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ORIGINAL ARTICLE

Posterior column ataxia with retinitis pigmentosa in a Japanese family with a novel mutation in FLVCR1

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Abstract Posterior column ataxia with retinitis pigmentosa (PCARP) is an autosomal recessive neurodegenerative disorder characterized by retinitis pigmentosa and sensory ataxia. Previous studies of PCARP in two families showed a linkage to 1q31–q32. However, detailed investigations on the clinical presentations as well as molecular genetics of PCARP have been limited. Here, we describe a Japanese consanguineous family with PCARP. Two affected siblings suffered from childhood-onset retinitis pigmentosa and slowly progressive sensory ataxia. They also showed mild mental retardation, which has not been described in patients with PCARP. Parametric linkage analysis using high-density single nucleotide polymorphism arrays supported a linkage to the same locus. Target capture and high-throughput sequencing technologies revealed a novel homozygous c.1477G>C (G493R) mutation in *FLVCR1*, which cosegregated with the disease. A recent study has identified three independent mutations in *FLVCR1* in the original and other families. Our results further confirmed that PCARP is caused by mutations in *FLVCR1*.

Keywords Posterior column ataxia with retinitis pigmentosa · Linkage analysis · Target capture · Massively parallel sequencing · FLVCR1

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Introduction

Posterior column ataxia with retinitis pigmentosa (PCARP, MIM 609033) is an autosomal recessive, childhood onset neurodegenerative disorder characterized by sensory ataxia and retinitis pigmentosa. Previous studies [1, 2] on American and Spanish families revealed a linkage to chromosome 1q31–q32 defined by D1S2692 (206.10M in NCBI36/hg18 assembly, http://genome.ucsc.edu/) and D1S2141 (213.26M). Because only two families have been reported with proven linkage to 1q31–q32, detailed investigations on the clinical presentations as well as the molecular genetics of PCARP have been limited. We have recently identified a Japanese family with PCARP with supportive linkage to 1q31–q32. Employing target capture and high-throughput sequencing technologies, we herein identified a novel mutation in *FLVCR1*.

Patients and methods

Patients

The pedigree chart of the Japanese family with PCARP is shown in Fig. 1. Two affected siblings and an unaffected sibling were born to consanguineous parents. Written informed consent was obtained from all the participants. All the participants were clinically evaluated by a neurologist (T.S.). The study was approved by the ethical committee of The University of Tokyo.

Linkage analysis

Genomic DNAs were extracted from peripheral blood leukocytes according to standard protocols. Five of the

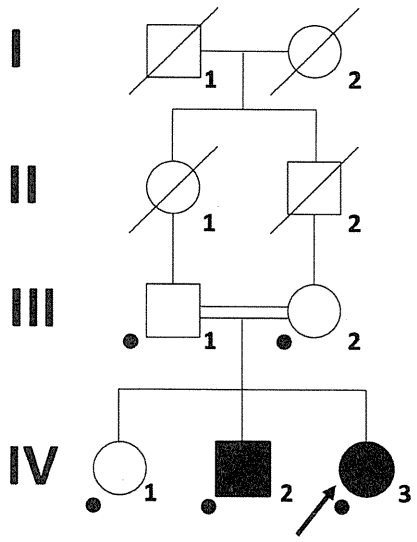

Fig. 1 Pedigree chart. Pedigree chart of a Japanese family with PCARP. *Squares and circles* indicate males and females, respectively. Affected persons are designated with filled symbols. A *diagonal line* through a symbol represents a deceased person. A person *with the arrow* is the index patient. Persons with available genomic DNAs are indicated by *dots*

family members were genotyped using Affymetrix 50K Xba and 50K Hind arrays (Affymetrix, Santa Clara, CA) following the manufacturer's instructions. Using pipeline software SNP-HiTLink [3], single nucleotide polymorphisms (SNPs) with a p value of >0.05 in the Hardy–Weinberg test, a call rate of >0.95, a confidence score of genotyping <0.1, a minor allele frequency in the controls >0, and intermarker distances of 80 to 120 kb were selected for the linkage analysis. Parametric multipoint linkage analysis (autosomal recessive model with complete penetrance) was performed with Allegro version 2 [4]. Haplotypes were reconstructed using Allegro.

Target capture

Using NimbleGen's custom human sequence capture 2.1M array (Roche NimbleGen, Madison, WI), we designed probes corresponding to the target regions (chromosome 1: 200,106,833–213,208,193 and chromosome 20: 15,311,130–32,500,997) avoiding repetitive sequences in the regions. Twenty micrograms of genomic DNA of an affected person (IV-2) was captured according to the manufacturer's instructions [5], followed by quantification of average fold enrichment of the captured sample.

Massively parallel sequencing

Since the target capture procedure was optimized for 454 Sequencer (454 Life Sciences, Branford, CT), the enrichment sample was nebulized for 16 min for further fragmentation to obtain appropriate lengths of DNA fragments suitable for sequencing using Genome Analyzer IIx (GAIIx, Illumina, San Diego, CA). We then carried out single-end library preparation for GAIIx. Massively parallel sequencing was accomplished using two lanes of GAIIx (100-bp-long single-end read).

Short read alignment and variant calling

After removing the tag sequences designed for 454 sequencing system, short reads were aligned to the reference genome (NCBI36/hg18 assembly) with bwa [6] using default parameters. After removing multiple aligned reads (mapping quality of 0), single nucleotide variants (SNVs) and short insertion/deletion variants (indels) were called with SAMtools [7]. Quality threshold for SNVs and indels were set to 20 and 50, respectively.

Annotation and confirmation of variant calls

After annotation with RefSeq (http://www.ncbi.nlm.nih.gov/projects/RefSeq/) and dbSNP130/dbSNP131 (http://www.ncbi.nlm.nih.gov/projects/SNP/), all the novel nonsynonymous variant calls were subjected to direct nucleotide sequence analysis for confirmation. Confirmed amino acid changes were then subjected to PolyPhen (http://genetics.bwh.harvard.edu/pph/) for prediction of functional effects.

Direct nucleotide sequence analysis for confirmation of mutation in *FLVCR1*

Polymerase chain reaction was performed using a primer pair of FLVCR1-F 5′-GCAATTCGCCTACCTCAACT-3′ and FLVCR1-R 5′-ACACAAGTCCTTTTGCCAGG-3′ and LATaq (TaKaRa, Ohtsu, Shiga, Japan). Direct nucleotide sequence analysis was performed using ExoSAP-IT (USB, Cleveland, OH), a BigDye Terminator v3.1 kit, and XTerminator employing an ABI PRISM3100 sequencer (Life Technologies Corporation, Carlsbad, CA).

Results

Clinical manifestations of the family

The index patient (IV-3 in Fig. 1) was a 31-year-old female, who was noted to be night-blind at the age of five by her mother. Thereafter, she developed gait disturbance. She consulted with an ophthalmologist at the age of 31. Ophthalmologic examinations revealed retinitis pigmentosa of the bone corpuscle type with optic atrophy. On neurological examination, she was found to be mildly

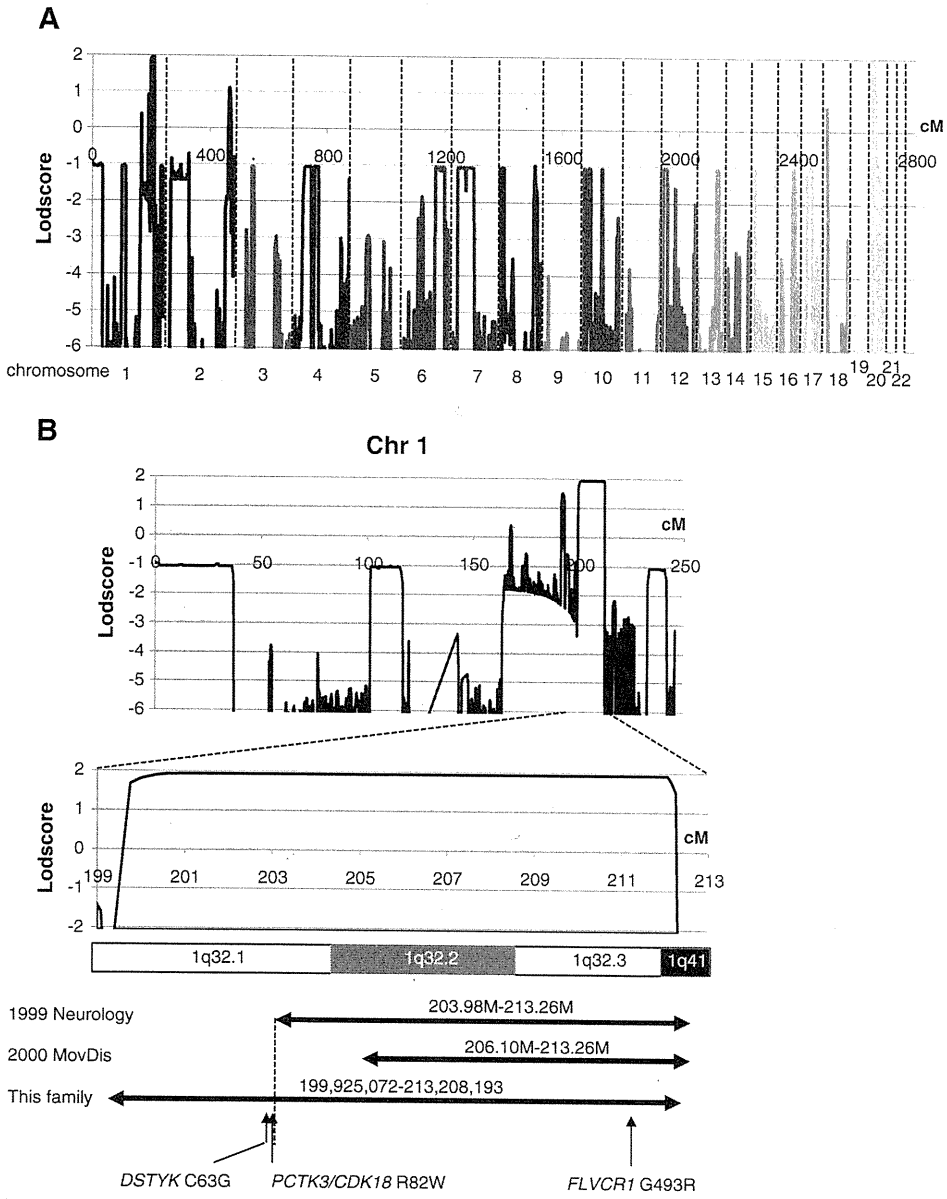

Fig. 2 Multipoint linkage analysis and candidate regions. **a** Parametric multipoint linkage analysis (autosomal recessive model) of the family revealed linked regions on chromosomes 1 and 20. Multipoint LOD scores spanning all the chromosomes are shown. The *horizontal axis* is the cumulative genetic distance (centimorgan) starting at the short arm of chromosome 1. The vertical axis represents LOD scores. Regions on chromosomes 1 and 20 give the highest multipoint LOD scores of 1.93. **b** Parametric multipoint linkage analysis of chromosome 1. Regions with a multipoint LOD score of 1.93 are enlarged below. The *horizontal axis* is the genetic distance (centimorgan) starting at the short arm of chromosome 1. The *vertical axis* shows multipoint LOD scores. *Below the graphs*, the candidate regions demonstrated by this study as well as by previous studies [1, 2] are shown along with the diagram of chromosome 1q32.1–q41. Novel nonsynonymous variants detected in this study are also shown. *FLVCR1* G493R is the only variant that is located inside the minimum candidate region

retarded. Muscle tone was decreased in the limbs with normal strength. Coordination was preserved in the arms and legs, but with moderately ataxic gait and truncal titubation. Romberg's sign was positive. Deep tendon reflexes were decreased in the arms and absent in the legs with flexor plantar responses. Superficial sensations were intact, whereas vibratory and position senses were lost in the toes. Normal values were found in the following tests: complete blood count, blood vitamin E level, and plasma phytanic acid level. Her peripheral blood smears showed no acanthocytes. Axial T2-weighted images of the cervical spinal cord on magnetic resonance imaging demonstrated a hyperintense signal in the posterior half of the cord. Her brother (IV-2 in Fig. 1) was examined early in his thirties and was found to have mental retardation, retinitis pigmentosa, and posterior column ataxia. The other family members were neurologically normal.

Table 1 Variants in target regions of chromosomes 1 and 20

	No. of variants	No. of variants in exon/SS	No. of novel variants in exon/SS	No. of novel nonsynonymous variants in exon/SS
chr1	13,616	60	5	4
chr20	10,545	30	1	1

SS splice site (splice donor and acceptor sites including two adjacent nucleotides in introns)

Table 2 Novel nonsynonymous variants detected in target regions

Chr	Physical position	Variant	Gene	Amino acid change	Polyphen
1	203447100	A>C (homo)	*DSTYK*	C63G	Probably damaging
1	203759347	C>T (homo)	*PCTK3/CDK18*	R82W	Possibly damaging
1	211129174	G>C (homo)	*FLVCR1*	G493R	Possibly damaging

Chr chromosome, *homo* homozygous

Linkage analysis

Multipoint parametric linkage analysis revealed the highest LOD scores of 1.93 spanning regions on chromosome 1 (defined by rs950114 and rs10494988) and chromosome 20 (defined by rs2876404 and rs6082269, Fig. 2a). The region on the chromosome 1 overlapped the previously defined locus of PCARP (Fig. 2b).

Massively parallel sequencing analysis

Average fold enrichment for QC loci of the captured library was 129. From two lanes of GAIIx, we obtained 37,165,950 reads. Of these, 15,865,704 reads (42.7%) had tag sequences for 454 in the first 20 bases. In these reads, tag sequences were eliminated and we used them as 80 bp sequences. Aligned uniquely to the reference genome were 32,332,900 reads (87.0%), and 29,693,695 reads (79.9%) were aligned to the target region. The average coverage of target regions was 89.6X.

In the 30.3 Mb of target region on chromosomes 1 and 20, 24161 variants were called. Of these, 90 were located in coding regions and splice sites in the target regions, six of which were not registered in dbSNP131 (http://www.ncbi.nlm.nih.gov/projects/SNP/), and five of which were concluded to be novel nonsynonymous SNV (Tables 1 and 2). Two of the five novel variant calls were heterozygous, and direct nucleotide sequence analysis revealed that they were false positives.

Considering previous linkage studies [1, 2], two of the three novel nonsynonymous SNVs were located outside the overlapping candidate region (Fig. 2b and Table 1). Thus, the only novel nonsynonymous variant within the minimum candidate region was a homozygous c.1477G>C (G493R) of *FLVCR1* (Fig. 3a). The mutation was further confirmed by direct nucleotide sequence analysis (Fig. 3b). The two

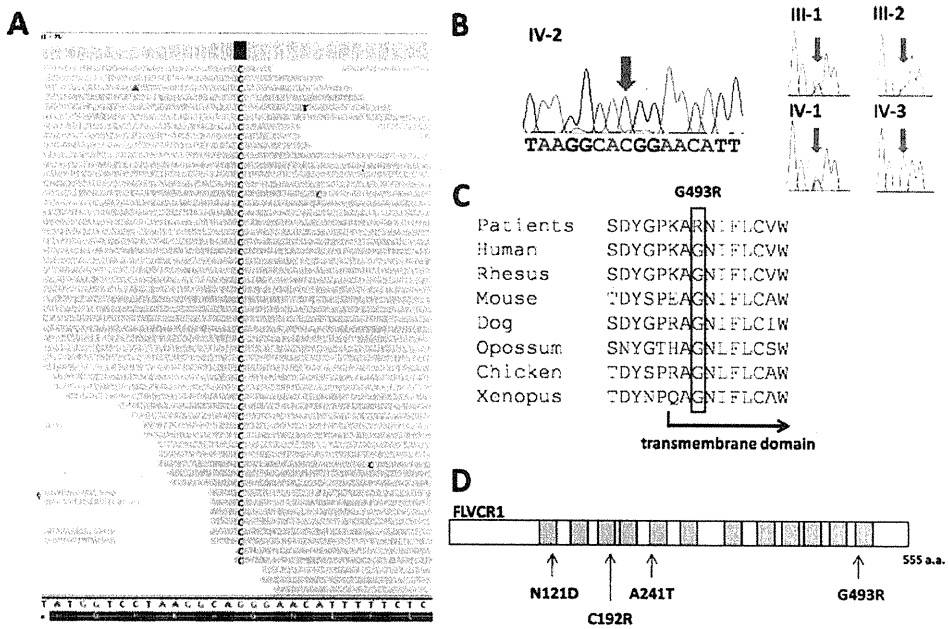

Fig. 3 Identification of causative mutation in *FLVCR1*. **a** Aligned short reads showing homozygous *FLVCR1* c.1477G>C mutation. Aligned reads are viewed using Integrative Genomic Viewer (http://www.broadinstitute.org/igv/). Each short read is represented as a horizontal bar. Only mismatched bases are explicitly shown. All the 52 reads aligned in the position show the C allele, suggesting a homozygous mutation. **b** Direct nucleotide sequence analysis confirms the mutation, which cosegregates with the disease. **c** Partial FLVCR1 amino acid sequence alignment reveals that G493 is evolutionally conserved among species. A putative transmembrane domain is also indicated by an arrow. **d** Schematic representation of FLVCR1 protein. Mutations detected to date are shown. Putative transmembrane domains are *shaded*

affected individuals carried the homozygous mutation, whereas the parents and the unaffected sibling carried the heterozygous mutation. Because R493 is evolutionally well conserved (Fig. 3c) and the amino acid change was not observed in 192 control chromosomes, we concluded it as a pathogenic mutation of PCARP.

Discussion

We described two cases of a Japanese family with PCARP. Linkage analysis supported the linkage to the previously defined locus, and we identified a novel mutation in *FLVCR1* employing targeted capture and massively parallel sequencing as the cause of PCARP. Very recently, Rajadhyaksha et al. have conducted massively parallel sequencing and found independent mutations in *FLVCR1* (N121D, A241T, and C192R) in three families [8]. Our report further confirmed that PCARP is caused by mutations in *FLVCR1*.

FLVCR1 is a 555 amino acid protein that has 12 transmembrane domains. Intriguingly, three previously reported mutations are located in the first, third, and fifth putative transmembrane domains of FLVCR1. The mutation which we found is also located in the 12th transmembrane domain (Fig. 3d). Moreover, all the mutations in FLVCR1 found in PCARP are substitution of a hydrophilic amino acid for a hydrophobic amino acid (A214T) or substitutions of charged amino acids for uncharged amino acids (N121D, C192R, and G493R). These finding suggest a possibility that disruption of transmembrane domains of FLVCR1 is involved in the pathogenesis of PCARP.

Although childhood-onset retinitis pigmentosa and sensory ataxia found in the affected siblings were characteristics of PCARP, they also had mild mental retardation. Because no cognitive deficits have been reported in the original PCARP families [2], careful interpretation would be necessary. One possibility is that the clinical presentations can be more heterogeneous depending on mutations and G493R mutation in *FLVCR1* is associated with mental retardation. Another possibility is that other gene(s) are responsible for mental retardation. Because there are at least two other novel homozygous amino acid changes in the candidate regions as determined on the basis of the linkage analysis of this family under an autosomal recessive model (Table 2), some of these substitutions may contribute to mental retardation.

Previous studies suggested that FLVCR1 is a heme transporter, and *FLVCR1* null mice present a phenotype with a lack of erythropoiesis and craniofacial and limb deformities resembling Diamond–Blackfan anemia [9]. Because neither changes in the shape of erythrocytes nor anemia was observed in the index patient, the discrepancy between human disease and mouse model should be further investigated in the future.

In conclusion, we identified a novel mutation in *FLVCR1* in a Japanese PCARP family. The study showed that target capture and massively parallel sequencing technologies enable us to identify causative genes even in a small family and they are expected to further unveil molecular pathogeneses of neurodegenerative disorders.

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【原　著】

神経難病患者・介護者における補完代替医療利用の実態調査

Use of Complementary and Alternative Medicine by Intractable Neurodegenerative Patients and Caregivers

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【要　旨】
神経難病患者における補完代替医療 (CAM) の利用に関する実態調査の報告は極めて少ない．本研究の目的は，神経難病患者の CAM 利用の実態を把握し今後の難病療養の基礎資料として役立てることである．対象は，和歌山県内の筋萎縮性側索硬化症，パーキンソン病と関連疾患，脊髄小脳変性症，スモン患者1,406名と，介護者（対照）とした．あんま・マッサージ・指圧，鍼灸，柔道整復，漢方，健康補助食品について質問票を郵送し，無記名回答で回収した．回収率は患者 33.7%，対照 30%で，回収率から求めた CAM 利用割合は，神経難病患者 20.5%，対照 9.8%であった．「あんま・マッサージ・指圧」が神経難病患者に最も利用されており，利用患者の 51.3%が「痛みの軽減や動きの改善などに効果あり」と回答した．本療法は対照でも 32.4%で利用され，その 62.8%で効果ありとされた．効果ありと回答した神経難病患者および対照では主観的健康感が良好である者が有意に多かった．根治療法が未だない疾患を有する患者と介護者において療養生活上での症状や心身の負担軽減に対して CAM 利用が選択肢の一つとして有用と考えられた．

【キーワード】
神経変性疾患患者，パーキンソン病，利用頻度，効果，介護者

はじめに

補完代替医療 (CAM) は西洋現代医学領域以外の全ての医療の総称で，一般的に毒性や侵襲性の低い治療法との印象があるため日常的に受け入れられやすい傾向がある．Eisenberg らは，アメリカ国民の CAM 利用率は 42.1%，一方日本では 65.6%と報告している[1]．神経難病を有し療養中の患者では，現代医療と併用して症状緩和のため CAM を利用する機会が増加していると推察される．しかしながら，我々の渉猟した限り神経難病患者における CAM 利用の実態に関する報告は極めて少ない．本研究は，CAM 利用の実態と効果に関する利用者の評価を把握し，今後の神経難病療養上での CAM の有用性を考える基礎資料として役立てる事を目的とした．

方法

和歌山県内の厚生労働科学省難治性疾患克服研究事業で指定されている特定疾患のうち，筋萎縮性側索硬化症 96名，パーキンソン病 1,048名，パーキンソン病関連疾患（多系統萎縮症，進行性核上性麻痺，大脳皮質基底核変性症）89名，脊髄小脳変性症 155名，スモン 18名の患者，計 1,406名と対照として健常な介護者もしくは患者の家族 1,406名を本研究の対象とした．本研究では CAM として「あんま・マッサージ・指圧」，「鍼灸」，「柔

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道整復（接骨）」,「漢方（医院や薬局で処方されるもの）」,「健康補助食品」について調査した．質問票には 1) 年齢, 2) 性別, 3) 疾患名, 4) 介護度, 5) 日常生活の状況（1：仕事や家事ができる，2：近所までの外出は一人でできる，3：身の回りのことは一人でできる，4：身の回りのことに介助が必要，5：寝たり起きたり，6：座れるが主として寝たきり，7：胃瘻造設している，8：呼吸器を使用している），6) 現在の全般的な健康状態（1：療養中であるが病状は落ち着いており，気分は良い，2：療養中であるが，気分はまあまあ良い，3：全般的にあまり良くない，4：良くない），7) 一年前と比べて日常生活の状況の変化（漠然とした体の不調，病状の進行，それぞれについて 1：増えた（悪くなった），2：変わらない，3：減った（良くなった）），8) 最近一年間で利用した補完代替医療について（利用した補完代替医療の種類，頻度，効果（1：あり，2：なし，3：不明），9) 効果の内容，10) 補完代替医療を利用するに際して主治医に相談したか，などの質問項目，全 10 問とした．本アンケート用紙を郵送法にて患者宅に送付し，回答は個人情報保護のため無記名とし返信用封筒にて回収した．患者住所は，和歌山県庁福祉保健部健康局難病・感染症対策課から「特定疾患治療研究事業における臨床調査個人票の研究目的使用に関する要綱」に基づき住所ラベルの提供を受けた．調査期間は平成 20 年 6 月 1 日から同年 8 月 31 日とした．本研究は平成 20 年度関西医療大学倫理審査会で承認された．

結果

I. 神経難病患者の CAM 利用実態について

全対象者のうち，神経難病患者では 33.7%から，対照では 30.2%から有効回答が得られた．回答した患者と対照の属性と日常生活状況を表 1 に示した．患者と対照の男女比に有意差を認めなかった．患者の平均年齢は対照のそれより有意に高齢であった．疾患別ではパーキンソン病が 62.5%と最も多く,次いで脊髄小脳変性症，パーキンソン病関連疾患，ALS がそれぞれ 10%前後であった．患者の日常生活状況については，身の回りのことに介助を要する者が 44.1%と最も多く, 次いで寝たり起きたり，あるいは座れるが主として寝たきりの者が約 20%，胃瘻造設 11.4%，呼吸器使用 7.4%の順であった．患者の介護度は，要支援（社会的支援を要する）と要介護度 1 または 2（部分的か軽度の介護を要する）の患者を合わせて 50.2%，他は要介護 3 以上であった．患者では，現在の健康状態について「良い」,「まあまあ良い」と回答した者は 55.3%，「あまり良くない」「良くない」が44.6%であった．

表 1　回答した患者と介護者の属性と日常生活状況

	患者（%）	対照（%）
回収率	33.7	30.2
年齢　男性 (Mean±S.D.)	69.1±11.3 歳	64.8±14.1 歳
女性	72.4±10.4 歳	61.7±13.7 歳
<50	4	12.7
50–59	10.5	23.6
60–69	22.5	28.8
70–79	37.7	28.4
80 歳以上	22.3	6.5
性　男性	43.9	36.2
女性	56.1	63.8
特定疾患の病名		
パーキンソン病	62.5	
脊髄小脳変性症	12.8	
パーキンソン病関連疾患	9.6	
筋萎縮性側索硬化症	8.6	
スモン	1.6	
その他	1.4	
日常生活状況（複数回答可）		
仕事や家事ができる	17.6	
近所まで外出は一人でできる	27.3	
身の回りのことは一人でできる	37.6	
身の回りのことに介助が必要	44.1	
寝たり起きたり	19.3	
座れるが主として寝たきり	17.3	
胃瘻を造設している	11.4	
呼吸器を使用している	7.4	
患者の介護度		
要支援	17	
要介護度 1	11.6	
要介護度 2	21.6	
要介護度 3	12.8	
要介護度 4	10.9	
要介護度 5	26.1	
主観的健康状態		
良い	17.2	6.5
まあまあ良い	38.1	56.5
あまり良くない	37.5	30.7
良くない	7.1	6.3

神経難病患者の CAM 利用実態を把握するため，月 1 回以上利用する CAM の利用割合を表 2 に，疾患別の CAM 利用頻度について図 1 に示した．

疾患別に「効果あり」と回答（複数回答可）した CAM の種類を図 2 に示した．

患者が「効果あり」と回答した具体的内容については図 3 に示した．

次に患者の介護度と「あんま・マッサージ・指圧」の効果については図 4 に示した．

西洋医学による医療と CAM を併用している患者で

表 2　月 1 回以上利用する CAM の種類

	患者		対照	
	月 1 以上利用する割合（%）	効果あり（%）	月 1 以上利用する割合（%）	効果あり（%）
あんま・マッサージ・指圧	60.8	51.3	32.4	62.8
鍼灸	30.9	32.4	14.1	29
柔道整復	18.8	26.8	14.2	37.6
漢方	31.8	42.2	17.4	35.3
健康補助食品	40.5	25.8	42.5	35.9

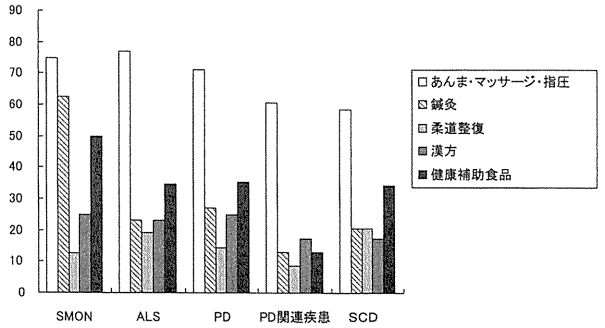

図 1　疾患別 CAM の利用状況の比較
スモン，ALS，パーキンソン病，パーキンソン病関連疾患，脊髄小脳変性症の全てで，「あんま・マッサージ・指圧」の利用が最も多かった．スモンでは次に「鍼灸」の利用が多いことが特徴であった．ALS，パーキンソン病，パーキンソン病関連疾患，脊髄小脳変性症では，「あんま・マッサージ・指圧」の次に「健康補助食品」の利用が多く，「鍼灸」「漢方」がほぼ同様の利用頻度であった．SMON：スモン，ALS：筋萎縮性側索硬化症，PD：パーキンソン病，SCD：脊髄小脳変性症．

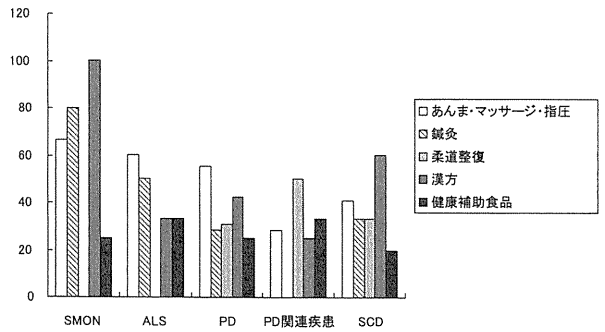

図 2　疾患別「効果有り」と回答した CAM の割合
スモンでは回答者全員が「漢方」は有効と回答した．ALS では「あんま・指圧・マッサージ」が 60%，「鍼灸」は 50%で「効果あり」と回答したが，「漢方」や「健康補助食品」では 33%にとどまった．パーキンソン病では 55%の患者が「あんま・指圧・マッサージ」，42%の患者が「漢方」は「効果あり」と回答した．パーキンソン病関連疾患では，50%の患者が「柔道整復」は「効果あり」と回答した．脊髄小脳変性症では「漢方」が最も「効果あり」とされ，次いで「あんま・指圧・マッサージ」の順であった．SMON：スモン，ALS：筋萎縮性側索硬化症，PD：パーキンソン病，SCD：脊髄小脳変性症．

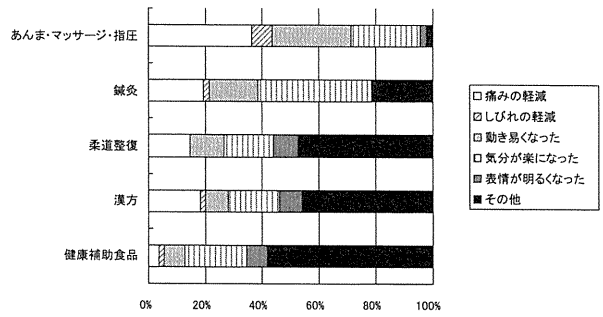

図 3　患者が「効果有り」と回答した効果の内容
「あんま・指圧・マッサージ」では，「痛みが軽くなった」が 36.2%と最も多く，次いで「動き易くなった」27.7%，「気分が楽になった」24.5%等であった．「鍼灸」では，「気分が楽になった」が 40.4%と最も多く，次いで痛みの軽減（19.1%）や動き易さ（17.0%）に効果があると回答された．「柔道整復」，「漢方」，「健康補助食品」では，選択肢以外の「その他」がそれぞれ 47.1%，46.0%，58.2%等となっていた．

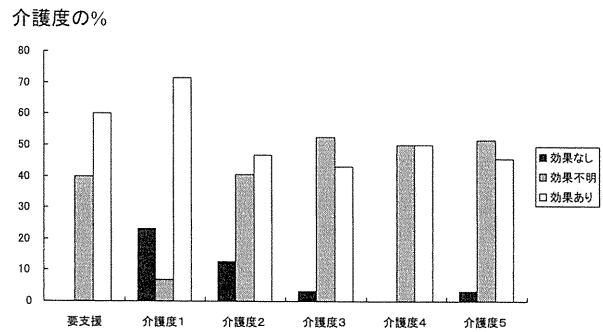

図 4　介護度と「あんま・マッサージ・指圧」の効果
患者の介護度と「あんま・マッサージ・指圧」の効果について示した．要支援と介護度 1 の患者では「効果あり」と回答する者が 60–70%と高く，一方介護度 3–5 では「効果あり」との回答は平均 46%に止まり，「効果不明」とする者が約半数であった．

は，CAM 利用について主治医に相談した者 50.2%，相談していない者 49.8%であった．

II. CAM の利用と全般的健康感について―患者と対照の比較

「あんま・マッサージ・指圧」の利用割合は，神経難病患者では60.8%，対照32.4%であり，神経難病患者で有意に多かった ($p<0.05$)（表 2）．CAM の利用頻度の最も高かった「あんま・指圧・マッサージ」について効果と健康感の関連を検討した．「あんま・指圧・マッサージ」の「効果あり」と回答した神経難病患者のうち 66%において，全般的な健康状態が「病状は落ち着いており気分が良い・気分はまあ良い」と回答し，これは「良くない・あまり良くない」と回答した患者割合に比し有意に多かった ($p<0.05$)（表 3）．パーキンソン病患者に限っても，「効果あり」と回答した患者のうち 65.2%が「病状は落ち着いており気分が良い・気分はまあ良い」と回答し，「あまり良くない・良くない」と回答した患者割合 34.8%に比し有意に多かった ($p<0.05$)（表 3）．さらに，「あんま・マッサージ・指圧」に「効果あり」と回答した患者では，患者全体での「現在の全般的な健康状態が良い・まあ良い」の割合 55.3%に比べても高く，対照の 63%とほぼ同様であった（表 1）．患者では，「あんま・マッサージ・指圧」について，「効果の有無」と「全般的な健康状態の良しあし」との間にゆるい正相関を認めた（Spearman 相関係数 0.205, $p=0.005$）．一方，対照では，「あんま・マッサージ・指圧」の効果と「現在の全般的な健康状態の良しあし」には有意な相関を認めなかった．

一方利用割合が 2 番目に多かった「健康補助食品」の効果については，69.9%の患者が「効果不明」と回答した．主観的健康感と「健康補助食品」，「漢方」，「鍼灸」，「柔道整復」の各々の効果の有無に関して有意な相関関係を認めなかった．

最近一年間の患者の健康状態の変化と「あんま・指圧・マッサージ」の利用頻度を比較した．利用患者では 1 年前と比較して漠然とした体の不調が増えた者の割合 73.8%で，病状悪化したと回答した者の割合 69.7%であったが，一方非利用患者でも体の不調が増えたと回答した者の割合 67.8%，病状悪化したと回答した者の割合 62.4%であり，両者に有意差を認めなかった．最近一年間の漠然とした体の不調の増加や，「病状悪化した」と感じたことと「あんま・指圧・マッサージ」の利用頻度とは関連が認められなかった．

「鍼灸」，「柔道整復」の利用は患者，対照で有意差を認めず，「効果あり」の割合も患者と対照で有意差を認めなかった．「漢方」の利用は患者で対照に比し多い傾向で，「効果あり」の回答は対照に比し患者の方が多い傾向であったが有意差を認めなかった．「健康補助食品」の利用割合は患者，対照でほぼ同数で，「効果あり」の回答に有意差を認めなかった（表 2）．

考察

本研究では，神経難病患者の CAM 利用実態を把握することを第一の目的にした．また，神経難病に対する CAM の作用機序が必ずしも明らかでないため，本研究では患者の自覚的有効性と全般的健康感を CAM の評価に使用した．

本研究による回答率から換算した CAM の利用割合は，神経難病患者では 20.5%，対照では 9.8%（それぞれ回答者の約 60.8%，30.2%）と推察された．本研究の結果からは，神経難病患者において，対照に比し有意差をもって効果のある CAM は認められなかったが，神経難病患者では，「あんま・マッサージ・指圧」が最も利用され，症状緩和や全般的健康感（気分が良いという状態）の向上に効果があると考えられた．

表 3 「あんま・マッサージ・指圧」の効果と主観的健康感

		健康状態			χ2 乗検定 (Fisher 直接法)
		良い・まあ良い	良くない・あまり良くない	合計	
全患者	効果あり	64	33	97	$p<0.05$
	効果なし	10	10	20	
	効果不明	30	40	70	
パーキンソン病患者	効果あり	45	24	69	$p<0.05$
	効果なし	8	5	13	
	効果不明	19	27	46	
対照	効果あり	57	30	87	$p<0.05$
	効果なし	4	9	13	
	効果不明	20	18	38	

これまで神経難病患者においてCAM利用と主観的効果について検討した研究は極めて少ない．パーキンソン病患者におけるCAMの普及の実態を調査した大越らの報告によると，運動療法・理学療法を実施している患者は55%であり，一方漢方薬の服用11.7%，鍼灸3.3%，あんま・マッサージ治療11.7%などとされている[2]．日本神経内科専門医を対象としたアンケート調査では，76.4%の医師がパーキンソン病患者に運動療法・理学療法を実施し，さらに29.8%の医師があんま・マッサージ治療の紹介あるいは推奨，14.7%で鍼灸治療の紹介・推奨，5.9%で漢方薬の処方が行なわれていた[2]．このように，パーキンソン病に対するあんま・マッサージ療法は比較的多く，主に自覚的改善を目的として利用されている．これは，本研究とほぼ同様の結果と考えられた．

本研究において，神経難病患者では「あんま・マッサージ・指圧」，「健康補助食品」，「漢方」の利用割合が多く，自覚的効果も高かった．さらに，「一年前に比して病状が進行・悪化している」かどうかとは関係なく，「全般的健康感」改善に有効であったことから，CAM療法は神経難病患者の症状緩和に有効と考えられた．今後，客観的効果判定と作用機序の解明が課題と考えられる．また本調査から，我が国では主治医に相談せずにCAMを利用している患者が約半数いることが明らかになった．患者が主治医にCAM利用に関して躊躇無く相談できるためにも，一般人のみならず医療関係者についてもCAMの理解と普及の啓発活動が必要と考えられた．

文献的には，癌患者を対象としたCAM研究が多く，米国，英国，ドイツなどでは癌患者の40–60%でCAMの利用が報告されている[3,4]．これらの国では，CAMに関する各種データベース作成や研究機関・研究者間ネットワーク構築が進められ，さらに癌患者向けのCAM指導書の提供など積極的な研究と啓発がなされている．英国では，癌患者のペインコントロールのため，医師の約70%が鍼灸治療を利用しているとされ，その他マッサージやアロマテラピー，リフレクソロジーなども70–80%の臨床現場で活用が報告されている．日本においては，癌患者の44.6%が1種類以上のCAMを利用していることが示され，また，日本の特徴として健康食品の利用頻度が96.2%と極めて高いことが指摘されている[4,5]．CAMは癌の進行抑制，症状緩和などの他，生活習慣病，アレルギー，感染症，自己免疫疾患など広い領域にわたって効果が期待されている[6]．さらに，健康成人におけるマッサージ療法の効果の検討では，不安の軽減効果，免疫力および血清脂質濃度に影響を与えることが示されている[7]．今後，神経疾患においても疾患別にCAMの効果を自覚的のみならず科学的に実証していく必要がある．

結論

本研究では，回答率から換算したCAMの利用割合は神経難病患者では20.5%，対照とした介護者では9.8%であった．患者に利用されているCAMの内訳は，「あんま・マッサージ・指圧」が60.8%と最も多く，次いで「健康補助食品」40.5%，「漢方」31.8%であり，自覚的な症状緩和や全般的健康感（気分が良いという状態）の向上に有効と考えられた．神経難病患者では，「鍼灸」や「柔道整復」の利用割合はそれぞれ30.1%，18.8%と多くはなかったが，利用者では効果ありの回答が多い傾向を認めた．さらにCAMの「効果あり」と回答した患者では，主観的健康状態が良好と感じている者が多かった．根治療法が未だ確立されていない疾患を有する患者において，CAMが症状緩和に有効との結果が得られたことから，神経難病患者の身体的及び精神的な負担の軽減に対しCAMの利用が選択肢の一つとして有用性を示すものと考えられた．

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ABSTRACT

Use of Complementary and Alternative Medicine by Intractable Neurodegenerative Patients and Caregivers

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Objective: We aimed to characterize patterns of use of complementary and alternative (CAM) therapies on patients with intractable neurodegenerative diseases and their caregivers.

Methods: We sent questionnaires to 1,406 patients with subacute myelo-optico-neuropathy (SMON), amyotorophic lateral sclerosis (ALS), Parkinson's disease (PD), Parkinson related disease, or spino-cerebellar degeneration (SCD). We also send questionnaires to the 1,406 caregivers of these patients. The participants were asked to answer questions about current use of Annma/Massage/ Shiatu, acupuncture, Zyudoseifuku, Chinese medicine or Supplementary food. Other questions including reasons for the use, subjective effectiveness of the CAM and subjective wellness were also asked.

Results: 33.7% of patients and 30% of caregivers responded to the questionnaires. Anna/Massage/Shiatu and Chinese medicine were most frequently used by patients (60.8%), and 51.3% of them answered that these therapies were effective. The caregiver's response showed supplementary food and Anna/Massage/Shiatu were most frequently used (42.5%), and 35.9% of them answered that these were effective.

Conclusion: The present study showed that use of CAM was 20.5% in patients with intractable neurodegenerative diseases and 9.8% among caregivers when calculated using collection rates. Annma/Massage/Shiatu was most frequently used and was regarded effective on subjective wellness both in the patients and caregivers.

Key words: Patients with neurodegenerative disease, Parkinson's disease, usage, caregivers

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Genetic polymorphisms involved in dopaminergic neurotransmission and risk for Parkinson's disease in a Japanese population

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Abstract

Background: Parkinson's disease (PD) is characterized by alterations in dopaminergic neurotransmission. Genetic polymorphisms involved in dopaminergic neurotransmission may influence susceptibility to PD.

Methods: We investigated the relationship of catechol-O-methyltransferase (COMT), monoamine oxidase B (MAOB), dopamine receptor (DR) D2 and DRD4 polymorphisms and PD risk with special attention to the interaction with cigarette smoking among 238 patients with PD and 369 controls in a Japanese population.

Results: Subjects with the AA genotype of *MAOB* rs1799836 showed a significantly increased risk of PD (odds ratio (OR) = 1.70, 95% confidence interval (CI) = 1.12 - 2.58) compared with the AG and GG genotypes combined. The AA genotype of *COMT* rs4680 was marginally associated with an increased risk of PD (OR = 1.86, 95% CI = 0.98 - 3.50) compared with the GG genotype. The *DRD2* rs1800497 and *DRD4* rs1800955 polymorphisms showed no association with PD. A *COMT* -smoking interaction was suggested, with the combined GA and AA genotypes of rs4680 and non-smoking conferring significantly higher risk (OR = 3.97, 95% CI = 2.13 - 7.41) than the AA genotype and a history of smoking (P for interaction = 0.061). No interactions of smoking with other polymorphisms were observed.

Conclusions: The *COMT* rs4680 and *MAOB* rs1799836 polymorphisms may increase susceptibility to PD risk among Japanese. Future studies involving larger control and case populations and better pesticide exposure histories will undoubtedly lead to a more thorough understanding of the role of the polymorphisms involved in the dopamine pathway in PD.

Background

Dopamine is one of the major modulatory neurotransmitters in the central nervous system (CNS) [1]. As dysfunction of dopaminergic neurotransmission in the CNS has been implicated in development of PD [2], it has been suggested that genetic polymorphisms involved in the biosynthesis and degradation of dopamine and related compounds influence susceptibility to PD. Catechol-O-methyltransferase (COMT) is an enzyme, which by methylation inactivates neurotransmitters and toxic catechols such as the immediate precursor of dopamine. Monoamine oxidase B (MAOB) is one of the primary enzymes regulating metabolism of neurotransmitters such as dopamine. There are five known dopamine receptors (DRD1-5) grouped into D-1 like (DRD1 and DRD5) and D-2 like (DRD2, DRD3 and DRD4) receptors based on their pharmacological profiles and sequence homology. Of these, DRD2 and DRD4 govern the signaling effect and modulate the motor behavior and activity of nigrostriatal neurons [3]. Genetic variation in these proteins, which are responsible for

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dopaminergic neurotransmission, may influence susceptibility to PD.

Decreased COMT activity may result in increased metabolism of dopamine to neuromelanin that can enhance the formation of cytototoxic radicals contributing to neuronal degeneration [4]. As the A allele of *COMT* rs4680 is associated with low COMT activity of soluble COMT [5], the A allele of *COMT* rs4680 may be linked to an increased risk of PD. It has been suggested that MAOB inhibition may prevent degeneration of the dopaminergic system in PD [6]. It is well-documented that cigarette smoking is associated with reduced MAOB activity and confers beneficial effects against PD [7]. Therefore, low MAOB activity may play a preventive role in PD development. Although the *MAOB* rs1799836 polymorphism is a synonymous substitution, this single nucleotide polymorphism (SNP) is associated with varying enzyme activity. In fact, synonymous SNPs can cause inactivation of the native splicing donor site, which results in a premature stop codon or exon skipping, yielding a shorter mRNA [8]. The shorter mRNA results in a truncated protein that is likely rapidly degraded or functionally inactive [9]. As the G allele of *MAOB* rs1799836 polymorphism is associated with lower activity of brain MAOB activity [10], the G allele may be involved in PD susceptibility (protective). The *DRD2* rs1800497 T allele (formerly *DRD2* TaqI A1) showed reduced DRD2 density in the postmortem brain [11], decreased receptor binding in positron emission tomography *in vivo* [12] and reduction of dopaminergic activity in the CNS [13]. However, the impact of *DRD2* rs1800497 on D2 receptor density has recently been questioned [14]. The functional significance of *DRD2* rs1800497 is not clear at this time, and there may be linkage disequilibrium between the other polymorphisms. The *DRD4* rs1800955 SNP is thought to influence promoter activity with the T allele exhibiting a 40% reduction in promoter activity relative to the C allele *in vitro* [15]. As the T allele of *DRD4* rs1800955 is considered to be involved in defects in dopaminergic neurotransmission, the T allele may play a deleterious role in PD development.

Studying gene-environment interactions in relation to PD risk may be valuable because positive findings would clearly implicate disease-causing exposures, clarify PD etiology, and elucidate environmental modifications for disease prevention. This study aimed to determine the impact of polymorphisms involved in dopaminergic neurotransmission on PD risk alone or in combination with smoking in a Japanese population.

Methods

Study subjects

PD patients were recruited at three university hospitals and one national hospital in Fukuoka Prefecture, a metropolitan area of Kyushu Island in southern Japan, and in three university hospitals, three national hospitals and one municipal hospital in Osaka, Kyoto, and Wakayama Prefectures. Eligible (prevalent) cases were patients who were within 6 years of the onset of PD and who presented at one of the 11 collaborating hospitals between April 1, 2006 and March 31, 2008. The mean duration (± SD) of PD was 38.8 (16.7) months. The mean age of onset (± SD) was 65.76 (± 8.82) years. There were no patients with juvenile PD. During the same period, hospital controls, without a previous diagnosis of a neurodegenerative or malignant disease, were recruited from departments other than the department of neurology because hospital controls are more motivated and are more easily accessible for obtaining DNA samples. Controls were not, individually or in larger groups, matched to cases. Details of the study subjects have been documented elsewhere [13].

Six hundred and eleven subjects (240 PD patients and 371 controls) agreed to donate buccal samples. Data on smoking and pesticide use were insufficient for two cases and one control. In total, 238 cases and 369 controls were enrolled in this study. The ethics committees of the eleven collaborating universities/hospitals approved the research protocol, and all subjects signed informed consent.

Genetic analysis

Genomic DNA was extracted from buccal samples. Genetic determinations were blinded to PD status. TaqMan SNP Genotyping Assays (ABI) were used for the following (gene, SNP, assay ID): *COMT*, rs4680, C_25746809_50; *MAOB*, rs1799836, C_8878790_10; *DRD2*, rs1800497, C_7486676_10; *DRD4*, rs1800955, C_7470700_30.

Statistical analysis

To test for associations between SNPs and PD, we defined the ancestral allele using the National Center for Biotechnology Information SNP database as the major allele. We assessed Hardy-Weinberg equilibrium (HWE) via a goodness-of-fit χ^2 test (Pearson) to compare the observed and expected genotype frequencies among controls. Based on the results from functional studies (SNPs other than *DRD2* rs1800497) and our results of associations between SNPs and PD, we designated the genotype presumed to increase the risk of PD as the "at-risk" genotype. The trend of association was assessed by a logistic regression model assigning ordinal scores to the levels of the independent variable. As *MAOB* is located on the X chromosome (Xp11.23), the genotypes were assessed separately in men and women. Although men are in a hemizygous state, the genotypes of *MAOB* rs1799836 for men were coded as

homozygous. All "at-risk" alleles were classified into six categories (0-2 and 3, 4, 5, 6, and 7+). Alternatively, all "at-risk" alleles were classified into four categories (0-3 and 4, 5, 6 +). Unconditional logistic regression was used to compute the odds ratios (ORs) and their 95% confidence intervals (CIs), with adjustments for potential confounders. The potential confounders included age (continuous variable), sex (male/female), region of residence (Fukuoka/Kinki), smoking status (ever/never), alcohol consumption [long-term consumption of alcoholic beverages (continuing consuming for ≥49 years, which is a cutoff point at the 90th percentile of controls)/short-term consumption of alcoholic beverages (continuing consuming for < 49 years)] and pesticide, herbicide or fungicide exposure (ever/never). Modeling different mechanisms of action of a particular allele was conducted by grouping individuals with one or two particular genotypes regarding the chosen model (dominant model: scored as 1 for heterozygous and homozygous of the possible risk allele for PD and 0 otherwise; recessive model: scored as 1 for homozygous of the possible risk allele for PD and 0 otherwise). The interaction between SNPs and cigarette smoking on the risk of PD was statistically evaluated based on the likelihood test, comparing the models with and without a term for interaction (multiplicative scale).

All statistical analyses were implemented in STATA Version 10.1. All P values were two-sided, with those less than 0.05 considered statistically significant.

Results

The distributions of selected characteristics among subjects are summarized in Table 1. Two hundred and thirty-eight patients with PD and 369 controls were enrolled in the study. The sex ratio, the prevalence of first degree family history of PD and the region of residence did not differ significantly between cases and controls. Compared with control subjects, cases were more likely to be older (P = 0.007) and report long-term alcohol consumption (P = 0.041). PD patients were less likely to report a history of smoking compared to the control subjects (P < 0.0001). Unexpectedly, the PD patients tended to have less frequent home or occupational pesticide exposure.

The distributions of polymorphisms involved in dopaminergic neurotransmission among cases and controls are shown in Table 2. Four SNPs did not deviate from HWE in controls (P_{HWE} = 0.077 for *COMT* rs4680, P_{HWE} = 0.443 for *MAOB* rs1799836 among women, P_{HWE} = 0.111 for *DRD2* rs1800497, P_{HWE} = 0.083 for *DRD4* rs1800955). As *MAOB* is located on the X chromosome, rs1799836 among men (no heterozygotes) and women combined deviated from HWE. There were nonsignificant differences in genotypic

Table 1 Selected characteristics of Parkinson's disease cases and controls

Characteristics	Cases (n = 238)	Controls (n = 369)	*P*
Age, year (95% CI)	68.5 (67.4 - 69.6)	66.6 (65.7 - 67.4)	0.007
Sex, n (%)			
Male	91 (38.2)	140 (38.0)	
Female	147 (61.8)	228 (62.0)	0.96
First degree family history of PD, n (%)	11 (4.62)	12 (3.25)	0.39
Smoking status, n (%)			
Current smoker	7 (2.94)	50 (13.6)	
Former smoker	57 (24.0)	97 (26.3)	
Non-smoker	174 (73.1)	222 (60.2)	< 0.0001
Consumption of alcoholic beverages, n (%)*			
Short-term	195 (81.9)	320 (87.9)	
Long-term	43 (18.1)	44 (12.1)	0.041
Home pesticide use, n (%)			
Yes	117 (49.2)	202 (54.7)	
No	121 (50.8)	167 (45.3)	0.18
Occupational pesticide use, n (%)			
Yes	20 (8.4)	33 (8.9)	
No	218 (91.6)	336 (91.1)	0.82
Either home or occupational pesticide use, n (%)			
Yes	122 (51.3)	210 (56.9)	
No	116 (48.7)	159 (43.1)	0.17
Region of residence, n (%)			
Fukuoka	89 (37.4)	154 (41.7)	
Kinki	149 (62.6)	215 (58.3)	0.29

95% CI, 95% confidence interval
Five cases were missing.

frequencies between case and control subjects for all of the polymorphisms (P = 0.106 - 0.460). The AA genotype of *COMT* rs4680 was marginally associated with an increased risk of PD compared with the GG genotype (OR = 1.86, 95% CI = 0.98 - 3.50). There was a significant trend in increasing risk with the number of the A alleles of *COMT* rs4680 (P_{trend} = 0.044). A dominant effect of the A allele on PD risk was suggested. Subjects with the AA genotype had a significantly increased risk of PD compared with those with at least one G allele (adjusted OR = 1.70, 95% CI = 1.12 - 2.58). There was a significant trend in decreasing risk with the number of the G alleles of *MAOB* rs1799836 (P_{trend} = 0.016). A recessive effect of the A allele of *MAOB* rs1799836 on PD risk was suggested. The C allele of *DRD2* rs1800497 was associated with an increased risk of PD and appeared to act in a recessive mode in this study. Similarly, the T allele of *DRD4*

Table 2 Associations of polymorphisms involved in dopaminergic neurotransmission and Parkinson's disease

Polymorphism	Cases (%) (n = 238)	Controls (%) (n = 369)	P	Adjusted* OR (95% CI)
COMT rs4680				
GG (ancestral)	98 (41.2)	179 (48.5)		1.0
GA	116 (48.7)	166 (45.0)		1.26 (0.88 - 1.79)
AA	24 (10.1)	24 (6.5)	0.106	1.86 (0.98 - 3.50)
		P_{HWE} = 0.077		P_{trend} = 0.044
GA + AA vs. GG	140 (58.8)	190 (51.4)		1.33 (0.95 - 1.87)
MAOB rs1799836				
AA (A) (ancestral)	192 (80.7)	273 (74.0)		1.0
AG	34 (14.3)	68 (18.4)		0.61 (0.37 - 0.99)
GG (G)	12 (5.0)	28 (7.6)		0.55 (0.26 - 1.16)
		P_{HWE} < 0.0001	0.154	P_{trend} = 0.016
AA (A) vs. AG + GG (G)				1.70 (1.12 - 2.58)
Female				
AA (ancestral)	110 (74.8)	156 (68.1)		1.0
AG	34 (23.1)	68 (29.7)		0.60 (0.36 - 0.99)
GG	3 (2.13)	5 (2.18)		0.90 (0.21 - 3.97)
		P_{HWE} = 0.443	0.368	P_{trend} = 0.084
Male				
A	82 (90.1)	117 (83.7)		1.0
G	9 (9.89)	23 (16.3)	0.166	0.45 (0.19 - 1.09)
DRD2 rs1800497				
TT (ancestral)	29 (12.2)	52 (14.1)		1.0
TC	117 (49.2)	192 (52.0)		1.04 (0.61 - 1.77)
CC	92 (38.7)	125 (33.9)	0.460	1.32 (0.77 - 2.28)
		P_{HWE} = 0.111		P_{trend} = 0.204
CC vs. TC + TT				1.28 (0.90 - 1.82)
DRD4 rs1800955**				
TT (ancestral)	81 (34.0)	136 (37.0)		1.0
TC	122 (51.3)	162 (44.0)		1.23 (0.85 - 1.79)
CC	35 (14.7)	70 (19.0)	0.173	0.88 (0.54 - 1.47)
		P_{HWE} = 0.083		P_{trend} = 0.928
TT + TC vs. CC				1.27 (0.80-2.00)

*Adjusted for age, sex, first degree family history of PD, region, smoking status, drinking status and pesticide exposure.
**One control was missing.

rs1800955 was regarded as the putative risk allele and appeared to behave in a dominant fashion.

We assessed interactions between polymorphisms involved in dopaminergic neurotransmission and smoking (Table 3). To achieve adequate statistical power, current and former smokers were combined (ever smokers). The OR of a history of smoking was 0.40 (95% CI = 0.25 - 0.64) after adjustment for age, sex, first degree family history of PD, region, alcohol consumption and pesticide use (data not shown). As shown in Table 3, non-smokers with at least one A allele of *COMT* rs4680 (adjusted OR = 3.97, 95% CI = 2.13 - 7.41) had a higher risk of PD than those with the GG genotype (adjusted OR = 3.70, 95% CI = 1.95 - 7.02), relative to ever smokers with the GG (non-risk) genotype (reference). Ever smokers with the GA and AA genotypes combined had a significantly increased risk of PD (adjusted OR = 2.19, 95% CI = 1.17 - 4.10). Evidence of interaction between the *COMT* rs4680 genotypes and smoking was suggested (P = 0.061). Similarly, non-smokers with the "at-risk" AA genotype of *MAOB* rs1799836 (adjusted OR = 5.74, 95% CI = 2.16 - 15.2) had a higher risk of PD than those with the AG and GG genotypes combined (adjusted OR = 3.68, 95% CI = 1.30 - 10.4), relative to

Table 3 Interaction between smoking and polymorphisms involved in dopaminergic neurotransmission in Parkinson's disease

Polymorphism	Genotype	Non-smokers		Ever smokers		$P_{inteaction}$
		Cases/controls	Adjusted OR* (95% CI)	Cases/controls	Adjusted OR* (95% CI)	
COMT	No risk† (GG)	77/103	3.70 (1.95 - 7.02)	21/76	1.0 (reference)	0.061
rs4680	At-risk† (GA + AA)	97/119	3.97 (2.13 - 7.41)	43/71	2.19 (1.17 - 4.10)	
MAOB	No risk‡ [GG (G) + AG]	40/70	3.68 (1.30 - 10.4)	6/26	1.0 (reference)	0.434
rs1799836	At- risk‡ [AA (A)]	134/152	5.74 (2.16 - 15.2)	58/121	2.39 (0.91 - 6.27)	
DRD2	No risk‡ (TC + TT)	108/153	2.32 (1.34 - 3.99)	38/91	1.0 (reference)	
rs1800497	At- risk‡ (CC)	66/69	3.16 (1.75 - 5.70)	26/56	1.12 (0.61 - 2.08)	0.608
DRD4	No risk† (CC)	25/37	2.98 (1.18 - 7.56)	10/33	1.0 (reference)	
rs1800955**	At- risk† (TT + TC)	149/184	3.50 (1.57 - 7.80)	54/114	1.48 (0.67 - 3.28)	0.637

*Adjusted for age, sex, first degree family history of PD, region, drinking status and pesticide exposure.

**One control was missing.

† Based on the dominant model.

‡ Based on the recessive model.

ever smokers with the AG and GG genotypes combined. Smokers with the AA genotype presented a nonsignificantly increased risk of PD (adjusted OR = 2.39, 95% CI = 0.91 - 6.27). Interaction between the *MAOB* rs1799836 genotypes and smoking was far from significant. As for the *DRD2* rs1800497 and *DRD4* rs1800955 SNPs, the significantly high ORs were attributed largely to the effect of non-smoking. The interaction measure between smoking and either *DRD2* rs1800497 or *DRD4* rs1800955 did not reach statistical significance.

There were no polymorphism-polymorphism interactions in any possible combination (data not shown).

We examined the cumulative effect of putative "at-risk" alleles of four SNPs involved in dopaminergic neurotransmission on PD risk (Table 4). Increasing numbers of putative "at-risk" alleles increased PD risk in a dose dependent manner (P_{trend} = 0.007). The risk was more than doubled in subjects with seven or eight putative "at-risk" alleles (adjusted OR = 2.66, 95% CI = 1.03 - 6.88), compared to those with one or two putative "at-risk" alleles. Alternatively, for carriers with more than five putative "at- risk" alleles, PD risk was increased ~2-fold (OR = 1.80, 95% CI = 1.07 - 3.05), compared with carriers with less than or equal to three putative "at-risk" alleles.

Discussion

The polymorphisms involved in dopaminergic neurotransmission such as *COMT* rs4680, *MAOB* rs1799836, *DRD2* rs1800497 and *DRD4* rs1800955 were determined in a total of 607 Japanese subjects (238 PD cases and 369 controls). As compared with the GG genotype of

Table 4 Relationship of total number of "at-risk" genotypes of polymorphisms involved in dopaminergic neurotransmission to Parkinson's disease

Number of "at-risk"* alleles	Subjects, n (%)		Adjusted† OR (95% CI)		
	Cases (n = 238)	Controls** (n = 368)			
0	0 (0.0)	0 (0.0)	-	-	-
1	2 (0.84)	6 (1.63)	1.0 (reference)	1.0	
2	8 (3.36)	16 (4.35)	1.41 (0.22 - 9.06)	(reference)	1.0 (reference)
3	25 (10.5)	46 (12.5)	1.59 (0.29 - 8.74)	1.23 (0.49 - 3.09)	
4	51 (21.4)	100 (27.2)	1.65 (0.31 - 8.78)	1.28 (0.54 - 2.99)	1.10 (0.64 - 1.90)
5	73 (30.7)	107 (29.1)	2.13 (0.41 - 11.2)	1.64 (0.71 - 3.79)	1.42 (0.85 - 2.39)
6	51 (21.4)	65 (17.7)	2.39 (0.45 - 12.7)	1.84 (0.78 - 4.36)	
7	25 (10.5)	26 (7.07)	3.47 (0.62 - 19.4)	2.66	1.80 (1.07 - 3.05)
8	3 (1.26)	2 (0.54)	3.25 (0.28 - 37.8)	(1.03 - 6.88)	
			P_{trend}= 0.007	P_{trend}= 0.007	P_{trend}= 0.012

* Based on our results, we designated the allele that is presumed to increase the risk of PD as the "at-risk" allele.

**One control was missing.

†Adjusted for age, sex, first degree family history of PD, region, smoking status, drinking status and pesticide exposure.

COMT rs4680, the AA genotype was marginally associated with an increased risk of PD. The AA genotype of *COMT* rs4680 has been reported to be a genetic risk factor for PD in Japanese populations [16,17] but the studies among ethnic populations other than Japanese failed to confirm any significant association [5,18-25]. This ethnic difference might be partly due to the difference in the allelic frequency of *COMT* rs4680. According to the HapMap SNP database [26], the A allele frequency is more common among Caucasians (51.7%) and less common among Japanese (23.3%), Han Chinese (25.6%) and Yorubas (a West African ethnic group, 29.2%). The frequency of the A allele in this study (29.0%) was somewhat higher than that of the HapMap SNP database but similar to that of other Japanese populations (28.8% and 31.1%) [16,17]. Generally, the low frequency of the "at risk" allele reduces the statistical power. As the prevalence of the A allele was lower in Japanese than in Caucasians, this is not the case. Given the lower frequency of the A allele in Japanese subjects, if this allele is associated with an increased risk of PD, then the prevalence of PD would be lower among Japanese than Caucasians. In fact, the prevalence of PD is generally lower in Asian and African-American populations than in Caucasian populations [27,28]. The reason why Japanese studies found a significant association between *COMT* rs4680 and PD risk is not clear. The ethnic difference may reflect different gene-environment interactions, gene-gene interactions, or different linkages to the polymorphisms determining PD risk.

As compared with individuals with at least one G allele of *MAOB* rs1799836, those with the AA genotype had a significantly increased risk of PD. A number of studies have examined *MAOB* rs1799836 and PD risk with conflicting results. Some studies reported that the G allele of *MAOB* rs1799836 was significantly associated with an increased risk of PD [22,29,30]. Similarly, the presence of the G allele was associated with a modest increased risk of PD [22,25,31]. On the contrary, a significant association between the A allele of *MAOB* rs179986 and an increased PD risk was observed [32]. Other studies found no association between this polymorphism and PD risk [23,24,33-36]. A meta-analysis based on six studies published before November 1999 reported that there was no significant association of *MAOB* rs1799836 with PD [37]. Each population may have its own set of environmental and genetic factors that contribute to PD risk. The lack of replication can in part be accounted for as the role of *MAOB* rs179986 on PD risk differs with environmental factors such as smoking.

The *DRD2* rs1800497 and *DRD4* rs1800955 SNPs were not associated with PD risk in this study. No significant association of *DRD2* rs1800497 and PD risk has been reported in different populations [30,36,38-40]. However, two studies of Caucasians found that the T allele of *DRD2* rs1800497 was associated with a significantly increased risk of PD [41,42]. As the conflicting results may be attributed to linkage disequilibrium (LD) between the other polymorphisms, there is a possibility that other polymorphisms such as -141 Ins/Del (rs1799732), Ser311Cys (rs1801028), Taq IB (rs1079597) and C957T (rs6277), which may be in LD with rs1800497, may play a causative role in PD development. The differences in LD would be observed among different populations [43] and different historical stages of the same population [44]. Therefore, it is more likely that the ethnic differences of the association between *DRD2* rs1800497and PD exist. As reproducibility of the results is important in genetic association studies, additional studies with a large sample size are needed to clarify the pivotal role of *DRD2* rs1800497 in PD development. Furthermore, the association of the T allele of *DRD2* rs1800497 with receptor availability was not always replicated. Future mechanistic studies are needed to verify the functional significance of different *DRD2* rs1800497 alleles. To the best of our knowledge, no studies on the association between *DRD4* rs1800955 and PD have been previously reported. PD risk associated with the 48 bp tandem repeat polymorphism of *DRD4* at the third exon, which may also be functional, has been evaluated, and one [45] of four studies [45-48] found a significant association. This tandem repeat polymorphism is probably not the main determinant in developing PD. Again, testing replication in different populations is an important step. Additional studies are warranted to corroborate the null association among Japanese samples suggested in the present study.

It is widely accepted that PD development requires environmental factors acting on a genetically predisposed individual. A gene-environment interaction was suggested, with the GA and AA combined genotype of *COMT* rs4680 and non-smoking conferring significantly higher risk (OR = 3.97, 95% CI = 2.13 - 7.41), compared with the GG genotype and a history of smoking (P for interaction = 0.061). In other words, the impact of the combined genotype of GA and AA on PD risk was marginally different between ever smokers (2.19) and non-smokers (3.97/3.70 = 1.07). In contrast to our results, two studies have reported no interaction between cigarette smoking and *COMT* rs4680 in relation to PD risk [23,25]. Our results suggest that interaction between *COMT* rs4680 and smoking is likely to vary in different races. Additional epidemiological studies are warranted to determine the smoking-*COMT* polymorphism interaction. There were no interactions between *MAOB* rs1799836, *DRD2* rs1800497 or *DRD4* rs1800955 polymorphisms and smoking. Conflicting results regarding

the modifying effect for *MAOB* rs1799836 on PD risk have been reported. *MAOB* rs1799836 modified the association between PD risk and smoking [31,49]. A significant interaction was observed in men but not in women [50]. Several studies, including the studies with the same data or overlapping data by the overlapping authors [23,39], also reported no interaction between *MAOB* rs1799836 and smoking in PD risk [23,35,36,39]. Other environmental factors may reduce MAOB and such phenotypic determinants may vary across populations. Given the possibility of environmental effects on MAOB activity, further work on interactions between the *MAOB* polymorphism and smoking is needed. There was no interaction between smoking and *DRD2* rs180049 in two studies [36,39]. No studies examining the interactions between smoking and the *DRD4* rs1800955 in PD have been published to date. Ethnicity might also play a role when studying the role of genetic factors in the association between smoking and PD.

Accumulation of multiple "at-risk" alleles markedly increased the risk of PD in a dose dependent manner, although each "at-risk" allele was associated with a small increase in risk (Table 4). Thus, individuals may have several nonsignificant "at-risk" genotypes" whose combined effect results in a high-risk. Compared with known nongenetic risk factors, smoking (1/0.40 = 2.50) and a combination of "at-risk" alleles (2.66, seven or eight "at-risk" alleles vs, less than four "at-risk" alleles) provided the same impact in PD risk prediction. Our study therefore suggests that the combined effect of multiple variant alleles may be more important than the investigation of a SNP in modulating PD risk although "at-risk" allele combinations are rare in the general population. However, although an "at-risk" allele (genotype) may confer a small individual risk, this small increase in risk translates to a large number of excess PD cases in the population. Therefore, the polymorphisms, even those not significantly associated with PD, should be considered an important public health issue.

One strength of our study is that cases were identified according to strict diagnostic criteria, and thus the possibility of misclassification of PD is negligible. Several limitations of the study also warrant mention. Our study may have included a bias due to the self-reporting of smoking habits. However, discrepancies between self-reported smoking habits and biochemical verification are minimal among the general population [51,52]. Another problem with case-control studies is recall bias. As risk factors for PD are poorly characterized, study subjects have few systematic preconceived ideas regarding their disease etiology. Any recall bias was likely to be non-differential given the many pesticides reported, the complex temporal pattern of their use, and the fact that subjects were not informed of the study hypotheses.

Conclusions

Our results suggest that the *MAOB* rs1799836 played an important role in PD susceptibility in our Japanese population. To the best of our knowledge, this is the first report on the relationship between *DRD4* rs1800955 with PD. Our study provides evidence of the interaction between *COMT* rs4680 polymorphisms and smoking. The previous studies have failed to confirm any significant association between PD and rs1799836/rs4680 [53], however. Replication of findings is very important before any causal inference can be drawn. In order to confirm our findings, consortia of investigators working on PD may need to be established. Future studies involving larger control and case populations and better pesticide exposure histories will undoubtedly lead to a more thorough understanding of the role of the polymorphisms involved in dopaminergic neurotransmission in PD development.

Appendix

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