)	
90–	14 (0.9)		4 (0.4)	
Smoking status, n (%)				
Current-smokers	345 (21.3)		30 (3.3)	
Ex-smokers	1004 (62.1)		53 (5.9)	
Never-smokers	267 (16.5)		822 (90.8)	
Mean age according to smoking status, years (± s.d.)				
Current-smokers	72.1 (± 6.0)	0.002[a]	70.8 (± 5.0)	0.065[a]
Ex-smokers	73.4 (± 6.0)		71.8 (± 6.4)	
Never-smokers	73.3 (± 7.0)		73.2 (± 6.4)	
Mean age at start of smoking, years (± s.d.)				
Ever-smokers	19.6 (± 3.5)		33.9 (± 12.4)	
Current-smokers	19.9 (± 4.3)	0.298[b]	36.1 (± 13.1)	0.196[b]
Ex-smokers	19.6 (± 3.2)		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]	12.2 (± 6.0)	0.604[b]
Ex-smokers	22.7 (± 13.7)		13.9 (± 9.0)	
Mean numbers of CPD × years (± s.d.)				
Ever-smokers	854 (± 582)		402 (± 357)	
Current-smokers	852 (± 466)	0.057[b]	428 (± 304)	0.257[b]
Ex-smokers	855 (± 617)		386 (± 386)	
Mean FTND score (± s.d.)				
Ever-smokers	3.58 (± 2.20)		2.35 (± 2.01)	
Current-smokers	3.61 (± 2.08)	0.526[b]	2.17 (± 1.76)	0.733[b]
Ex-smokers	3.57 (± 2.24)		2.47 (± 2.17)	
Mean TDS score (± s.d.)				
Ever-smokers	3.07 (± 2.48)		2.87 (± 2.47)	
Current-smokers	3.75 (± 2.41)	<0.001[b]	3.82 (± 2.56)	0.022[b]
Ex-smokers	2.84 (± 2.47)		2.43 (± 2.33)	
Mean number of trial times for quitting smoking in current-smokers (± s.d.)	1.36 (± 1.64)		1.24 (± 1.55)	
Mean number of trial times for quitting smoking before succeeding in ex-smokers (± s.d.)	2.10 (± 1.54)		1.66 (± 1.25)	
Drinking status, n (%)[c]				
Current drinkers	853 (52.9)		178 (19.7)	
Ex-drinkers	319 (19.8)		50 (5.5)	
Never-drinkers	442 (27.4)		676 (74.8)	
Lung cancer history, n (%)				
Yes	47 (2.9)		12 (1.3)	
No	1569 (97.1)		893 (98.7)	

Abbreviations: CPD, cigarettes smoked per day; FTND, Fagerström Test for Nicotine Dependence; TDS, 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 1614 male subjects and 904 female subjects.

Table 2 Subjects distribution according to smoking status, lung cancer history and the rs5320 polymorphism of *DBH*

			Smoking status				*Lung cancer history*		
	Genotype	*Total* n *(%)*	*Current-smokers* n *(%)*	*Ex-smokers* n *(%)*	*Never-smokers* n *(%)*	P-*value*[a]	*Yes* n *(%)*	*No* n *(%)*	P-*value*[b]
Males	GG	1275 (78.9)	277 (80.3)	788 (78.5)	210 (78.7)		41 (87.2)	1234 (78.6)	
	AG	319 (19.7)	65 (18.8)	203 (20.2)	51 (19.1)	0.639	5 (10.6)	314 (20.0)	0.195
	AA	22 (1.4)	3 (0.9)	13 (1.3)	6 (2.2)		1 (2.1)	21 (1.3)	
Females	GG	693 (76.6)	22 (73.3)	41 (77.4)	630 (76.6)		7 (58.3)	686 (76.8)	
	AG	200 (22.1)	7 (23.3)	12 (22.6)	181 (22.0)	0.688	5 (41.7)	195 (21.8)	0.277
	AA	12 (1.3)	1 (3.3)	0 (0)	11 (1.3)		0 (0)	12 (1.3)	

[a]The χ^2 tests were performed based on 3×3 tables.
[b]The χ^2 tests were performed based on 3×2 tables.

Table 3 Subjects distribution according to smoking status and lung cancer history

			Smoking status			
	Lung cancer history	*Total* n *(%)*	*Current-smokers* n *(%)*	*Ex-smokers* n *(%)*	*Never-smokers* n *(%)*	P-*value*[a]
Males	Yes	47 (2.9)	2 (0.6)	44 (4.4)	1 (0.4)	<0.001
	No	1569 (97.1)	343 (99.4)	960 (95.6)	266 (99.6)	
Females	Yes	12 (1.3)	0 (0)	1 (1.9)	11 (1.3)	0.689
	No	893 (98.7)	30 (100)	52 (98.1)	811 (98.7)	

[a]The χ^2 tests were performed based on 2×3 tables.

females). Although it may be possible that some kind of population bias exist in this rural population, we tested this genotype if it is in accordance with the Hardy–Weinberg equilibrium. Actually the genotype distribution obeyed the Hardy–Weinberg equilibrium ($\chi^2 = 0.179$, $P = 0.914$ for males and $\chi^2 = 0.332$, $P = 0.842$ for females). Smoking status (current-smokers, ex-smokers and never-smokers) was not significantly different between the three genotypes, nor was lung cancer history.

Table 3 shows subjects distribution according to smoking status and lung cancer history. It shows a significant relationship between lung cancer history and ex-smokers in males ($P < 0.001$); however, there was no significant relationship between the *DBH* rs5320 polymorphism and lung cancer history in male ex-smokers. Most cases of lung cancer history occurred in male ex-smokers with GG genotype, but not statistically significant (Table 4).

The *DBH* rs5320 polymorphism was shown to be of significance in both males and females with regard to FTND, and in males only with regard to CPD (Table 5). Males with AA genotype smoked less cigarettes per day, while those with GG and AG smoked similar number of cigarettes per day. Male ever-smokers with AA genotype also had lower FTND scores than those with AG and GG genotypes, while female ever-smokers with AG genotype had the lowest FTND scores.

Table 4 Subjects distribution according to lung cancer history and the rs5320 polymorphism of *DBH* in male ex-smokers

		Lung cancer history		
Genotype	*Total* n *(%)*	*Yes* n *(%)*	*No* n *(%)*	P-*value*[a]
GG	788 (78.5)	38 (86.4)	750 (78.1)	
AG	203 (20.2)	5 (11.4)	198 (20.6)	0.208
AA	13 (1.3)	1 (2.3)	12 (1.3)	

[a]The χ^2 test was performed based on 3×2 table.

DISCUSSION

The dopaminergic brain pathway is one that has been studied extensively in regards to addictive behaviour due to the shared characteristics of drug abuse to elicit the release dopamine. Because of this, genes involved in dopamine metabolism represent good candidates in the study of addictive behaviours, such as smoking.

This study specifically shows a significant correlation between the CPD in males and FTND in males and females and the *DBH* polymorphism. Males with AA genotype tend to be the least dependent on nicotine, having the lowest CPD and FTND scores, while females with AG genotype had the lowest FTND scores.

DBH polymorphisms have been investigated from the standpoint of DBH deficiency, several neurological diseases, from migraine[18] to attention-deficit hyperactivity disorder,[19] hypertension[20] and cocaine dependence.[21] The promoter polymorphism has been reported to be associated with Alzheimer's disease.[22]

Several polymorphisms of *DBH* have been studied in terms of the association of smoking behaviour. Among them the promoter polymorphism −1021C/T (rs1611115) is the most extensively

Table 5 Comparison of smoking index of ever-smokers according to the rs5320 polymorphism of *DBH*

	Males			*Females*		
Index	n	*Mean ± s.d.*	P-*value*[a]	n	*Mean ± s.d.*	P-*value*[a]
Age at start of smoking						
GG	1062	19.6 ± 3.3		62	33.9 ± 13.2	
AG	268	19.9 ± 4.3	0.593	19	33.9 ± 10.1	0.949
AA	16	20.4 ± 3.0		1	35.0	
CPD						
GG	1065	21.3 ± 13.1		63	13.8 ± 8.4	
AG	268	20.9 ± 12.5	0.007	19	11.2 ± 6.6	0.389
AA	16	13.4 ± 6.1		1	20.0	
CPD × years						
GG	1061	862 ± 598		62	419 ± 377	
AG	268	840 ± 528	0.055	19	334 ± 289	0.557
AA	16	549 ± 226		1	620	
FTND						
GG	983	3.62 ± 2.23		57	2.60 ± 2.02	
AG	251	3.53 ± 2.07	0.044	19	1.53 ± 1.84	0.030
AA	15	2.20 ± 1.42		1	4.00	
TDS						
GG	934	3.08 ± 2.47		51	2.94 ± 2.48	
AG	229	3.10 ± 2.57	0.215	17	2.59 ± 2.58	0.692
AA	15	1.87 ± 1.46		1	4.00	
Times of trial for quitting smoking in current-smokers						
GG	276	1.37 ± 1.68		17	1.38 ± 1.69	
AG	64	1.34 ± 1.48	0.415	7	0.71 ± 1.11	0.557
AA	3	0.33 ± 0.58		1	2.00	
Times of trial for quitting smoking before succeeding in ex-smokers						
GG	698	2.10 ± 1.54		35	1.74 ± 1.28	
AG	182	2.12 ± 1.52	0.916	12	1.42 ± 1.17	0.317
AA	12	2.17 ± 1.59		0	–[b]	

[a]Kruskal–Wallis test.
[b]Not applicable.

investigated as Zabeitian *et al.*[23] reported this polymorphism as a functional polymorphism explaining inter-individual difference of plasma DBH activity. As to its possible association with smoking behaviour, Freier *et al.*[24] reported that individuals who had at least one *DBH*-1021 T allele smoked fewer cigarettes per day than CC homozygotes in relatively small numbers of the European smokers ($n = 220$). Another polymorphism, *DBH* polymorphism T1368A (rs77576840), has been shown to be associated with cigarette consumption.[25]

The tobacco and genetics consortium did a meta-analysis totalling 74 053 subjects and found *DBH* rs3025343 [G] is associated with smoking cessation.[26] Siedlinski *et al.*[27] reported the genome-wide study of chronic obstructive pulmonary disease. They could replicate the result by the above consortium; that is, there was an association between a candidate genotype rs3025343 and smoking cessation in their subjects. The other polymorphisms of *DBH* have been also attempted to correlate with smoking. For example, the rs77905 was investigated in terms of the association with smoking status and nicotine level in 1518 adolescent subjects in United Kingdom, but no association was found.[28] Actually, Breitling *et al.*[29] reported this rs77905 polymorphism did not influence smoking cessation programme including 577 heavy smokers.

The rs5320 polymorphism of *DBH* was investigated as haplotype analysis of Parkinson's disease. Haplotypes rs1611115T > C–rs1108580A > G–rs5320A > G–rs129882C > T are reported to be associated with Parkinson's disease.[13] Our findings that *DBH* rs5320 genotype are associated with smoking behaviour remind us of a well-known observation that Parkinson's disease is less prevalent in smokers and there may be a common genetic root for these status, Parkinson's disease and (addicted) smoking.[30–32] Haplotype block structure of Japanese and Han Chinese population is shown in the Figure 1. Actually, this haplotype block indicates the relatively strong linkage ($r^2 = 0.63$) between rs1108589 and rs5320. Among the single-nucleotide polymorphisms mentioned above, the rs77576840 is not so common and is not listed in the HapMap database. It is between rs302530 and rs1108580. The rs3025343 is outside the figure covers (far 5′ upstream).

As shown previously, numerous polymorphic sites of *DBH* were investigated in various populations. We picked up rs5320 because the prevalence in Japanese is feasible and this is a non-synonymous variation. These polymorphic sites including rs5320 are linked with each other depending on populations. Most of them do not have mechanistic rationale for why these polymorphisms are apparently associated with smoking behaviour, which awaits further investigation. The polymorphism at rs5320, G allele in GCG (Ala) vs A allele in ACG (Thr) at the position 211 of this protein may not have a severe biological effect probably, considering these two amino acids exist alternatively from each other in some of the primates (Figure 2). In regard to our result that males with AA genotype had the lowest CPD and FTND score, the reason why this A allele behaves in a recessive manner is unknown. Effect of amino-acid substitution may influence only when both alleles are variants. An exploration on functional rationale would be warranted.

Interestingly, unlike the FTND scores, no relation was found between the TDS and any of the rs5320 allelotype in our study. This could be due to the fact that the traits detected by the scores of the FTND and TDS are different from each other.[33] Smoking is a personal behaviour that can be attributed to many genetic and environmental factors. The questions in the FTND detect the physical aspects of nicotine dependence, including the one on the value of CPD, whereas those in the TDS tend to focus on the mental aspects of smoking. The TDS is a questionnaire for screening tobacco/nicotine dependence according to some criteria of mental disorder. These characteristics of scales may have led to the result that the CPD and FTND, not TDS in males, were related to the DBH polymorphism significantly. Regarding female subjects, the number of smoker with AA genotype was only one, so the relations between the FTND and this polymorphism should be examined further.

Limitations of this study and its interpretation include the fact that the participants were recruited from a rural city, where demographical and occupational characteristics are different from those living in urban cities or agricultural communities. To validate our observations, replication of this study would need to be done with larger and different populations. This would allow for a larger representation of the various genotypes associated with the *DBH* rs5320 polymorphism and also a larger female sample size, so that one can be able to make a clearer conclusion whether the AA genotype of this locus corresponds

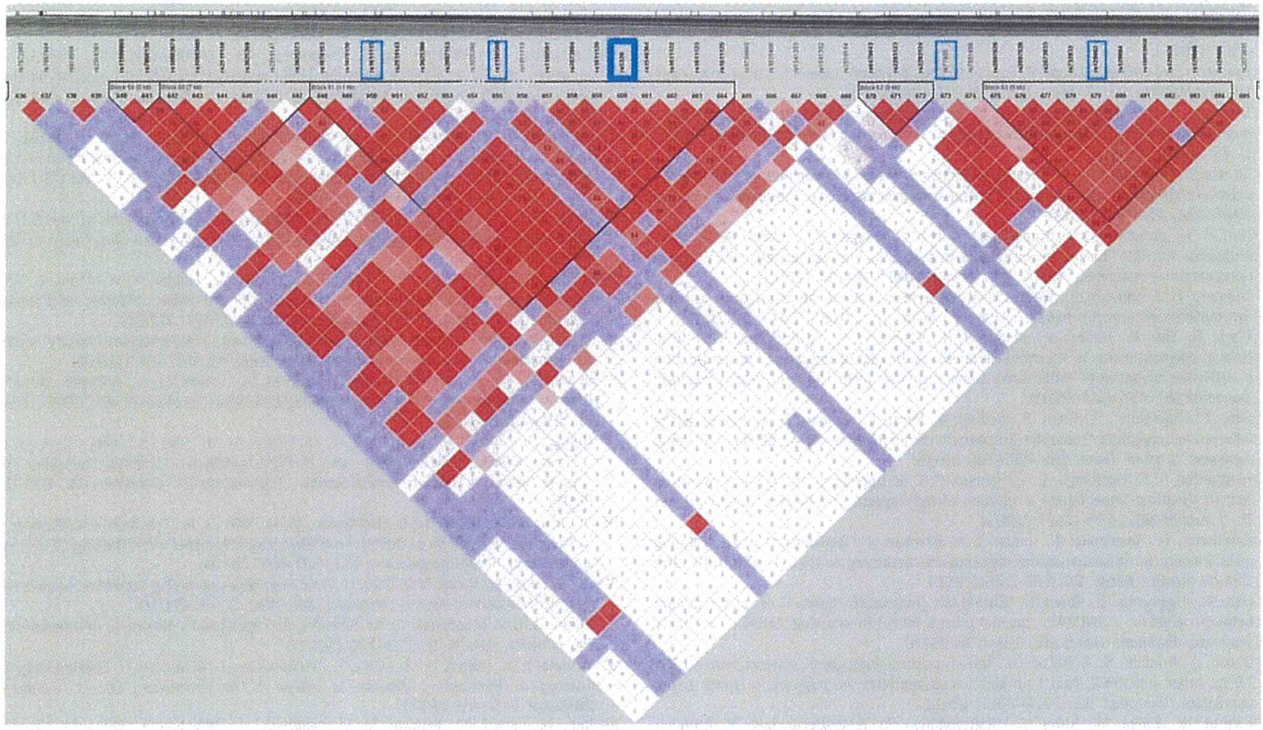

Figure 1 The state of linkage disequilibrium (LD) between the single-nucleotide polymorphisms in the region around the rs5320 polymorphism, which is shown in thick rectangle. The rs1611115, rs77905, rs129882 and rs1108580 are shown in thin rectangles. The strength of LD was calculated based on the genotype data of the Japanese and Han Chinese population extracted from the position 135,474,113 to 135,522,004 on chromosome 9 in the Hapmap database (http://hapmap.ncbi.nlm.nih.gov/index.html.ja).[34] Numbers in squares represent percentage of the r^2 values. Squares without numbers represent $r^2 = 1$. The colour scheme was according to the 'Standard Color Scheme' of the Haploview v.4.1 software (Lod $\geqslant 2$, $D' = 1$: bright red; Lod $\geqslant 2$, $D' < 1$: shades of pink/red; Lod < 2, $D' = 1$: blue; Lod < 2, $D' < 1$: white).[35] The LD blocks were defined based on the default algorithm by Gabriel *et al*.[36]

Homo sapiens	P	S	D	A	C	T	M
Pan troglodytes	P	S	D	A	C	T	M
Pongo abelii	P	S	D	T	Y	T	M
Nomascus leucogenys	P	S	D	A	C	T	M
Macaca mulatta	P	S	D	T	Y	T	M

rs5320, exon4

Figure 2 Amino-acid alignment surrounding the position 211 in human and the corresponding positions in the other primates. Though the information on genetic polymorphism in the primates is limited, the database shows this position is Ala or Thr in several primates including human being.

with having a higher FTND score. Recruitment of sample population from a wider demography that is, urban cities and agricultural villages would be needed. This would take into account the different smoking characteristics. Despite these shortcomings, our data has provided a major clue in understanding the smoking behaviour of humans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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