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Association study between reward dependence temperament and a polymorphism in the phenylethanolamine *N*-methyltransferase gene in a Japanese female population

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Abstract

Cloninger's theory is that specific dimensions of temperament are associated with single neurotransmitter systems, and it is based on neurophysiologic and genetic approaches to the human traits. It suggests that overexpression of temperament could cause psychiatric illness. Based on this theory, we examined the correlation between reward dependence (RD) trait, measured with the Temperament and Character Inventory, and 5 polymorphisms in genes of norepinephrine pathways, *ADRB1*, *COMT*, *PNMT*, *SLC18A1*, and *SLC6A2*, in 85 Japanese female nursing students. We found that rs3764351 in *PNMT* was significantly associated with RD on Fisher's exact test (P = .029, P_{corr} = .236). When haplotype analysis was performed for rs3764351 and rs876493 polymorphisms in the 5′ flanking region of *PNMT*, 3 haplotypes were identified. Rs3764351 itself appeared to be correlated with RD in the present study of a specific population, although we could not demonstrate an association between RD and any of the haplotypes. Our findings have implications for the understanding of temperament using neurophysiologic approaches.
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1. Introduction

Correlations between traits as behavioral predispositions and mental disorders have recently been increasingly studied. However, it has been difficult to assess the effect of psychologic factors on mental disorders. In this context, Cloninger's theory is unique in suggesting that specific dimensions of temperament are associated with single neurotransmitter systems on the basis of neurophysiology and genetic findings [1,2]. Based on this psychobiologic model of personality, the Temperament and Character Inventory (TCI) was developed [1]. The questionnaire comprises 4 dimensions of temperament: novelty seeking (NS), harm avoidance (HA), reward dependence (RD), and persistence (P). Novelty seeking is hypothesized to be a tendency to be impulsive, exploratory, fickle, excitable, quick tempered, and extravagant. Harm avoidance is related to be cautious, to be tense, to be fearful, and to exhibit apprehensive worrying. Reward dependence refers to the preference for acting in a group, loathing isolation from others, and the concern about evaluation by others. These dimensions are suggested to be correlated with neurotransmitter pathways [1,2]. Although persistence appears to involve a tendency toward perseverance despite frustration and fatigue, its associated neurotransmitter pathways remain unclear. Moreover, overexpression of temperament has been suggested to be associated with mental disorders [3], and studies to investigate such associations have been reported [4-6]. Indeed, regarding RD, a study revealed a significant association between RD and eating disorder [5]. Based on

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physiologic and pharmacologic studies using animal models [7,8] and by measurement of norepinephrine metabolites in human [9,10], the RD behavioral pattern was shown to refer to norepinephrine pathways.

The studies on the effect of interaction with specific genes on such traits are increasing [11,12]. Among them, many studies using TCI examined correlations between genetic variations in specific genetic loci involved in neurotransmission and NS and HA. Indeed, the associations of D4 dopamine receptor gene (*D4DR*) polymorphisms with NS were demonstrated [13]. In addition, Katsuragi et al [14] reported the association between the serotonin transporter gene promoter region (*5-HTTLPR*) and HA. As for RD, the association between RD and norepinephrine pathways is still controversial partly because of limited number of studies, differences in study design, insufficient number of subjects, and ethnic differences [12,15].

On the basis of Cloninger's original theory, we focused on the correlation between RD and genetic polymorphisms of norepinephrine metabolism-related genes. Investigation of the genetic association of single nucleotide polymorphisms (SNPs) in these genes with RD could further the understanding of temperament as a behavioral predisposition at a molecular level. We hope this has implications for the use of TCI as a tool for measuring temperament, which is the basis of behavioral patterns as phenotypes, and detecting susceptibility genes or SNPs for mental disorders with characteristically inappropriate behavioral patterns.

2. Materials and methods

2.1. Subjects

A total of 85 healthy unrelated female volunteers with a mean age of 22 ± 2.7 years (mean ± SD) were recruited from among nursing students. All participants were ethnically Japanese and gave written informed consent to voluntarily participate in this study after receiving full explanation of the procedures. They completed a family and medical questionnaire and were ascertained to be free of major mental disorders among themselves and their families. The participants themselves were also confirmed to have no history of physical illnesses by responding to the questionnaire. The study protocol was approved by the Institutional Review Board and was carried out in accordance with the latest version of the Declaration of Helsinki.

2.2. Candidate genes and SNPs

We checked the genes related to systems for metabolism of norepinephrine using the NCBI db SNP (build 126; http://www.ncbi.nlm.nih.gov/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/) [16,17]. We used SNPs in the target genes as candidates, drawing on previous studies and the reference pages of them at the above websites on Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim), which exhibit correlations between the SNPs and mental disorders such as affective, anxiety, and panic disorders as well as schizophrenia, which met the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision*, criteria [18].

2.3. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes of the volunteers using a DNA extraction kit (QIAamp DNA Blood Kit; QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was carried out using 10 to 20 ng of genomic DNA. Genotyping of polymorphisms was performed by the TaqMan method, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR amplification was carried out on 10 to 20 ng of DNA using 2.5 μL of 2× TaqMan universal PCR master mix (no AmpErase UNG), 0.25 μL of 20× SNP Genotyping Assay Mix, and 2.25 μL of DNase-free water in a 5-μL reaction volume. The ABI 9700 dual plate thermal cycle (Applied Biosystems) was programmed as follows: 1 cycle of 95°C for 10 minutes, followed by 60 cycles of 92°C for 15 seconds and 58°C for 1 minute.

2.4. Psychometric evaluation

The Japanese version of the TCI has 4 dimensions of temperament and 3 of character. The TCI has been increasingly used to investigate the correlations between genetic polymorphism and personality traits in the Japanese population [14,15,19-21]. It is a self-rating instrument with a 125-item questionnaire, with the original true-false response scale modified to a 4-point scale. Its reliability and validity have been verified [22,23]. In the present study, we used the 125-item, 4-point scale version and applied only with the RD subscale to the subjects. Reward dependence score consisted of RD1, sentimentality; RD3, attachment; and RD4, dependence.

2.5. Statistical analysis

2.5.1. Single SNP analyses

Allele frequencies were calculated and tested for agreement with Hardy-Weinberg equilibrium at each SNP locus. The differences in allele frequencies of each SNP between case and control subjects were compared using Fisher's exact test with one degree of freedom. Odds ratios (ORs) were also calculated with 95% confidence intervals (95% CIs). P values of less than .05 (2 tailed) were considered significant.

2.5.2. Haplotype analyses

Single nucleotide polymorphisms exhibiting statistically suggestive findings on Fisher's exact test were subjected to haplotype analyses using Haploview ver 3.2 (http://www.broad.mit.edu/mpg/haploview/). For multiple comparisons, P values were corrected by permutation test (no. of iterations = 10 000).

3. Results

3.1. Reward dependence score

The score was not normally distributed as a continuous variable. We observed a tendency toward bimodal distribution with the cutoff point at 31. The mean score was 31, with a SD of 0.5 and a median score of 31. Hence, the participants were divided into 2 groups (t = 11.25, P < .001). They were the "high-score group" (RD score >31, n = 38) and "low-score group" (RD score ≤31, n = 47).

3.2. Target genes and SNP analyses

The 5 genes we chose were as follows: β_1-adrenergic receptor, *ADRB1*; catechol-*O*-methyltransferase, *COMT*; phenylethanolamine *N*-methyltransferase, *PNMT*; solute carrier family 18, member 1, *SLC18A1*; and solute carrier family 6, member 2, *SLC6A2*.

We tested 5 SNPs in the 5 target genes: rs1801252 (S49G) in *ADRB1* [24], rs4680 (V158M) in *COMT* [25], rs3764351 in *PNMT* [26], rs2270641 (T4P) in *SLC18A1* [27], and rs2242446 in *SLC6A2* [28]. All SNPs were in Hardy-Weinberg equilibrium (P > .3). On Fisher's exact test, we found that, among the 5 SNPs, rs3764351 in *PNMT* appeared to be associated with RD (raw P = .029, P_{corr} = .236). The other 4 SNPs were not statistically significant (P > .05), as shown in Table 1. On determination of ORs, we found that rs3764351 genotype (OR, 3.07; 95% CI, 1.16-8.11) and its allele (OR, 2.04; 95% CI, 1.09-3.82) also appeared to be associated with RD.

3.3. Haplotype analyses

On single SNP analyses, we found that rs3764351 in *PNMT* might be correlated with RD. This SNP was consequently subjected to haplotype analyses by constructing a 2-marker haplotype of *PNMT*, with rs3764351 and rs876493 polymorphisms in the 5′ flanking region. As with rs3764351, rs876493 polymorphism in *PNMT* was also reported to be correlated with mental disorders [26,29]. These 2 SNPs as markers and 3 haplotypes identified (C-C, T-T, T-C) were included in the association test (Table 2). Although rs3764351 itself was statistically significant (raw P = .029), the other 3 patterns were significantly under-represented (P > .035).

Again, rs3764351 exhibited a weak association with RD (raw P = .029; genotype OR = 3.07, 95% CI, 1.16-8.11; allele OR = 2.04, 95% CI, 1.09-3.82). The other 4 SNPs (rs1801252, rs4680, rs2270641, and rs2242446) failed to exhibit associations with RD (P > .05), as summarized in Tables 1 and 2.

Table 2
Structure and frequencies of 2-loci haplotypes in *PNMT*

Haplotype	rs3764351 [a]	rs876493 [a]	Haplotype frequencies		χ^2	P [b]
			Case	Control		
1	C	C	0.33	0.49	4.44	.035
2	T	T	0.48	0.36	2.43	.119
3	T	C	0.19	0.14	0.85	.356

Haplotypes 1, 2, and 3 consist of 2 SNPs, rs3764351 and rs876493.
[a] refSNP ID: NCBI Build 36.1.
[b] Nominal P value before adjustment for multiple testing.

4. Discussion

Exploring the association between RD and 5 polymorphisms (rs1801252, rs4680, rs3764351, rs2270641, and rs2242446) in genes of the norepinephrine pathways, we found that polymorphism in the 5′ flanking region of *PNMT* (rs3764351) statistically suggests correlation with RD (raw P = .029) before correction. When we examined *PNMT* gene haplotypes, rs3764351 itself exhibited some effect on RD, compared with the haplotypes identified. These findings implied that no other SNPs in *PNMT* acted in combination on RD. It, thus, appears that the apparent correlation with rs3764351 in *PNMT* was possibly caused by the single effect of this SNP rather than that of haplotypes. In this regard, Yuan et al [30] reported that a significant difference in

Table 1
Associations of 5 selected polymorphisms in the genes examined

Gene	db SNP ID	Location	Allele frequency [a]				P [b]	OR (95% CI)	
			Case (n = 38)		Control (n = 47)			Genotype [c]	Allele [d]
			Ref.	Var.	Ref.	Var.			
ADRB1	rs1801252	Exon 2	0.88	0.12	0.88	0.12	.907	1.11 (0.39-3.11)	0.96 (0.37-2.45)
COMT	rs4680	Exon 4	0.65	0.35	0.59	0.41	.513	1.06 (0.32-3.48)	0.77 (0.40-1.46)
PNMT	rs3764351	5′ flanking	0.33	0.67	0.50	0.50	.029 [e]	3.07 (1.16-8.11)	2.04 (1.09-3.82)
SLC18A1	rs2270641	Exon 3	0.71	0.29	0.64	0.36	.402	1.81 (0.45-7.32)	0.74 (0.38-1.46)
SLC6A2	rs2242446	5′ flanking	0.40	0.60	0.35	0.65	.632	1.90 (0.55-6.54)	1.21 (0.65-2.25)

[a] Ref. and Var. were defined by db SNP.
[b] Nominal P value before adjustment for multiple testing.
[c] Genotype 11 vs 12 + 22.
[d] Allele 1 vs allele 2.
[e] Raw P value, statistically significant.

activity was observed in the haplotype of rs3764351 and rs876493 polymorphisms, in the ability to drive transcription, compared with that of the wild-type haplotype in the 5′ flanking region. They also suggested that this raised the possibility of inherited variation in the ability to form epinephrine from norepinephrine. In a search of the TRANSFAC database (http://www.gene-regulation.com/index.html), the polymorphism rs3764351 of the *PNMT* 5′ flanking region was not located in a putative transcription factor binding element. We proceeded to analyze 5 SNPs based on raw P values. On multiple testing, P_{corr} value of rs3764351 after permutation testing was .236, which did not indicate correlation between the polymorphism and RD at a significance level of .05 (2-tailed). This method of correction is considered conservative, and applying this critical value to the result of an association study reduces the significance of associations that are detected. Our study did not yield a statistically strong association, but it does suggest the possibility of a correlation with RD. Using ORs, weak association with RD was also observed for rs3764351 in *PNMT* (Table 1). PNMT (*PNMT* [MIM* 171190]) catalyzes the synthesis of epinephrine from norepinephrine, the last step of catecholamine biosynthesis, and has been reported to be a candidate gene for a range of mental disorders [26,29]. Therefore, we show the possibility of weak genetic contributions of unidentified polymorphisms in *PNMT* in this specific population of female nursing students. The present study may not exclude true or false negativity with regard to RD because of the small sample size. Mendlowicz et al [31] reported that sex may influence the RD score. As for RD, females tend to score higher than males. Evaluation of the effect of sex on temperament and character could be of importance in further research. It was relatively difficult to measure the definitive temperament to make objective evaluations. An investigation of both nonclinical and clinical populations, as in a case-control study, would enable direct comparisons of differences in clinical phenotype. To recognize phenotype clearly on the basis of clinical information, we need to clearly determine the pathophysiology of mental disorders. Further understanding of the mechanisms of mental disorders will contribute to confirmation of associations between specific neurotransmitters and temperaments based on Cloninger's theory.

Several limitations of the present study must be noted. First, the subjects were all female nursing students, whose mean age was 22 years, with an SD of 2.7 years. The results of the study might have been different for participants with different sex, different backgrounds, and different ethnicities. Second, the previous studies have shown the difficulty in defining an a priori cutoff point, which successfully applies to other studies [5,14,15,19-21]. In the present study, we used the score, 31, as the cutoff point to divide groups into 2 because of the observed score distribution. Third, our findings were based on a small sample size. Further large-scale investigations of other populations will draw clearer conclusions.

In conclusion, we found that rs3764351 in *PNMT* may be a variable in the RD behavioral pattern in the present study of a specific population. Our findings have implications for understanding temperament using neurophysiologic approaches.

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Identifying Pathogenic Genetic Background of Simplex or Multiplex Retinitis Pigmentosa Patients: A Large-Scale Mutation Screening Study

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ABSTRACT

Background and purpose: More than half of the retinitis pigmentosa (RP) cases are genetically simplex or multiplex. To date, 37 causative genes of RP have been identified; however, the elucidation of gene defects in simplex or multiplex RP patients/families remains problematic. The aim of our study was to identify the genetic causes of RP in patients with unknown or non-Mendelian inheritance.

Methods and results: Since 2003, 52 simplex RP patients, 151 patients from 141 multiplex RP families, 6 sporadic patients with retinal degeneration were studied. A total of 108 exons of 30 RP-causing genes that harbored the reported mutations were screened by an efficient denaturing high-performance liquid chromatography (dHPLC)-based assay. Aberrant fragments were subsequently analyzed by automatic sequencing. Twenty-six mutations, including 2 frameshift mutations, one single amino acid deletion, and 23 missense mutations, were identified in 28 probands (14.07%). Eighteen mutations have not been reported to date. Three pairs of combined mutations in different genes were identified in 2 sporadic cases and 1 multiplex family, indicating the possibility of novel digenic patterns. Of the 23 missense mutations, 21 were predicted as deleterious mutations by computational methods using PolyPhen, SIFT, PANTHER, and PMut programs.

Conclusion: We elucidated the mutation spectrum in Japanese RP patients and demonstrated the validity of the mutation detection system using dHPLC-sequencing for genetic diagnosis in RP patients independent of familial incidence, which may provide a model strategy for identifying genetic causes in other diseases linked to a wide range of genes.

KEY WORDS: retinitis pigmentosa; simplex; multiplex; mutation; dHPLC

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KEY POINTS

- Retinitis pigmentosa (RP) is the most common genetic disorder in human eye. However, large proportion of RP patients are in fact simplex (sporadic) or multiplex (non-Mendelian) RP, and genetic background of these patients have not yet been explored due to a large number of causative genes and variable, or sometimes even complicated, inheritance patterns.
- In the present report, we specifically aimed to identify the genetic causes of simplex and multiplex RP through a large-scale screening of 30 RP causing genes.
- We identified genetic causes in as frequently as 14.07% of these patients. Intriguingly, we also revealed 3 potential digenic patterns in 2 unrelated patients and in a multiplex family. Our findings elucidated the mutation spectrum in Japanese RP patients and facilitated the screening strategy as a clinical applicable tool for genetic diagnosis.

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INTRODUCTION

Retinitis pigmentosa (RP) is a degenerative disorder of retinal photoreceptors, which play pivotal roles in human visual function. RP occurs worldwide with a prevalence rate of 1/4000 and to date has affected up to 1.5 million patients. In Japan, RP has become the major disorder of visually handicapped patients during the past 15 years [1].

RP is both genetically and clinically heterogeneous. Genetically, there are different traits of inheritance of this disorder, including autosomal dominant (ADRP), autosomal recessive (ARRP), and X chromosome-linked (XLRP). RP has been generally considered as a monogenetic disease until the discovery of *RDS*/peripherin, which can pair with *ROM1* as a digenic cause [2]. To date, at least 37 genes have been identified as the causative genes of RP (RetNet), thereby making genetic analysis difficult. In addition, the determination of causative genes becomes more difficult due to marked clinical heterogeneity of RP with incompatible disease penetrance as well as extensive intra- and inter-familial phenotypic variances [3-5]. Another challenge in understanding the genetic background of RP patients is multiplex and simplex RP, which comprise more than 50% of all RP disorders in different populations [6-9]. The "multiplex RP" family refers to the RP family with more than one affected individual without predictable specific Mendelian patterns of inheritance; on the other hand, a simplex RP involves an individual RP patient with no family history of RP and consanguinity in the pedigree. These 2 types of RP exclude a large proportion of RP patients with unresolved segregation pattern of candidate genes. Furthermore, recent trends of decreasing birthrate as well as socio-ethic considerations prevented us from obtaining sufficient family history of RP, resulting in conceivable increase in the number of simplex and multiplex RP cases. Despite these complexities, patients are often greatly concerned regarding the possible transmission of the disease to their children as well as prognosis of their diseases. Particularly in simplex or multiplex RP cases, genetic diagnosis could provide the only clue to meet these needs of patients. Therefore, it is essential to establish a universal method to screen mutation on an individual basis, irrespective of stringent family background information.

In a large-scale molecular screening study, clinically affected patients may show many nonsynonymous single base substitutions, which may or may not lead to altered protein function. The functional test in vivo or ex vivo is generally performed to determine whether the variant causes phenotypic differences; however, these tests are not definitive and are time-consuming and costly to apply for each detected gene alteration in clinical diagnosis. Under these circumstances, considerable efforts have been made to develop computational methods for predicting the functional impact of missense variants. Conventional programs such as PolyPhen, SIFT, PANTHER, and PMut are freely

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available online and have relatively high accuracy of prediction [10-13]. Each algorithm performs prediction through multiple sequence alignment, structure-based calculation, and/or evolutionary evaluation. A recent study has showed that predictive accuracy can be improved by combining these methods [13].

In order to regularize genetic diagnosis for each patient with RP or retinal degeneration in our clinic, we developed a systematic procedure consisting of semi-automated denaturing high-performance liquid chromatography (dHPLC), followed by sequencing method for molecular screening of RP that allows efficient, high-throughput, and wide spectrum of mutation screening. Detected mutations were then analyzed by 4 computational programs to predict pathogenicity. In the present study, we specifically aimed to identify the genetic causes of simplex and multiplex RP by recruiting 203 RP patients from 193 unrelated families with unknown inheritance pattern and 6 unrelated patients with retinal degeneration; furthermore, we performed a comprehensive survey of all previously reported mutations containing exons. Mutations were identified in approximately 14.07% of these patients, and surprisingly, 3 potential digenic patterns were observed in 2 unrelated patients and in a multiplex family.

SUBJECTS AND METHODS

Assessment of patients and study approval

This study was performed in accordance with the tenets of the Declaration of Helsinki, and an approval was obtained from our institutional ethical review board. A total of 203 RP patients from 193 unrelated families and additional 6 sporadic patients with clinically suspected areolar atrophy (2), cone dystrophy (3) or Stargart's disease (1) were recruited in the study. Informed consent was obtained from all patients before the collection of blood samples. All patients were diagnosed and followed-up at the Kyoto University Hospital, Kyoto, Japan. The doctors who participated in this project were asked to enquire the family history of patients in as much detail as possible and to obtain accurate patient information. Among 203 RP patients, 52 patients were sporadic (simplex); the inheritance of the remaining 151 multiplex RP patients from 141 families could not be determined from their familial background. A total of 115 adult healthy volunteer were recruited as normal controls.

Screening targets

We retrospectively summarized 30 RP-causing genes and the reported mutations through a web-based search on Retinal International (www.retina-international.org/) or Medline. The genes and coding regions that harbored the reported mutations during the time when our experiments were initiated in January 2003 were included in the screening

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process (Table 1).

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Table 1. The genes and targeted exons in this study

Trait	Gene	Targeted Exon(s)
ADRP	RP1	4
	RHO	1, 3, 5
	FSCN2	1
	RDS	1, 2, 3
	CRX	2, 3
	HPRP3	11
	IMPDH1	7
	NRL	1
	PRPF8	42
	ROM1	1, 2
	RP9	5, 6
	RP11	5, 6, 7, 8, 11
ARRP	RLBP-1	3, 6, 7, 8
	LART	1, 2
	MERTK	10, 14, 15
	RPE65	1, 3, 4, 5, 6 ,7, 10 ,11, 13
	CRB1	2, 3, 6, 7, 8, 9
	PDE6B	1, 3, 4, 5, 12, 13, 14, 15, 17, 18, 22
	PDE6A	7, 13, 14
	ABCA4	13, 23, 30, 40
	CNGA1	7, 9, 12a, 12b
	CNGB1	31
	NR2E3	9
	RGR	1, 6
	RHO	2, 4
	SAG	11
	TULP1	2, 5, 9, 11, 12, 13
	USH2A	2a, 2b, 3, 9
XLRP	RP2	1, 2, 4
	RPGR	1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, ORF15-1, ORF15-2, ORF15-4

Note the ORF15-3, a GC-rich region of exon ORF15 in the *RPGR* gene, was failed to amplify and only a part of the exon 4 of *RP1* gene which spans 5684 bps was screened.

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DNA and PCR

Genomic DNA was extracted from peripheral blood by using the MagNA Pure LC system (Roche, Germany) according to the manufacturer's instructions. In order to simplify the PCR procedure, primers (Supplemental table 1) were optimized to allow amplification under a common set of conditions (Tm 60°C). After PCR amplification, each sample was subjected to duplex formation.

DHPLC analysis

DHPLC analysis was performed using the WAVE DNA Fragment Analysis System (Transgenomic Inc., USA) according to the manufacturer's instructions. Briefly, 5 µl of each sample was loaded on a DnaSep column (Transgenomic Inc., USA) and eluted with an acetonitrile gradient formed by mixing buffers A and B (WAVE Optimized; Transgenomic Inc., USA). To increase the detection sensitivity in order to identify additional unreported mutations within the amplicons, the analysis was performed at 2 or 3 different temperatures for each duplexed sample, based on the predicted denaturing temperature calculated using the Wave software program. The flow rate was 0.9 ml/min, and DNA was detected based on absorbance at 260 nm.

Since the elution wave pattern changes only in the presence of heterodimers, homozygous mutations can only be detected by mixing them with wild-type amplicons. Thus, to detect recessive gene mutations, aliquots of PCR products from DNA of 3 patients were mixed prior to the heteroduplex reaction. Each amplicon was analyzed both individually and as a part of the mixture, thereby allowing the detection of both hetero- and homozygous mutations.

Sequencing of aberrant fragments

Direct sequencing was performed in both directions by using an ABI Prism Genetic Analyzer. The amplicons were purified with a 96-well Mini-Elute PCR purification kit (Qiagen, USA) and labeled with BigDye according to the manufacturer's protocol. The labeled amplicons were washed with the 96-well Mini-Elute PCR purification kit, suspended in 30 µl distilled water, and run on the analyzer. Comprehensive sequencing of all CNGA1, CRB1 and PDE6B exons was carried out in patients 0103, 0040 and 0093, 0116, 0117, 0118, respectively.

Computational test for missense mutations

Four computational algorithms, namely, PolyPhen, SIFT, PANTHER, and PMut, were used to predict the functional impact of missense mutations identified in this study. None of these mutations was identified in 115 healthy individuals. We classified the variants into 3 categories based on the combined computational analysis. When all or most (3 of 4 or 2 of 3) of the analytical results of computational programs were

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"pathological", the variant was classified as a pathogenic mutation. If one or more programs predicted the case as nonfunctional variant or missense mutation and other program(s) yielded a boundary score between "pathogenic" and "benign," the case was classified as a possible pathogenic mutation. A sequence variant predicted as a "neutral change" by all or most of the available algorithms was considered as indeterminate. The details of each program are as follows:

PolyPhen. The PolyPhen method has been described previously in detail [10]. In brief, ID or accession numbers of targeting protein sequences were derived from SWALL database. The ID or accession numbers together with the position and 2 amino acid variants were input into the PolyPhen server with default settings. Results for each variant were classified into "probably damaging," "possibly damaging," "benign," and "unknown." In the present study, both "probably damaging" and "possibly damaging" were classified as suspected pathogenic mutation.

SIFT. The methodology of SIFT has been described elsewhere [11]. In brief, the protein query sequences and the substitutions of interest were entered into the web server (http://blocks.fhcrc.org/sift/SIFT.html). The prediction test was run automatically under default settings. The SIFT output shows a normalized probability score. Positions with normalized probabilities of <0.05 are predicted to be deleterious (affecting protein function), while those with normalized probabilities of ≥ 0.05 are predicted to be tolerated. In this study, "affected protein function" was considered as suspected pathogenic mutation. The normalized probability score was considered as a reference. The score of 0.00 or 1.00 suggests extremely likely pathogenic mutation or benign variant, respectively.

PANTHER. The PANTHER website provides online service of predicting the functional effect of single amino acid substitutions in proteins. The output of PANTHER, i.e., the subPSEC score, is the negative logarithm of the probability ratio of the wild-type and mutant amino acids at a specific position, ranging from 0 (benign) to approximately -10 (most likely to be deleterious). Thus, smaller subPSEC scores indicate a higher probability of deleterious functional effect. In this study, we defined the score of -3 as a cutoff point for prediction according to the recommendation described previously [12].

PMut. The methodology of PMut developed by Ferrer-Costa et al. has been described previously in detail [13]. This program is based on the use of different types of sequence information to label mutations along with neural networks to process this information. By inputting the reference sequence and the position and amino acid substitution of interest, this program provides a very simple output: a yes/no answer and a reliability index. The output greater than 0.5 is predicted as pathological mutation and that below 0.5 as neutral;

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the reliability is good enough with a score of 6 and more and "highly reliable" at the maximum score of 9.

RESULTS

Identified mutations

In 199 patients, 28 probands (32 patients in total) (14.07%) were found to contain 26 mutations that were not identified in 115 healthy individuals; 18 of these mutations have not been reported previously (Table 2). Among the 26 mutations, 3 were deletion mutations with or without frameshift, and 23 were nonsynonymous missense mutations. None of the deletion mutations has been reported previously. Notably, 2 patients (No. 0103 and 0040) were identified to have only one heterozygous mutation in the *CNGA1* and *CRB1* genes, respectively, either of which is responsible for ARRP. The patient 0106 was identified to have a compound mutation in the recessive gene, *PDE6B*, and a patient (0102) with clinically diagnosed areolar atrophy was identified to have a double

Table 2. Mutations identified in simplex and multiplex RP patients

Gene-exon	Mutation type	AA	Nucleotide change	Patients	Computational prediction					Report
					PolyPhen	SIFT	PANTHER	PMut	Remark	
CRX-exon2	Missense	D65H	c.193G>C (homo)	S: 0003	Prob.	A/0.00	-3.5	P*	Pathogenic	Present study
CRX- exon3	Missense	G122D	c.365G>A	S: 0275, M: 0269	Benign	A/0.02	NA	N	Neutral	29
CRX-exon3	Missense	S152Y	c.455C>A	S: 0107	Poss.	A/0.04	-3.4	P*	Pathogenic	Present study
CNGA1-exon12	Missense	R424H	c.1271G>A	S: 0103	Benign	T/0.11	-1.5	P	Possible pathogenic	Present study
CRB1-exon9	Missense	H991L	c.2972A>T	M: 0040	Prob.	T/0.07	NA	P**	Pathogenic	Present study
GRK1-exon3	Missense	P290L	c.869C>T	M: 0102	Poss.	A/0.02	-3.0	P	Pathogenic	Present study
GRK1-exon6	Missense	W460R	c.1378T>C	M: 0102	Prob.	A/0.00	-9.9	P*	Pathogenic	Present study
IMPDH1-exon7	Missense	R308H	c.923G>A	M: 0139	Prob.	A/0.00	-4.1	P	Pathogenic	Present study
PDE6B-exon3	Missense	R234H	c.699G>A	S: 0019	Prob.	A/0.00	NA	N	Pathogenic	Present study
PDE6B-exon5	Missense	G308S	c.922G>A	S: 0068	Benign	T/0.12	NA	N	Neutral	Present study
PDE6B-exon13	Missense	H557Y	c.1669C>T	M: 0106	Prob.	A/0.00	-9.3**	N	Pathogenic	30
PDE6B-exon14	Missense	T604I	c.1811C>T	M: 0093†, 0116†, 0117†, 0118†	Prob.	A/0.00	-3.4	P	Pathogenic	Present study
PDE6B-exon16	Missense	L671P	c.2012T>C	M: 0106	Prob.	A/0.00	-4.6	P	Pathogenic	Present study
RDS-exon1	Missense	G137D	c.410G>A	M: 0161	Poss.	A/0.01	-3.3	P	Pathogenic	Present study
RDS-exon1	Missense	M152V	c.454A>G	S: 085; M: 0056, 0146	Poss.	T/0.11	-1.4	N	Possible pathogenic	Present study
RDS-exon1	Missense	G167S	c.499G>A	M: 0124†, 0125†;	Prob.	A/0.00	-4.3	P*	Pathogenic	31

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				0093†, 0116†, 0117†, 0118†						
RDS-exon2	Missense	K197E	c.589A>G	M: 0195	Benign	T/0.12	-2.3	P	Possible pathogenic	32
RDS-exon2	Deletion	271delL	c.811_813delCTC	M: 0180†, 0204†	NA	NA	NA	NA	NA	Present study
RDS-exon3	Missense	W316G	c.946T>G	S: 0101	Prob.	T/0.05	NA	P*	Pathogenic	Present study
RHO-exon1	Missense	N15S	c.44A>G	M: 0171	Poss.	A/0.00	NA	N	Pathogenic	21
RHO-exon1	Missense	G89D	c.266G>A	M: 0157	Prob.	A/0.01	NA	P*	Pathogenic	22
RHO-exon2	Missense	R135W	c.403C>T	M: 0063	Prob.	A/0.00	-9.6**	P**	Pathogenic	23
RHO-exon2	Missense	G174S	c.520G>A	S: 0061, 0101	Benign	A/0.00	-5.3*	P*	Pathogenic	10
RHO-exon5	Frameshift	P327fs	c.979_982delCCAC	S: 0010	NA	NA	NA	NA	NA	Present study
RP11-exon7	Missense	G224R	c.670G>A	S: 0175	Prob.	A/0.00	-4.7	P*	Pathogenic	Present study
RPGR-ORF15	Frameshift	R196fs	568_571delAGAG	S: 0107	NA	NA	NA	NA	NA	Present study

A total of 26 mutations were identified in 28 proband patients with retinitis pigmentosa. AA, amino acid; fs, frameshift; S, simplex; M, multiplex; Prob., probable; Poss., possible; A, affected; T, tolerated; P, pathological; N, neutral; NA, not applicable. † indicates two individuals in the same family were identified mutation. Asterisk indicates the prediction result is high reliable; double-asterisk indicate very high reliability.

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mutation in the *GRK1* gene (Figure 2). The G174S mutation in the *RHO* gene was identified in the patients 0061 and 0101, which has been reported as a recessive mutation [14]. Unexpectedly, a homozygous mutation in the dominant gene *CRX* was identified in the sporadic patient 0003. Two distinct mutations in unrelated genes were identified in 2 patients (0101 and 0107), and two mutations in the *RDS* and *PDE6B* genes respectively were simultaneously existed in a multiplex family (0093, 0116, 0117 and 0118) with disease co-segregation. Additionally, in our group of patients, the proband of 3 patients was found to have a "frameshift" mutation in the *FSCN2* gene, c.72delG, which was excluded from our mutation list because this mutation was detected in 2 control patients and a recent report indicated that it might be a nonpathogenic change [15].

Computational analysis

Of the 26 mutations that were not identified in 115 healthy individuals, 23 mutations were missense mutations and were analyzed by the previously mentioned 4 software programs (Table2). Of these 23 missense mutations, 18 were consistently estimated as pathogenic mutations, 3 were predicted as possible mutations with functional impacts, and the remaining 2 mutations (the G122D and G308S in the *CRX* and *PDE6B* genes, respectively) were consistently estimated as "neutral" by 3 programs. Among the 8 previously reported missense mutations, 6 were predicted as pathogenic, 1 as possibly pathogenic, and 1 as neutral by computational analysis. Of 15 novel missense mutations, 12 were predicted as pathogenic, 2 as possibly pathogenic, and 1 as neutral. In addition, a nonsynonymous variant, c.346G>T (A116S) in exon 1 of the *RDS* gene, detected in 2 patients (0066 and 0161) was also identified in 1 healthy individual. This variant was consistently predicted as a nonfunctional alteration by the 4 algorithms.

DISCUSSION

In cases with simplex or multiplex RP, which comprise a large proportion of all RP patients, genetic diagnosis is the only approach to understand the genetic background of these patients. However, to the best of our knowledge, large-scale molecular screening studies that target simplex and multiplex RP have not been reported thus far; this could possibly be due to the anticipation of low detection rate and high costs. In the present study, we established a systematic molecular screening method that encompassed as many as 30 RP-causing genes and detected mutations in 28 probands (14.07%); this result is almost comparable to the detection rate found in clinically predictable ARRP and ADRP patients together [17,18]. This suggests that gene diagnosis in simplex and multiplex RP patients is equally important in understanding this disease.

DHPLC sequencing strategy