## Author Contributions

Conceived and designed the experiments: JC EAS RMP. Performed the experiments: JC EAS RMP. Analyzed the data: JC EAS RMP. Contributed reagents/materials/analysis tools: SS CM DD GT TR MUM HC KI CT YO SW JA HY SM AT KO FM TM NG MK AWM JDI AGW KLH MH MED P-PT JBAC IEvdH-B GJW PLCMvR MvdL H-JG NAS CFA TWJH REMT RPK SLB LAC LWM JEF NdV BES PLDJ SR MEW PKG XM AB LP MJHC EWK. Wrote the paper: JC EAS RMP. All authors reviewed and approved the manuscript.

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# Genome-wide association analyses in east Asians identify new susceptibility loci for colorectal cancer

Wei-Hua Jia[1,16], Ben Zhang[2,16], Keitaro Matsuo[3], Aesun Shin[4], Yong-Bing Xiang[5], Sun Ha Jee[6], Dong-Hyun Kim[7], Zefang Ren[1], Qiuyin Cai[2], Jirong Long[2], Jiajun Shi[2], Wanqing Wen[2], Gong Yang[2], Ryan J Delahanty[2], Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO)[8], Colon Cancer Family Registry (CCFR)[8], Bu-Tian Ji[9], Zhi-Zhong Pan[1], Fumihiko Matsuda[10], Yu-Tang Gao[5], Jae Hwan Oh[11], Yoon-Ok Ahn[12], Eun Jung Park[6], Hong-Lan Li[5], Ji Won Park[11], Jaeseong Jo[6], Jin-Young Jeong[7], Satoyo Hosono[3], Graham Casey[13], Ulrike Peters[14,15], Xiao-Ou Shu[2], Yi-Xin Zeng[1,17] & Wei Zheng[2,17]

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**To identify new genetic factors for colorectal cancer (CRC), we conducted a genome-wide association study in east Asians. By analyzing genome-wide data in 2,098 cases and 5,749 controls, we selected 64 promising SNPs for replication in an independent set of samples, including up to 5,358 cases and 5,922 controls. We identified four SNPs with association *P* values of $8.58 \times 10^{-7}$ to $3.77 \times 10^{-10}$ in the combined analysis of all east Asian samples. Three of the four were replicated in a study conducted in 26,060 individuals of European descent, with combined *P* values of $1.22 \times 10^{-10}$ for rs647161 (5q31.1), $6.64 \times 10^{-9}$ for rs2423279 (20p12.3) and $3.06 \times 10^{-8}$ for rs10774214 (12p13.32 near the *CCND2* gene), derived from meta-analysis of data from both east Asian and European-ancestry populations. This study identified three new CRC susceptibility loci and provides additional insight into the genetics and biology of CRC.**

CRC is one of the most commonly diagnosed malignancies in east Asia and many other parts of the world[1]. Genetic factors have an important role in the etiology of both sporadic and familial CRC[2]. However, less than 6% of CRC cases can be explained by rare, high-penetrance variants in the CRC susceptibility genes identified to date, such as the *APC, SMAD4, AXIN2, BMPR1A, POLD1, STK11, MUTYH* and DNA mismatch repair genes[2]. Over the past two decades, many candidate gene studies have evaluated common genetic risk factors for CRC; only a few of these have been replicated in subsequent studies[3]. Recent genome-wide association studies (GWAS) have identified approximately 15 common genetic susceptibility loci for CRC[4–12]. However, these newly identified genetic factors, along with known high-penetrance variations in CRC susceptibility genes, explain less than 15% of the heritability for this common malignancy[10,11]. Furthermore, with the exception of a small study conducted in Japan[12], all other GWAS have been conducted in populations of European ancestry, which differ from other populations in certain features of genetic architecture. Many of the variants discovered in populations of European ancestry show only weak or no association with CRC in other ancestry groups[13]. Therefore, additional GWAS are needed, particularly in populations not of European ancestry, to fully uncover the genetic basis for CRC susceptibility.

In 2009, we initiated the Asia Colorectal Cancer Consortium (ACCC), a GWAS in east Asians, to search for previously unknown genetic risk factors for CRC. The discovery stage (stage 1) consisted of five GWAS conducted in China, Korea and Japan, including 2,293 CRC cases and 5,780 controls (**Supplementary Table 1**). Cases and controls were genotyped using several SNP arrays, including the Affymetrix Genome-Wide Human SNP Array 6.0 (906,602 SNPs), the Affymetrix Genome-Wide Human SNP Array 5.0 (443,104 SNPs), the Illumina Infinium HumanHap610 BeadChip (592,044 SNPs), the Illumina Human610-Quad BeadChip (620,901 SNPs) and the Illumina HumanOmniExpress BeadChip (729,462 SNPs) (**Supplementary Table 1**). After quality control exclusions as described previously[14–17], 2,098 cases and 5,749 controls remained for this study (**Supplementary Tables 1** and **2**). Also excluded from the analyses were SNPs with call rate of <95%, genotype concordance rate of <95%

[1]State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-sen University, Guangzhou, China. [2]Division of Epidemiology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA. [3]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan. [4]Molecular Epidemiology Branch, National Cancer Center, Goyang-si, Korea. [5]Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China. [6]Institute for Health Promotion, Department of Epidemiology and Health Promotion, Graduate School of Public Health, Yonsei University, Seoul, Korea. [7]Department of Social and Preventive Medicine, Hallym University College of Medicine, Okcheon-dong, Korea. [8]A complete list of members is provided in the Acknowledgements. [9]Division of Cancer Epidemiology & Genetics, National Cancer Institute, Bethesda, Maryland, USA. [10]Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [11]Center for Colorectal Cancer, National Cancer Center, Goyang-si, Korea. [12]Department of Preventive Medicine, Seoul National University College of Medicine, Seoul, Korea. [13]Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA. [14]Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. [15]Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA. [16]These authors contributed equally to this work. [17]These authors jointly directed this work. Correspondence should be addressed to W.Z. (wei.zheng@vanderbilt.edu).

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**Table 1 Association of CRC risk with the top four risk variants identified in east Asian samples**

| SNP | Alleles[a] | Chr. | Gene[b] | Location (bp)[c] | Stage | Cases Sample size | Cases MAF | Controls Sample size | Controls MAF | Per-allele association OR (95% CI)[d] | Per-allele association $P_{trend}$ | Heterogeneity $P^e$ | Heterogeneity $I^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs10774214 | T/C | 12p13.32 | *CCND2* | 4238613 | GWAS | 2,098 | 0.373 | 5,749 | 0.348 | 1.20 (1.09–1.32) | $2.03 \times 10^{-4}$ | | |
| | | | | | Replication | 5,197 | 0.381 | 5,797 | 0.355 | 1.16 (1.09–1.23) | $5.80 \times 10^{-7}$ | | |
| | | | | | Overall | 7,295 | 0.379 | 11,546 | 0.352 | 1.17 (1.11–1.23) | $5.48 \times 10^{-10}$ | 0.615 | 0% |
| rs647161 | A/C | 5q31.1 | *PITX1* | 134526991 | GWAS | 2,098 | 0.353 | 5,749 | 0.308 | 1.22 (1.12–1.33) | $3.29 \times 10^{-6}$ | | |
| | | | | | Replication | 5,217 | 0.344 | 5,815 | 0.319 | 1.14 (1.07–1.21) | $1.15 \times 10^{-5}$ | | |
| | | | | | Overall | 7,315 | 0.347 | 11,564 | 0.313 | 1.17 (1.11–1.22) | $3.77 \times 10^{-10}$ | 0.444 | 0% |
| rs2423279 | C/T | 20p12.3 | *HAO1* | 7760350 | GWAS | 2,098 | 0.339 | 5,749 | 0.307 | 1.16 (1.07–1.26) | $4.96 \times 10^{-4}$ | | |
| | | | | | Replication | 5,227 | 0.315 | 5,811 | 0.297 | 1.13 (1.06–1.19) | $1.22 \times 10^{-4}$ | | |
| | | | | | Overall | 7,325 | 0.322 | 11,560 | 0.302 | 1.14 (1.08–1.19) | $2.29 \times 10^{-7}$ | 0.331 | 12% |
| rs1665650 | T/C | 10q26.12 | *HSPA12A* | 118477090 | GWAS | 2,098 | 0.346 | 5,749 | 0.310 | 1.20 (1.10–1.31) | $3.88 \times 10^{-5}$ | | |
| | | | | | Replication | 5,192 | 0.328 | 5,808 | 0.320 | 1.10 (1.04–1.17) | 0.0018 | | |
| | | | | | Overall | 7,290 | 0.333 | 11,557 | 0.315 | 1.13 (1.08–1.19) | $8.58 \times 10^{-7}$ | 0.404 | 4% |

Chr., chromosome; OR, odds ratio; CI, confidence interval.
[a]Minor/major allele for east Asians. OR was estimated for the minor allele. [b]Closest gene. [c]Location based on NCBI Human Genome Build 36.3. [d]Adjusted for age, sex, the first ten principal components (stage 1) and study site. [e]*P* for heterogeneity across studies in GWAS and replication was calculated using Cochran's *Q* test.

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between positive control samples, minor allele frequency (MAF) of <5% or *P* value for Hardy-Weinberg equilibrium of $<1.0 \times 10^{-5}$ in controls for each study. Imputation was conducted for each study following the MaCH algorithm[18] using phased HapMap 2 Han Chinese in Beijing, China (CHB) and Japanese in Tokyo, Japan (JPT) samples as the reference. No apparent genetic admixture was detected, except for one sample from KCPS-II (**Supplementary Fig. 1**). Associations between CRC risk and each of the genotyped and imputed SNPs were evaluated using logistic regression within each study after adjusting for age, sex and the first ten principal components using mach2dat[18]. Meta-analyses were conducted under a fixed-effects model using the METAL program[19]. There was little evidence for inflation in the association test statistics for any of the five studies (genomic inflation factor ($\lambda$) range of 1.02 to 1.04) or for all studies combined ($\lambda = 1.01$) (**Supplementary Fig. 2** and **Supplementary Table 1**). The observed number of SNPs with small *P* values was slightly larger than that expected by chance (**Supplementary Fig. 2**).

Multiple genomic locations were found that were potentially related to CRC risk (**Supplementary Fig. 3**). Nine SNPs identified from published GWAS conducted in populations of European ancestry showed associations with CRC risk at $P < 0.05$ in stage 1 (data not shown). To improve the statistical power for evaluating these SNPs, we genotyped 6,476 additional samples to bring the total sample size to 5,252 cases and 9,071 controls. Except for the 2 SNPs that are monomorphic in east Asians (rs6691170 and rs16892766), all 16 of the other SNPs identified from published GWAS conducted in European-ancestry populations showed association with CRC risk in the same direction as reported previously (**Supplementary Table 3**). A significant association with CRC risk at $P < 0.05$ was found for 13 SNPs, including rs6687758, rs10936599, rs10505477, rs6983267, rs7014346, rs10795668, rs3802842, rs4444235, rs4779584, rs9929218, rs4939827, rs10411210 and rs961523. Except for two SNPs (rs6983267 and rs4779584), no statistically significant heterogeneity at $P < 0.05$ was observed between east Asian and European-ancestry populations (**Supplementary Table 3**).

To identify new genetic factors for CRC, we selected 64 SNPs for replication in an independent set of 5,358 cases and 5,922 controls recruited in 5 studies conducted in China, Korea and Japan (**Supplementary Table 2**). SNPs were selected from among those that (i) had MAF of >5%; (ii) showed no heterogeneity across studies ($P_{het} > 0.05$ and $I^2 < 25\%$); (iii) were not in linkage disequilibrium (LD; $r^2 < 0.2$) with any known CRC risk variant reported from previous GWAS; (iv) had high imputation quality in each of the five studies (RSQ > 0.5); and (v) were associated at $P < 0.01$ in the combined analysis of all five studies included in stage 1. These criteria were used to prioritize SNPs for replication.

Of the 64 SNPs evaluated in stage 2, 7 showed association with CRC risk at $P < 0.05$ with a direction of association consistent with that observed in stage 1 (**Table 1** and **Supplementary Table 4**). In the combined analysis of data from stages 1 and 2, *P* values for associations with two SNPs (rs647161 at 5q31.1, odds ratio (OR) = 1.17, $P = 3.77 \times 10^{-10}$, and rs10774214 at 12p13.32, OR = 1.17, $P = 5.48 \times 10^{-10}$) were lower than the conventional genome-wide significance level of $5.0 \times 10^{-8}$, providing convincing evidence for an association of these SNPs with CRC risk (**Table 1**). An additional SNP, rs2423279, showed a significant association in stage 2 after Bonferroni correction (corrected $P < 7.8 \times 10^{-4}$) but did not reach the conventional GWAS significance level for association with CRC risk in the combined analysis of all samples (OR = 1.14, $P = 2.29 \times 10^{-7}$). The association between CRC risk and each of these three SNPs was consistent across most studies (**Fig. 1**). Results for the other four SNPs that replicated in stage 2 at $P < 0.05$ (rs1665650, rs2850966, rs1580743 and rs4503064) are also presented (**Supplementary Table 4**), including one SNP (rs1665650) with an association *P* value of $8.58 \times 10^{-7}$ in the combined analysis of all data from both stages (**Table 1**).

We next evaluated these top four SNPs (**Table 1**) using data from GWAS in the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) and the Colon Cancer Family Registry (CCFR), which together include 11,870 cases and 14,190 controls of European ancestry[4,20,21]. Three of the four SNPs were replicated in the GECCO and CCFR sample, although the strength of the associations was weaker than in east Asians (**Table 2**). These results provide independent support of our findings in the east Asian population. Meta-analyses of data from both east Asian and European-ancestry populations provided strong evidence for associations of CRC risk with three SNPs, with *P* values all below the genome-wide significance threshold of $5 \times 10^{-8}$ (**Table 2**). The weaker associations observed in European-ancestry populations could be explained, in part, by differences in LD patterns at these loci for east

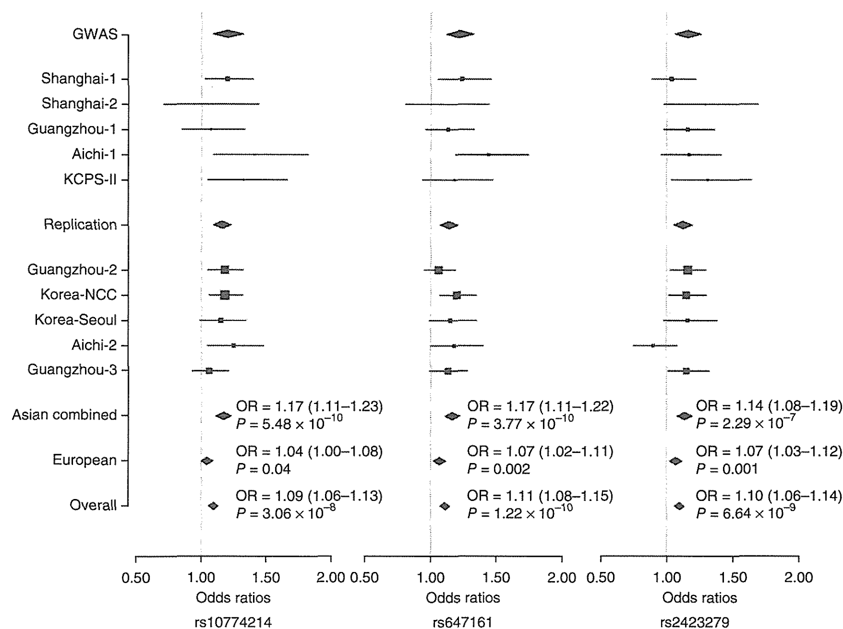


**Figure 1** Forest plots for the three SNPs showing evidence of an association with CRC risk. Per-allele ORs are presented, with the area of each box proportional to the inverse variance weight of the estimate. Horizontal lines represent 95% confidence intervals.

Asians and Europeans (**Supplementary Fig. 4**). It is possible that causal variants in these regions are tagged by different SNPs in these two populations or that there is allelic heterogeneity, in which different underlying causal variants exist in populations of Asian and European ancestry. The difference in LD structure between Asian and European descendants and possible allelic heterogeneity in these two populations might explain, in part, why these loci were not discovered in previous studies conducted in individuals of European ancestry. The fourth SNP evaluated in the GECCO and CCFR sample, rs1665650, however, was not replicated in individuals with European ancestry (OR = 0.96, $P = 0.05$).

Stratification analyses showed that the association of CRC risk with each of the three replicated SNPs was generally consistent in Chinese, Korean and Japanese individuals ($P_{het} > 0.05$), although the association with rs2423279 was not statistically significant in Japanese, perhaps owing to a small sample size (**Supplementary Table 5**). Associations of these three SNPs with CRC risk were similar for men and women ($P_{het} > 0.05$) (**Supplementary Table 6**).

The rs10774214 SNP is located just 15 kb upstream of *CCND2*, the gene encoding cyclin D2 (**Fig. 2a**), a member of the D-type cyclin family, which also includes cyclins D1 and D3. These cyclins have a critical role in cell cycle control (from G1 to S phase) through activation of cyclin-dependent kinases (CDKs), primarily CDK4 and CDK6 (ref. 22). *CCND2* is closely related to *CCND1*, a well-established human oncogene[22,23]. Although *CCND2* has been less well studied than *CCND1*, several studies, including The Cancer Genome Atlas (TCGA), have shown that *CCND2* is overexpressed in a substantial proportion of human colorectal tumors[22–25]. Overexpression of this cyclin may be an independent predictor of survival in individuals with CRC[24]. Several other genes, including *PARP11*, *FGF23*, *FGF6*, *C12orf5* and *RAD51AP1*, are also in close proximity to the SNP identified in our study, of which both *C12orf5* (also known as *TIGAR*, encoding TP53-induced glycolysis and apoptosis regulator) and *RAD51AP1* were found to be overexpressed in CRC tissue included in TCGA[25]. rs10774214 is in strong LD with several SNPs that are located in potential transcription factor–binding sites, as determined using the TRANSFAC database[26]. Additional research may be warranted regarding possible mechanisms by which this SNP is related to CRC risk.

The rs647161 SNP is located on chromosome 5q31.1, where a cluster of SNPs were associated with CRC risk (**Fig. 2b**). Of the genes in this region (including *PITX1*, *CATSPER3*, *PCBD2*, *MIR4461* and *H2AFY*), *PITX1* is the closest to rs647161 (approximately 129 kb upstream). The *PITX1* gene (encoding paired-like homeodomain 1) has been described as a tumor suppressor gene and may be involved in the tumorigenesis of multiple human cancers[27–31], including CRC[27,32]. *PITX1* has been reported to suppress tumorigenicity by downregulating the RAS pathway, which is frequently altered in colorectal tumors[27]. Inhibition of *PITX1* induces the RAS pathway and tumorigenicity, and restoring *PITX1* in colon cancer cells inhibits tumorigenicity[27]. It also has been reported that *PITX1* may activate *TP53* (ref. 33) and regulate telomerase activity[34]. Consistent with its possible function as a tumor suppressor gene, *PITX1* has been found to be downregulated in human cancer tissue samples and cell lines[27–30,32]. CRC tissue expressing wild-type KRAS showed significantly lower expression of *PITX1* than tissue with mutant KRAS[32]. Most recently, low *PITX1* expression was found to be associated with poor survival in individuals with CRC[35]. In addition, rs6596201, which is in moderate LD with rs647161 ($r^2 = 0.25$), is an expression quantitative trait locus (eQTL) ($P = 2.42 \times 10^{-28}$) for the *PITX1* gene[36]. Several other genes at this locus, including *C5orf24*, *H2AFY* and *NEUROG1*, were also found to be highly expressed in colorectal tumors included in TCGA ($P < 0.001$)[25]. Additional studies are warranted to explore a possible role for these genes in the etiology of CRC.

**Table 2 Association of CRC risk with the top three risk variants in European descendants and east Asian and European descendants combined**

| | | MAF[b] | | European-ancestry populations[c] | | | | East Asian and European-ancestry populations combined[c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | Alleles[a] | Cases | Controls | Cases | Controls | OR (95% CI) | $P_{meta}$ | Cases | Controls | OR (95% CI) | $P_{meta}$ |
| rs10774214 | T/C | 0.385 | 0.379 | 11,870 | 14,190 | 1.04 (1.00–1.09) | 0.040 | 19,165 | 25,736 | 1.09 (1.06–1.13) | $3.06 \times 10^{-8}$ |
| rs647161 | A/C | 0.680 | 0.667 | 11,870 | 14,190 | 1.07 (1.02–1.11) | 0.002 | 19,185 | 25,754 | 1.11 (1.08–1.15) | $1.22 \times 10^{-10}$ |
| rs2423279 | C/T | 0.263 | 0.252 | 11,870 | 14,190 | 1.07 (1.03–1.12) | 0.001 | 19,195 | 25,750 | 1.10 (1.06–1.14) | $6.64 \times 10^{-9}$ |

[a]Alelles (minor/major) for east Asians. [b]MAF in European-ancestry populations. [c]Summary statistics were generated using inverse variance–weighted fixed-effects meta-analysis.

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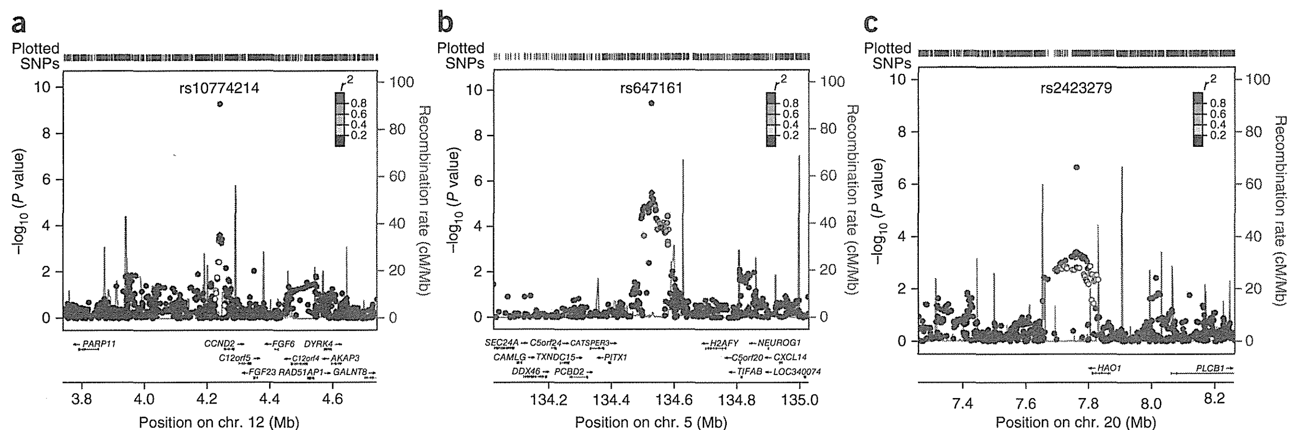


**Figure 2** Regional plots of association results and recombination rates for the three SNPs showing evidence of association with CRC risk. Genotyped and imputed data from GWAS samples are plotted on the basis of their chromosomal position in NCBI Human Genome Build 36.3. For each region, the SNP selected for stage 2 replication is denoted with a diamond, and the *P* value from the combined analysis of stage 1 and 2 data is provided. (**a–c**) Data are shown for rs10774214 (**a**), rs647161 (**b**) and rs2423279 (**c**).

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The rs2423279 SNP is located on chromosome 20p12.3, close to the *HAO1* and *PLCB1* genes (**Fig. 2c**). *HAO1* encodes hydroxyacid oxidase, which oxidizes 2-hydroxyacid. *PLCB1* encodes phospholipase C-β1, which has an important role in the intracellular transduction of many extracellular signals. Overexpression of the *PLCB1* gene has been observed in CRC tissue[25]. Possible mechanisms by which these genes are involved in CRC carcinogenesis are unknown. The rs2423279 SNP is 1,408,069 bp downstream of rs961253, a SNP previously identified in a European GWAS as being associated with CRC risk[10]. However, these two SNPs are not correlated in east Asians ($r^2 = 0$) or in Europeans ($r^2 = 0$). Adjustment for rs961253 did not change the results for rs2423279 (data not shown).

To our knowledge, this is the largest GWAS performed for CRC in east Asians, a population that differs from populations of European ancestry in CRC risk and certain aspects of genetic architecture. Results from our study, along with data from a large study conducted in a population of European ancestry, provide convincing evidence of associations with CRC risk for three new independent susceptibility loci at 5q31.1, 12p13.32 and 20p12.3. Results from this study provide new insights into the genetics and biology of CRC.

**URLs.** Cancer Genetic Markers of Susceptibility (CGEMS), http://cgems.cancer.gov/; Database of Genotypes and Phenotypes (dbGaP), http://www.ncbi.nlm.nih.gov/gap; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm; eqtl.uchicago.edu, http://eqtl.uchicago.edu/Home.html; GTEx eQTL Browser, http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi; Haploview, http://www.broad.mit.edu/mpg/haploview/; HapMap Project, http://hapmap.ncbi.nlm.nih.gov/; IntOGen, http://www.intogen.org/home; LocusZoom, http://csg.sph.umich.edu/locuszoom/; MaCH 1.0, http://www.sph.umich.edu/csg/abecasis/MACH/; mach2dat, http://genome.sph.umich.edu/wiki/Mach2dat:_Association_with_MACH_output; METAL, http://www.sph.umich.edu/csg/abecasis/Metal/; PLINK version 1.07, http://pngu.mgh.harvard.edu/~purcell/plink/; R version 2.13.0, http://www.r-project.org/; SAS version 9.2, http://www.sas.com/; SNAP, http://www.broadinstitute.org/mpg/snap/; TRANSFAC, http://www.gene-regulation.com/pub/databases.html; UCSC Genome Browser, http://genome.ucsc.edu/; WHI investigators, https://cleo.whi.org/researchers/SitePages/Write%20a%20Paper.aspx.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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University of Washington, Seattle, Washington, USA), Graham Casey (Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA), Andrew T. Chan (Division of Gastroenterology, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts, USA, and Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA), Jenny Chang-Claude (Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany), Stephen J. Chanock (Division of Cancer Epidemiology and Genetics, National Cancer Institute, US NIH, Bethesda, Maryland, USA), Lin S. Chen (Department of Health Studies, University of Chicago, Chicago, Illinois, USA), Gerhard A. Coetzee (Keck School of Medicine, University of Southern California, Los Angeles, California, USA), Simon G. Coetzee (Keck School of Medicine, University of Southern California, Los Angeles, California, USA), David V. Conti (Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA), Keith Curtis (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), David Duggan (Translational Genomics Research Institute, Phoenix, Arizona, USA), Todd L. Edwards (Division of Epidemiology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA), Charles S. Fuchs (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA, and Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA), Steven Gallinger (Department of Surgery, Mount Sinai Hospital, Toronto, Ontario, Canada, and Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada), Edward L. Giovannucci (Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA, and Departments of Epidemiology and Nutrition, Harvard School of Public Health, Boston, Massachusetts, USA), Stephanie M. Gogarten (School of Public Health, University of Washington, Seattle, Washington, USA), Stephen B. Gruber (Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California, USA), Robert W. Haile (Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA), Tabitha A. Harrison (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Richard B. Hayes (Division of Epidemiology, Department of Environmental Medicine, New York University School of Medicine, New York, New York, USA), Michael Hoffmeister (Division of Clinical Epidemiology and Aging Research, German Cancer Research Center, Heidelberg, Germany), John L. Hopper (Melbourne School of Population Health, The University of Melbourne, Melbourne, Victoria, Australia), Li Hsu (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, and Department of Biostatistics, University of Washington, Seattle, Washington, USA), Thomas J. Hudson (Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, and Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada), David J. Hunter (Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA), Carolyn M. Hutter (Division of Cancer Control and Population Sciences, National Cancer Institute, US NIH, Bethesda, Maryland, USA), Rebecca D. Jackson (Division of Endocrinology, Diabetes, and Metabolism, Ohio State University, Columbus, Ohio, USA), Mark A. Jenkins (Melbourne School of Population Health, The University of Melbourne, Melbourne, Victoria, Australia), Shuo Jiao (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Charles Kooperberg (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Sébastien Küry (Service de Génétique Médicale, CHU Nantes, Nantes, France), Andrea Z. LaCroix (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Cathy C. Laurie (Department of Biostatistics, University of Washington, Seattle, Washington, USA), Cecelia A. Laurie (Department of Biostatistics, University of Washington, Seattle, Washington, USA), Loic Le Marchand (Cancer Epidemiology Program, University of Hawaii Cancer Center, Honolulu, Hawaii, USA), Mathieu Lemire (Ontario Institute for Cancer Research, Toronto, Ontario, Canada), David Levine (School of Public Health, University of Washington, Seattle, Washington, USA), Noralane M. Lindor (Department of Health Sciences Research, Mayo Clinic, Scottsdale, Arizona, USA), Yan Liu (Stephens and Associates, Carrollton, Texas, USA), Jing Ma (Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA), Karen W. Makar (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Polly A. Newcomb (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, and Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA), Ulrike Peters (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, and Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA), John D. Potter (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA, and Centre for Public Health Research, Massey University, Palmerston North, New Zealand), Ross L. Prentice (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Conghui Qu (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), Thomas Rohan (Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York, USA), Robert E. Schoen (Department of Medicine and Epidemiology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA), Fredrick R. Schumacher (Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA), Daniela Seminara (Division of Cancer Control and Population Sciences, National Cancer Institute, US NIH, Bethesda, Maryland, USA), Martha L. Slattery (Department of Internal Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah, USA), Darin Taverna (Translational Genomics Research Institute, Phoenix, Arizona, USA), Stephen N. Thibodeau (Department of Laboratory Medicine, Mayo Clinic, Rochester, Minnesota, USA, and Department of Pathology and Laboratory Genetics, Mayo Clinic, Rochester, Minnesota, USA), Cornelia M. Ulrich (Division of Preventive Oncology, German Cancer Research Center, Heidelberg, Germany, Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, and Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA), Raakhee Vijayaraghavan (Genetic Basis of Human Disease Division, Translational Genomics Research Institute, Phoenix, Arizona, USA), Bruce Weir (Department of Biostatistics, University of Washington, Seattle, Washington, USA), Emily White (Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, and Department of Epidemiology, University of Washington School of Public Health, Seattle, Washington, USA) and Brent W. 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## AUTHOR CONTRIBUTIONS

W.Z. conceived and directed ACCC as well as the Shanghai-Vanderbilt Colorectal Cancer Genetics Project. W.-H.J., Y.-X.Z., K.M., A.S., Y.-B.X., S.H.J., D.-H.K., U.P.

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and G.C. directed CRC projects at Guangzhou, Aichi, Korea-NCC, Shanghai, KCPS-II, Korea-Seoul, GECCO and CCFR, respectively. B.Z., Q.C. and W.W. coordinated the project. Q.C. directed laboratory operations. J.S. performed genotyping experiments. B.Z., J.L. and W.W. performed statistical analyses. W.Z. wrote the manuscript with substantial contributions from B.Z., Q.C., J.L., X.-O.S. and R.J.D. Z.R., G.Y., B.-T.J., Z.-Z.P., F.M., Y.-T.G., J.H.O., Y.-O.A., E.J.P., H.-L.L., J.W.P., J.J., J.-Y.J. and S.H. contributed to data and biological sample collection in the original studies included in ACCC and contributed to manuscript revision. Members of GECCO and CCFR contributed to data and biological sample collection in studies included in these consortia.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Study populations.** After quality control filtering, 7,456 cases and 11,671 controls from 10 studies were included in the consortium (**Supplementary Table 2**). Detailed descriptions of participating studies and demographic characteristics of study participants are provided in the **Supplementary Note**. Briefly, the consortium included 10,730 Chinese participants, 5,544 Korean participants and 2,853 Japanese participants. Chinese participants were from five studies: the Shanghai Study 1 (Shanghai-1, $n$ = 3,102), the Shanghai Study 2 (Shanghai-2, $n$ = 485), the Guangzhou Study 1 (Guangzhou-1, $n$ = 1,613), the Guangzhou Study 2 (Guangzhou-2, $n$ = 2,892) and the Guangzhou Study 3 (Guangzhou-3, $n$ = 2,638). Korean participants were from three studies: the Korean Cancer Prevention Study-II (KCPS-II, $n$ = 1,301), the Seoul Study ($n$ = 1,522) and the Korea–National Cancer Center (Korea-NCC) Study ($n$ = 2,721). Japanese participants were from two studies: the Aichi Study 1 (Aichi-1, $n$ = 1,346) and the Aichi Study 2 (Aichi-2, $n$ = 1,507). We also evaluated associations for the top 4 SNPs using data from 11,870 CRC cases and 14,190 controls of European ancestry included in GECCO and CCFR, which included 14 studies from the United States, Europe, Canada and Australia[4,20,21]. Approval was granted from the relevant institutional review boards at all study sites, and all included participants gave informed consent.

**Genotyping and quality control procedures.** Detailed descriptions of genotyping and quality control procedures as well as design of plates and control samples are given in the **Supplementary Note**. Briefly, in stage 1, 481 cases and 2,632 controls from Shanghai-1 were genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 as described previously[14]. The average concordance percentage of quality control samples was 99.7%, with a median value of 100% in Shanghai-1 (refs. 14,37,38). Stage 1 genotyping for 296 cases and 257 controls in Shanghai-2 was performed using Illumina HumanOmniExpress BeadChips. The same method was used to genotype cases from the Guangzhou-1 ($n$= 694) and Aichi-1 ($n$ = 497) studies in stage 1. The positive quality control samples in these studies had an average concordance percentage of 99.41% and a median value of 99.97%. Cases and controls in KCPS-II were genotyped using the Affymetrix Genome-Wide Human SNP Array 5.0 (ref. 16). Controls for the Guangzhou-1 and Aichi-1 studies were genotyped previously using the Illumina Human610-Quad BeadChip[15] and Illumina Infinium HumanHap610 BeadChip[17] platforms, respectively. Details of quality control procedures for these samples have been described previously[15–17]. We excluded from the analysis samples that were genetically identical or duplicated, had a genotype-determined sex that was inconsistent with self-reported data, had unclear population structure, had close relatives with a PI-HAT estimate greater than 0.25 or had a call rate of <95%. Within each study, SNPs were excluded if (i) MAF was <5%, (ii) the call rate was <95%; (iii) the genotyping concordance percentage was <95% in quality control samples; (iv) the $P$ value for Hardy-Weinberg equilibrium was $<1.0 \times 10^{-5}$ in controls; or (v) SNPs were not on the 22 autosomes. The final numbers of cases, controls and SNPs remaining for analysis in each participating study are presented in **Supplementary Table 1**.

Genotyping for stage 2 was completed using the iPLEX Sequenom MassARRAY platform as described previously[14,39]. With the exception of samples from the Guangzhou-3 study, which were genotyped at Fudan University (Shanghai), all other samples were genotyped at the Vanderbilt Molecular Epidemiology Laboratory. The average concordance percentage of the genotyping data for positive control samples was >99% with a median value of 100% for each of the five studies. SNPs were excluded from the analysis if (i) the call rate was <95%, (ii) the genotyping concordance percentage was <95% in control samples, (iii) there was an unclear genotype call or (iv) the $P$ value for Hardy-Weinberg equilibrium was $<7.8 \times 10^{-4}$. The numbers of SNPs remaining for analysis in each participating study in stage 2 are presented in the **Supplementary Note**.

Genotyping for samples included in the GECCO and CCFR GWAS was conducted using Illumina BeadChip arrays, with the exception of the Ontario Familial Colorectal Cancer Registry study, for which Affymetrix arrays were used[4,20,21]. Details of the quality control procedures for these samples are presented in the **Supplementary Note**.

**SNP selection for replication.** SNPs were selected for stage 2 replication if (i) data were available in each of the five stage 1 studies; (ii) MAF was >5% in each stage 1 study; (iii) no heterogeneity was detected across the five studies included in stage 1 ($P_{het}$ > 0.05 and $I^2$ < 25%); (iv) there was no LD ($r^2$ < 0.2) with any known risk variant reported from previous GWAS; (v) there was no LD ($r^2$ < 0.2) with the other SNPs identified in this study; (vi) there was high imputation quality in each of the five studies (RSQ > 0.5); and (vii) $P$ < 0.01 in combined analysis of all stage 1 studies.

**Evaluation of population structure.** We evaluated population structure in each of the five participating studies included in stage 1 by using principal-components analysis (PCA). Genotyping data for uncorrelated genome-wide SNPs were pooled with data from HapMap to generate the first ten principal components using EIGENSTRAT software[40] (see URLs). The first two principal components for each sample were plotted using R (see URLs). We identified and excluded one participant of KCPS-II who was more than 6 s.d. away from the means of principal components 1 and 2 (**Supplementary Fig. 1**). The remaining 7,847 samples showed clear east Asian origin, and these samples were included in the final genome-wide association analysis. Cases and controls in each of the five studies were in the same cluster as HapMap Asian samples. The estimated inflation factor $\lambda$ ranged from 1.02 to 1.04 in these studies after adjusting for age, sex and the first ten principal components, with a $\lambda$ of 1.01 for combined stage 1 data (**Supplementary Fig. 2** and **Supplementary Table 1**).

**Imputation.** We used the MaCH 1.0 program[18] (see URLs) to impute genotypes for autosomal SNPs that were present in HapMap Phase 2 release 22 separately for each of the five studies included in stage 1. Genotype data from the 90 Asian subjects from HapMap were used as the reference. For Guangzhou-1 and Aichi-1, cases and controls were genotyped using different platforms. To improve imputation quality[41], we identified SNPs for which data were available in both cases and controls (250,612 SNPs in Guangzhou-1 and 232,426 SNPs in Aichi-1) and used them to impute genotyping data. A total of 1,636,380 genotyped SNPs or imputed SNPs with high imputation quality (RSQ > 0.50) in all five studies were tested for association with CRC. To directly evaluate the imputation quality for the top four SNPs identified in our study, we genotyped them in approximately 2,500 samples included in stage 1. The agreement of genotype calls derived from direct genotyping and imputation was very high, with mean concordance rates of 98.05%, 95.61%, 99.84% and 97.90% for rs647161, rs10774214, rs2423279 and rs1665650, respectively (**Supplementary Table 7**).

**Statistical analyses.** Dosage data for genotyped and imputed SNPs for participants in each stage 1 study were analyzed using the program mach2dat[18] (see URLs). We coded 0, 1 or 2 copies of the effect allele as the dosage for genotyped SNPs, and, for imputed SNPs, we used the expected number of copies of the effect allele as the dosage score. This approach has been shown to give unbiased estimates in meta-analyses[42]. Associations between SNPs and CRC risk were assessed using ORs and 95% CIs derived from logistic regression models. ORs were estimated on the basis of the log-additive model and adjusted for age, sex and the first ten principal components. PLINK version 1.07 (see URLs) also was used to analyze genotype data[43] and yielded results virtually identical to those derived from dosage data using mach2dat[18]. Meta-analyses were performed using the inverse-variance method, assuming a fixed-effects model, and calculations were implemented in the METAL package[19] (see URLs).

Similar to stage 1, we used logistic regression models to derive ORs and 95% CIs for the 64 selected SNPs in stage 2, assuming a log-additive model with adjustment for age and sex. We performed joint analyses to generate summary results for combined samples from all studies, with additional adjustment for study site. We also conducted stratification analysis for the top four SNPs by population ancestry (Chinese, Korean or Japanese) and by sex. We used Cochran's $Q$ statistic to test for heterogeneity[44] and the $I^2$ statistic to quantify heterogeneity[45] across studies as described elsewhere in detail[46]. Analyses for stage 2, as well as combined stage 1 and 2 data, were conducted using SAS, version 9.2 (see URLs), with the use of two-tailed tests. $P$ values of $<5\times10^{-8}$ in the combined analysis was considered statistically significant.

We used Haploview version 4.2 (see URLs; ref. 47) to generate a genome-wide Manhattan plot for results from the stage 1 meta-analysis. Forest plots

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and quantile-quantile plots were drawn using R. We drew regional association plots using the website-based tool LocusZoom, version 1.1 (see URLs; ref. 48). LD plots were generated using Haploview[47] and the UCSC Genome Browser (see URLs).


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# Evaluation of *SLC20A2* mutations that cause idiopathic basal ganglia calcification in Japan

Megumi Yamada, Masaki Tanaka, Mari Takagi, et al.

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# Evaluation of *SLC20A2* mutations that cause idiopathic basal ganglia calcification in Japan

Megumi Yamada, MD*
Masaki Tanaka, MD*
Mari Takagi, BA
Seiju Kobayashi, MD, PhD
Yoshiharu Taguchi, MD, PhD
Shutaro Takashima, MD, PhD
Kortaro Tanaka, MD, PhD
Tetsuo Touge, MD, PhD
Hiroyuki Hatsuta, MD
Shigeo Murayama, MD, PhD
Yuichi Hayashi, MD, PhD
Masayuki Kaneko, PhD
Hiroyuki Ishiura, MD, PhD
Jun Mitsui, MD, PhD
Naoki Atsuta, MD, PhD
Gen Sobue, MD, PhD
Nobuyuki Shimozawa, MD, PhD
Takashi Inuzuka, MD, PhD
Shoji Tsuji, MD, PhD
Isao Hozumi, MD, PhD

Correspondence to Dr. Hozumi: hozumi@gifu-pu.ac.jp

Supplemental data at www.neurology.org

## ABSTRACT

**Objective:** To investigate the clinical, genetic, and neuroradiologic presentations of idiopathic basal ganglia calcification (IBGC) in a nationwide study in Japan.

**Methods:** We documented clinical and neuroimaging data of a total of 69 subjects including 23 subjects from 10 families and 46 subjects in sporadic cases of IBGC in Japan. Mutational analysis of *SLC20A2* was performed.

**Results:** Six new mutations in *SLC20A2* were found in patients with IBGC: 4 missense mutations, 1 nonsense mutation, and 1 frameshift mutation. Four of them were familial cases and 2 were sporadic cases in our survey. The frequency of families with mutations in *SLC20A2* in Japan was 50%, which was as high as in a previous report on other regions. The clinical features varied widely among the patients with *SLC20A2* mutations. However, 2 distinct families have the same mutation of S637R in *SLC20A2* and they have similar characteristics in the clinical course, symptoms, neurologic findings, and neuroimaging. In our study, all the patients with *SLC20A2* mutations showed calcification. In familial cases, there were symptomatic and asymptomatic patients in the same family.

**Conclusion:** *SLC20A2* mutations are a major cause of familial IBGC in Japan. The members in the families with the same mutation had similar patterns of calcification in the brain and the affected members showed similar clinical manifestations. ***Neurology*® 2014;82:705–712**

## GLOSSARY

**DNTC** = diffuse neurofibrillary tangles with calcification; **FIBGC** = familial idiopathic basal ganglia calcification; **IBGC** = idiopathic basal ganglia calcification; **MMSE** = Mini-Mental State Examination; **PDGF** = platelet-derived growth factor; **PDGFRB** = platelet-derived growth factor receptor-β; **Pi** = inorganic phosphate; **PiB** = Pittsburgh compound B; **PiT** = type III sodium-dependent phosphate transporter; **PKC** = paroxysmal kinesigenic choreoathetosis.

Idiopathic basal ganglia calcification (IBGC), also known as Fahr disease, is thought to be a rare neuropsychiatric disorder characterized by symmetrical calcification in the basal ganglia and other brain regions. Clinical manifestations range widely from asymptomatic to variable symptoms including headaches, psychosis, and dementia.[1] The diagnosis of IBGC generally relies on the visualization of bilateral calcification mainly in the basal ganglia by neuroimaging and the absence of metabolic, infectious, toxic, or traumatic causes.[2,3]

The mode of inheritance of familial IBGC (FIBGC) has been thought to be autosomal dominant and, to date, 4 responsible chromosomal regions have been identified, namely 14q (IBGC1), 2q37 (IBGC2), 8p11.21 (IBGC3), and 5q32 (IBGC4).[3–14] The causative gene at the IBGC3 locus was identified as *SLC20A2* encoding type III sodium-dependent phosphate transporter 2 (PiT-2). Screening of a large series of patients with IBGC revealed that mutations in *SLC20A2* are a major cause of FIBGC[10]; moreover, other mutations in *SLC20A2* have recently been reported in China and Brazil.[11–13] The mutations of *PDGFRB* encoding platelet-derived growth factor

*These authors contributed equally to this work.

From the Laboratory of Medical Therapeutics and Molecular Therapeutics (Y.M., M. Takagi, Y.H., M.K., I.H.), Gifu Pharmaceutical University, Gifu; Department of Neurology (M. Tanaka, H. I., J.M., S. Tsuji), The University of Tokyo; Department of Neurology (N.A., G.S.), Nagoya University; Department of Neuropsychiatry (S.K.), Sapporo Medical University, Sapporo; Department of Neurology (Y.Y., S. Takashima, K.T.), Toyama University Hospital, Toyama; Department of Neurology (T.T.), Kagawa University Hospital, Kagawa; Department of Neurology (H.H, S.M.), Tokyo Metropolitan Institute of Gerontology, Tokyo; Department of Neurology and Geriatrics (M.Y., Y.H., T.I.), and Division of Genomic Research, Life Science Research Center (N.S.), Gifu University, Gifu, Japan.

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(PDGF) receptor-β (PDGFRB) and *PDGFB* have recently been reported to cause calcification in the brain.[14,15]

We have collected clinical information of patients with IBGC in a nationwide survey in Japan. Here, on the basis of a mutational analysis of *SLC20A2*, we aim to establish the molecular epidemiology of IBGC3 and evaluate clinically and genetically *SLC20A2* mutations in Japan.

**METHODS Subjects and samples.** We collected clinical information on patients with IBGC in a nationwide study. The criteria for the selection of patients in the initial survey were as follows: 1) conspicuous calcification is observed in the basal ganglia and/or dentate nucleus by CT scan; 2) calcification is bilateral and symmetrical; and 3) idiopathic (absence of biochemical abnormalities, and an infectious, toxic, or traumatic cause).[2,3] Neurologists enrolled patients in the survey. They examined the medical charts and performed the neurologic examinations again if necessary. The survey was approved by the Ethics Committee of the Gifu University Graduate School of Medicine. During the survey, some patients were found to have hypoparathyroidism, Aicardi-Goutières syndrome, and Cockayne syndrome, and these patients were excluded. For the genetic study, a total of 69 subjects from 41 hospitals provided written informed consent and were enrolled in the project. Of these patients, 46 came from families with a single affected member, and the other 23 came from 10 families with multiple affected members. We defined the former as sporadic patients and the latter as familial patients. The patients' mean age ± SD was 41.3 ± 23.6 years at registration. The patients comprised 32 males and 37 females.

**Standard protocol approvals, registrations, and patient consents.** All experiments on human DNA were approved by the Ethics Committees of both Gifu University and the University of Tokyo. After written informed consent was obtained, peripheral blood samples were collected.

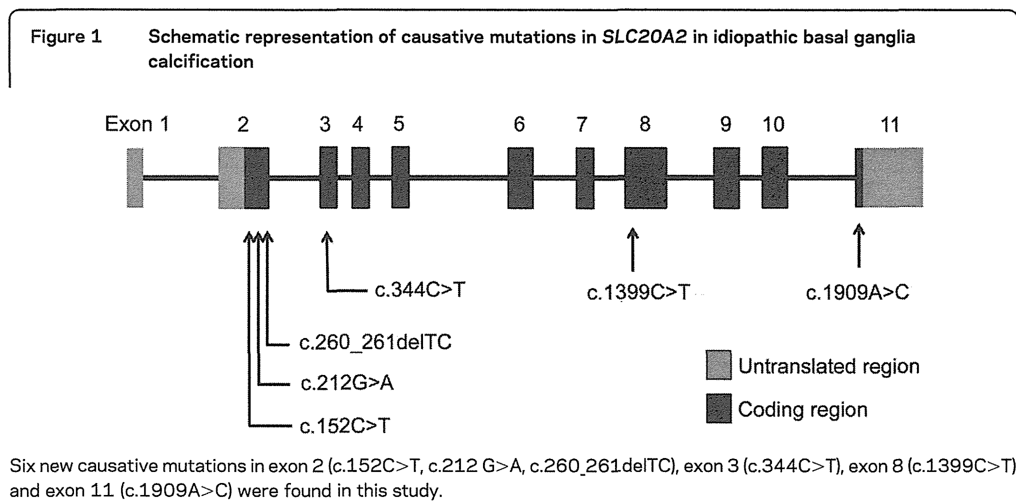
**Mutational analysis.** Genomic DNA was extracted from the whole blood samples. *SLC20A2* analysis was performed by Sanger sequencing of all coding regions, as described in detail in e-Methods and table e-1, A and B, on the *Neurology*® Web site at www.neurology.org. The pathologic potential of the identified variants was predicted using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph/).[16]

**RESULTS Mutational analysis.** We screened a total of 69 subjects including 23 subjects from 10 families in which multiple affected subjects were observed and 46 subjects in sporadic cases, all of whom were Japanese. Six new mutations in *SLC20A2* were found: 4 missense mutations, 1 nonsense mutation, and 1 frameshift mutation (figure 1). Electropherograms showed the individual heterozygous mutations (figure e-1). None of them were present in an in-house exome sequencing data set (358 Japanese control subjects), dbSNP 137 (www.ncbi.nlm.nih.gov/snp/), or the National Heart, Lung, and Blood Institute "Grand Opportunity" Exome Sequencing Project (ESP6500SI-V2). In silico analysis predicted deleterious consequences, as determined from the residue changes in figures 1 and e-1. When confined to the FIBGC patients, 5 of the 10 families (50.0%) showed mutations in *SLC20A2*. In contrast, 2 of the 46 patients (4.3%) with sporadic IBGC carried mutations in *SLC20A2* in this study.

**Clinical manifestations.** The clinical manifestations are summarized in table 1. A positive family history of IBGC was present in 5 families. Families 1 and 2 had the same mutation.

**Familial cases.** ***Case 1 (in family 1).*** The proband in family 1 was a 64-year-old woman who had dysarthria and gait disturbance for 5 years. She showed no dementia. Her neurologic examination revealed dysarthria, small steppage gait, rigidity at bilateral wrist joints, bradykinesia, and a pyramidal sign. Her CT images revealed severe calcification at the bilateral globus pallidus, caudate nuclei, thalamus, subcortical white matter, and dentate nuclei (figure 2C). Her son's CT showed similar brain calcification (figure 2D), although he was clinically asymptomatic. His DNA study revealed the

**Figure 1** **Schematic representation of causative mutations in *SLC20A2* in idiopathic basal ganglia calcification**




Six new causative mutations in exon 2 (c.152C>T, c.212 G>A, c.260_261delTC), exon 3 (c.344C>T), exon 8 (c.1399C>T), and exon 11 (c.1909A>C) were found in this study.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| Table 1 | Clinical features of 6 individuals (probands) with *SLC20A2* mutations | | | | | | |
|---|---|---|---|---|---|---|---|
| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 |
| Mutation | c.1909A>C | c.1909A>C | c.344C>T | c.212G>A | c.1399C>T | c.152C>T | c.260_261delTC |
| | S637R | S637R | T115M | R71H | R467X | A51V | L87Hfs*6 |
| Zygosity | Hetero | Hetero | Hetero | Hetero | Hetero | Hetero | Hetero |
| Exon | 11 | 11 | 3 | 2 | 8 | 2 | 2 |
| Proband information | | | | | | | |
| Age at detection of calcification, y | 60 | 51 | 60 | 73 | 23 | 71 | 74 |
| Age at onset, y | 58 | 50 | 60 | 71 | 15 | 71 | 57 |
| Onset symptom | Dysarthria | Dysarthria | Dementia | Parkinsonism | PKC | Dementia | Athetosis |
| Neurologic findings | | | | | | | |
| Cognitive impairment (MMSE) | 27 | 24 | 20 | 16 | 30 | 22 | 22 |
| Pyramidal sign | + | + | – | – | – | – | – |
| Extrapyramidal sign | + | + | – | + | – | – | + |
| Family information (except the proband) | | | | | | | |
| No. of other individuals with calcification | 1 | 2 | 5 | 1 | 1 | 0[a] | 0[a] |
| No. of other individuals with confirmed mutations | 1 | NE | 5 | NE | 1 | NA | NA |
| No. of other symptomatic individuals | 0 | 0 | 2 | 0 | 0 | NA | NA |
| Other symptoms (no.) in the family | — | — | Mental disorder (1), alcoholism (1) | — | — | NA | NA |

Abbreviations: MMSE = Mini-Mental State Examination; NA = not applicable; NE = not examined; PKC = paroxysmal kinesigenic choreoathetosis.
[a] Because there was no other family member who had any neurologic symptoms, brain CT screening of other family members was not performed.

same mutation in exon 11 that had been found in his mother.

*Case 2 (in family 2).* The proband in family 2 was a 54-year-old woman who had dysarthria and gait disturbance for 4 years. She showed mild mental deterioration in Mini-Mental State Examination (MMSE) score of 24 points, frontal signs, dysarthria, mild parkinsonism (rigidity of bilateral wrist joints and bradykinesia), adiadochokinesis, spasticity, and small steppage gait. Her CT images revealed severe calcification at the bilateral globus pallidus, caudate nuclei, thalamus, subcortical white matter, and dentate nuclei (figure 2E). Although her son and cousin also showed calcification in CT images, they were asymptomatic. Her DNA analysis revealed the same mutation as that in family 1.

*Case 3 and other symptomatic individuals (in family 3).* The proband was a 69-year-old woman (II-1 in the pedigree in figure 3). She was admitted to a hospital at the age of 65 because of forgetfulness since the age of 60 years. Her MMSE score was 20, which indicated a possibility of dementia (MMSE score below 22). Decreased blood flow was detected in the bilateral basal ganglia and thalamus and the right frontal lobe in particular by SPECT. She had a positive family history of brain calcification, as shown in figure 3A. The initial clinical diagnosis had been diffuse neurofibrillary tangles with calcification (DNTC),[17] although to our knowledge familial cases of DNTC have not been reported. Her son had psychological disorders including violent behavior; unfortunately, no brain CT had yet been performed on him. In the patients in family 3, the degree of calcification was mild compared with that observed in the other families (figure 3, B–G). Her brother with calcification in the brain (II-7) had a mental disorder and another (II-8) presented with alcoholism. The 3 other relatives with calcification were asymptomatic (II-5, II-9, and III-3). The symptomatic patients (II-1, II-7, and II-8) showed more apparent brain atrophy than the others (figure 3, B, D, and E, respectively). The individuals with calcification on the CT images (II-1, II-5, II-7, II-8, II-9, and III-3) had the same mutation in exon 3 in *SLC20A2*. However, the individuals with no calcification (III-2, III-5, and IV-1) revealed no mutation in *SLC20A2*. In summary, 6 patients had calcification among the 10 individuals examined by CT scan in family 3 and all of them carrying the *SLC20A2* mutation exhibited similar calcification on CT images. However, persons without the mutation did not show calcification.

*Case 4 (in family 4).* Family 4 had a mutation in exon 2. The proband developed clumsiness of her hands and gait unsteadiness at the age of 71 years, and she was diagnosed as having Parkinson disease. Visual


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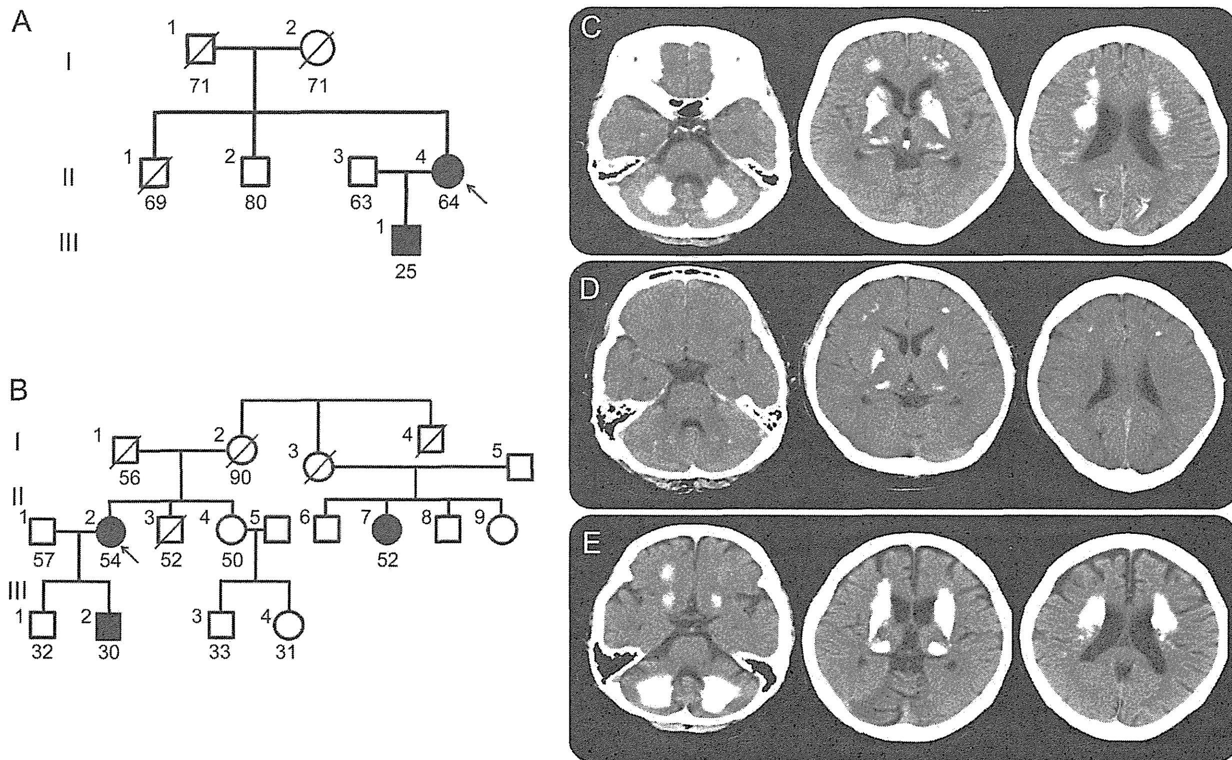
Figure 2 CT images and family trees of families 1 and 2




(A) Family tree of family 1. (B) Family tree of family 2. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. (C) CT images of proband (II-4 in pedigree of family 1, part A). (D) CT images of the proband's son (III-1 in pedigree of family 1, part A). (E) CT images of the proband (II-2 in pedigree of family 2, part B). All have mutation of S637R.

hallucinations started with the initiation of medication. She showed parkinsonism (rigidity, bradykinesia, and postural instability), which responded to levodopa. Her MMSE score was 16. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, and dentate nuclei, and her daughter, who was asymptomatic, also had intracranial calcification (figure e-2C). Brain CT was not performed in her other children. Her SPECT images showed decreased perfusion in the bilateral frontal, temporal, and parietal regions of the brain. She died of pneumonia at the age of 79. Neuropathologic examination revealed neuronal loss and Lewy bodies in the substantia nigra, locus ceruleus, amygdala, and parahippocampal gyrus indicative of Parkinson disease, and prominent deposition of calcium in the parenchyma and the wall of arteries in the globus pallidum and dentate nuclei compatible with the pathologic findings of IBGC.

*Case 5 (in family 5).* The proband was a 24-year-old man who had paroxysmal kinesigenic choreoathetosis (PKC). His laboratory data were normal except for CT findings. He presented with an attack of PKC after exercise and his symptom responded well to carbamazepine. His CT images revealed calcification at the globus pallidus, thalamus, subcortical white matter, and dentate nuclei (figure e-2B [A]). We had an opportunity to examine his parents, who had no symptoms or signs. Mutational analysis of *SLC20A2* of his parents with their informed consent revealed the same mutation in exon 8 in his mother as he had. Brain CT scan of his mother confirmed calcification at the globus pallidus, subcortical white matter, and dentate nuclei.

**Sporadic cases.** *Case 6.* The patient had a mutation in exon 2. She was a 72-year-old woman who noticed forgetfulness at the age of 71. She had no motor deficits. Her MMSE score was 22, and her score on the revised Hasegawa Dementia Scale was 24. Her Frontal Assessment Battery score at bedside was 5, indicating a frontal lobe deficit (cutoff score, 11/12). The index scores of the revised Wechsler Memory Scale were as follows: attention and concentration, 86; verbal memory, 89; general memory, 85; attention/concentration, 71; and delayed recall, 75. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, thalamus, subcortical and periventricular white matter, and dentate nuclei (figure e-2B [B]). Her SPECT images showed decreased perfusion in the left frontal,


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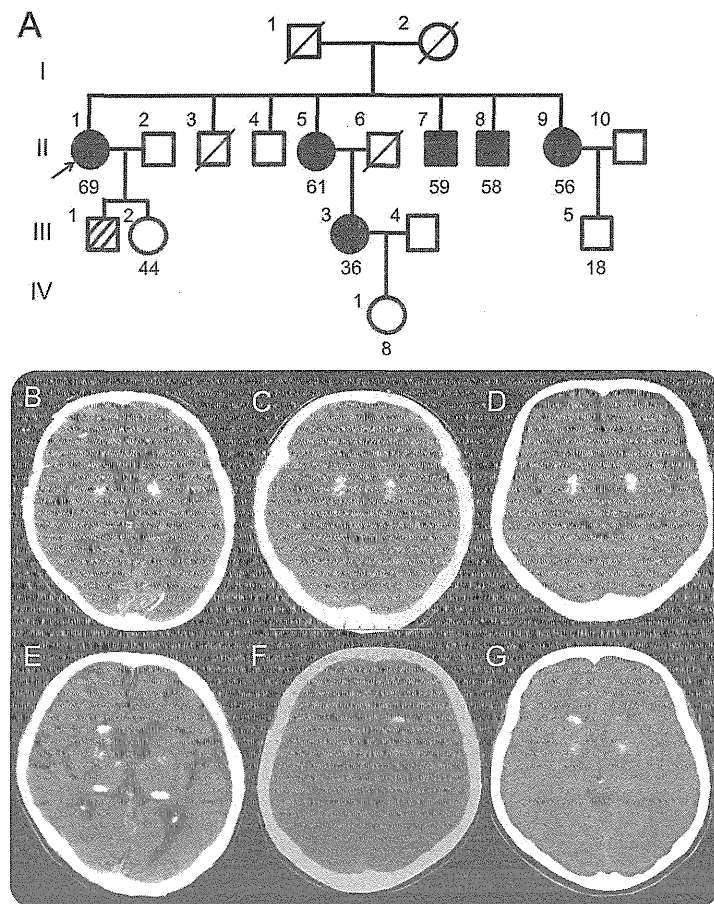
Figure 3 Pedigree and CT images of family 3




(A) Pedigree of family 3. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. The striped symbol represents a symptomatic patient, although his CT image and DNA sample were not available for the study. (B) CT image of the proband (II-1 in pedigree of family 3). (C) CT image of asymptomatic II-5. (D) CT image of symptomatic II-7. (E) CT image of symptomatic II-8. (F) CT image of asymptomatic II-9. (G) CT image of asymptomatic III-3. All have mutation of T115M.

temporal, and parietal regions of the cerebrum and bilateral cerebellum. [$^{11}$C] Pittsburgh compound B (PiB) retention was not observed by [$^{11}$C]PiB PET. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

*Case 7.* The patient was a 78-year-old man who had a frameshift in exon 2. Involuntary movement of the left thumb and index finger like "pill-rolling" began in his sixth decade. His family first noticed memory impairment at the age of 75. Gait disturbance appeared at the age of 77 and oral dyskinesia and left shoulder shrugging appeared at the age of 78. His scores on the MMSE and Frontal Assessment Battery were 22 and 10, respectively. His brain CT images showed calcification at the globus pallidus, thalamus, subcortical and periventricular white matter, and dentate nuclei (figure e-2B [C]). His SPECT images showed decreased perfusion in the bilateral (predominantly in the left) frontal and temporal regions of the cerebrum and bilateral cerebellum. [$^{11}$C]PiB retention was not observed by [$^{11}$C]PiB PET, which was performed at the age of 81. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

**DISCUSSION** We have obtained clinical information of 161 patients with brain calcification in a nationwide study. We discovered that 3 patients had hypoparathyroidism, Aicardi-Goutières syndrome, and Cockayne syndrome during the survey. CT images revealed varying degrees of calcification, from marked calcification in the basal ganglia to patchy calcification in various regions, suggesting diversity in the etiologies. Some patients were incidentally found to have calcification by CT performed for head injury caused by accidents. Because our previous survey revealed a considerable frequency (1%–2%) of patchy calcification in the CT images of all patients in 2 university hospitals in Japan,[18] more asymptomatic IBGC patients with patchy calcification may exist than the number that we had previously assumed to be present in the population in Japan. After the examination by neurologists, we collected 69 DNA samples from patients who met the criteria for IBGC.[2,3] Symptoms and neurologic findings varied widely from asymptomatic to variable symptoms including headaches, psychosis, and dementia.

In this study, we investigated mutations in *SLC20A2* in 69 patients with IBGC in Japan and identified 4 new mutations in 10 familial cases (the same mutation in 2 families) and 2 other new mutations in 46 sporadic cases. The frequency of families with mutations in *SLC20A2* was 50% (5 of the 10 families), and that of sporadic patients was 4.3% (2 of the 46 patients). The frequency of the mutations in *SLC20A2* in FIBGC in Japan was as high as in other countries in a previous report.[10] Case 5 indicates that it is difficult to reliably determine sporadic cases without brain CT scans and genetic studies of all members in the family.

The mutations in our study existed in exons 2, 3, 8, and 11. One of these mutations (R467X) in exon 8 resulted in a substitution to a TGA stop codon, and the other (c.260_261delTC) in exon 2 was a frameshift. None of the mutations were reported previously, indicating heterogeneities of the mutations in *SLC20A2*. Taken together with other reports, causative mutations identified in *SLC20A2* include 6 mutations in exon 2, 1 in exon 3, 3 in exon 4, 1 in exon 5, 1 in exon 7, 10 in exon 8, 2 in exon 9, 4 in exon 10, and 4 in exon 11.[9–12] It does not seem that there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are mutation hot spots in *SLC20A2*. The in silico analysis using PolyPhen-2 for the missense mutations predicted all to be likely damaging, as determined from the residue changes. We drew the structure model of the PiT-2 protein using the TOPO2 software (http://www.sacs.ucsf.edu/TOPO/top.html). The schematic structure of the PiT-2 protein with the mutations is shown in figure 4.

Although the clinical features varied widely among the families with IBGC with *SLC20A2* mutations, the patients in families 1 and 2 with the same *SLC20A2* mutation exhibited similar clinical manifestations including dysarthria, mild cognitive decline, pyramidal signs, and extrapyramidal signs as well as similar ages at detection of calcification and onset of symptoms. Of note, the CT images among the affected individuals in the 2 families are similar (figure 2). In family 3, in contrast, 3 symptomatic patients presented with dementia, psychological disorder, and alcoholism, accompanied with brain atrophy in CT images. None of them showed movement disorders such as those in families 1 and 2.

Although mutational analysis and CT scan were not performed in other familial members of cases 6 and 7, concordance of the presence of mutations of *SLC20A2* and brain calcification were confirmed in 15 individuals, and we did not observe any individuals who carried the mutation and did not show brain calcification. These observations strongly support a high penetrance of the *SLC20A2* mutations regarding brain calcification.

Correlations of genotypes and neurologic phenotypes, however, have been controversial. *SLC20A2* mutations in patients with FIBGC have been described to show variability in clinical manifestations among the families. In the present study, the 2 affected individuals in families 1 and 2, who carried the same mutation, exhibited quite similar neurologic manifestations and clinical courses, suggesting a genotype-phenotype correlation of the S637R mutation. Of note, 2 individuals aged 56 and 61 years in family 3 did not exhibit any neurologic manifestations despite carrying the mutation and having brain calcification, indicating that penetrance regarding the neurologic manifestations is incomplete.

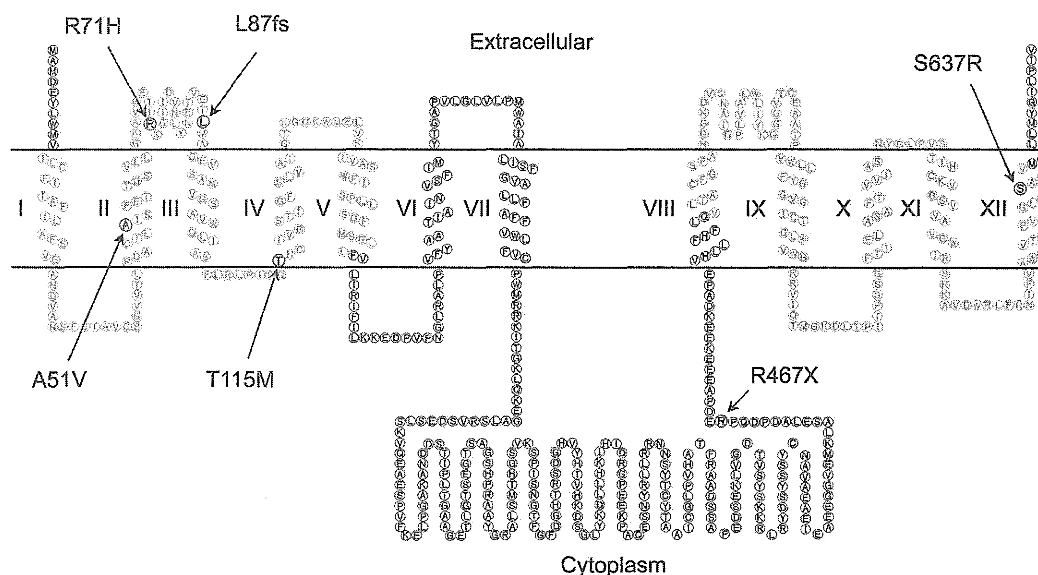
In case 4, interestingly, the proband showed pathologic findings of both IBGC and Parkinson disease. Because Parkinson disease is a common disorder in aged people, there remains a possibility that the presence of IBGC and Parkinson disease is coincidental.

Case 5 had a mutation that leads to a premature stop codon, making an incomplete structure of PiT-2. His neurologic symptom was PKC controllable by carbamazepine. Intriguingly, several patients with IBGC have been reported to present with PKC or paroxysmal nonkinesigenic dyskinesia.[19,20] For these cases of PKC or paroxysmal nonkinesigenic dyskinesia, mutational analyses of not only *SLC20A2* but also *PRRT2* and MRI will be indispensable.[21,22]

Herein, we have reported 5 cases of FIBGC and 2 cases of IBGC with *SLC20A2* mutations in Japan. We could not find any characteristic features of Japanese patients, although we had discovered that each case has a new mutation in *SLC20A2*, respectively.

The mechanisms of calcification and cell damage remain to be elucidated. Despite that the expression of PiT-2 encoded by *SLC20A2* is distributed widely in the human body,[23] mutations in *SLC20A2* cause

**Figure 4** **Schematic structure of PiT-2 (type III sodium-dependent phosphate transporter) with the mutations**





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calcification only in the brain. Mutations in *SLC34A2* have been reported to cause pulmonary alveolar microlithiasis.[24] Because Npt2b encoded by *SLC34A2* is the only phosphate transporter that is highly expressed in the lungs,[25] the mutations in *SLC34A2* are compatible with the lesion of the alveolar type II cells in the lungs.[24] Because the limitation of calcification to the brain cannot be explained by only the mutation in *SLC20A2* followed by abnormalities of inorganic phosphate (Pi) transport via PiT-2, there might be some other genes responsible for calcification in the brain, or the mutations in *SLC20A2* may take some toxic gain of function. The dysfunction of Pi transport can explain the accumulation of various metals in regions of the brain and the abnormal distribution of metals, which we observed in CSF[26] and hair in the patients with IBGC.[27] We have recently shown that PiT-2 immunopositivity was expressed predominantly in neurons, astrocytes, and vascular endothelial cells in the mouse brain.[28] PDGF-B is expressed in endothelial cells and neurons.[29] PDGF-B homodimer (PDGF-BB) enhanced the expression of PiT-1 mRNA encoded by *SLC20A1* in human aortic smooth muscle cells.[30] The hypomorph of PDGF-B in mice has recently been revealed to cause brain calcification through pericyte and blood-brain barrier impairment.[15] Recently, simple knockout of *SLC20A2* has also been shown to lead to calcification in the mouse brain.[31] PiT-2, PDGF, and as yet undetermined other molecules are considered to have pivotal roles in blood vessel–associated calcification and neuronal death in patients with IBGC. Elucidation of the molecular basis underlying IBGC will contribute to the development of therapeutic measures for patients with calcification in the brain.

## AUTHOR CONTRIBUTIONS

Principal investigator: Isao Hozumi. Study supervision: Shoji Tsuji, Gen Sobue, Takashi Inuzuka, and Kortaro Tanaka. Manuscript draft preparation: Megumi Yamada and Masaki Tanaka. Acquisition and collection of data: Seiju Kobayashi, Yoshiharu Taguchi, Shutaro Takashima, Tetsuo Touge, Hiroyuki Hatsuta, and Shigeo Murayama. Analysis and interpretation: Megumi Yamada, Masaki Tanaka, Mari Takagi, Yuichi Hayashi, Masayuki Kaneko, Naoki Atsuta, Nobuyuki Shimozawa, Hiroyuki Ishiura, and Jun Mitsui.

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## DISCLOSURE

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**Evaluation of *SLC20A2* mutations that cause idiopathic basal ganglia calcification in Japan**

Megumi Yamada, Masaki Tanaka, Mari Takagi, et al.

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RESEARCH PAPER

# Autosomal-recessive complicated spastic paraplegia with a novel *lysosomal trafficking regulator* gene mutation

Haruo Shimazaki,[1] Junko Honda,[1] Tametou Naoi,[1] Michito Namekawa,[1] Imaharu Nakano,[2] Masahide Yazaki,[3] Katsuya Nakamura,[3] Kunihiro Yoshida,[3] Shu-ichi Ikeda,[3] Hiroyuki Ishiura,[4] Yoko Fukuda,[4] Yuji Takahashi,[4] Jun Goto,[4] Shoji Tsuji,[4] Yoshihisa Takiyama[5]

[1]Division of Neurology, Department of Internal Medicine, Jichi Medical University School of Medicine, Tochigi, Japan
[2]Department of Neurology, Tokyo Metropolitan Neurological Hospital, Tokyo, Japan
[3]Department of Medicine (Neurology and Rheumatology), Shinshu University School of Medicine, Nagano, Japan
[4]Department of Neurology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[5]Department of Neurology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

**Correspondence to**
Dr Haruo Shimazaki, Division of Neurology, Department of Internal Medicine, Jichi Medical University School of Medicine, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan; hshimaza@jichi.ac.jp

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## ABSTRACT

**Background** Autosomal-recessive hereditary spastic paraplegias (AR-HSP) consist of a genetically diverse group of neurodegenerative diseases characterised by pyramidal tracts dysfunction. The causative genes for many types of AR-HSP remain elusive. We tried to identify the gene mutation for AR-HSP with cerebellar ataxia and neuropathy.

**Methods** This study included two patients in a Japanese family with their parents who are first cousins. Neurological examination and gene analysis were conducted in the two patients and two normal family members. We undertook genome-wide linkage analysis employing single nucleotide polymorphism arrays using the two patients' DNAs and exome sequencing using one patient's sample.

**Results** We detected a homozygous missense mutation (c.4189T>G, p.F1397V) in the *lysosomal trafficking regulator* (*LYST*) gene, which is described as the causative gene for Chédiak–Higashi syndrome (CHS). CHS is a rare autosomal-recessive syndrome characterised by hypopigmentation, severe immune deficiency, a bleeding tendency and progressive neurological dysfunction. This mutation was co-segregated with the disease in the family and was located at well-conserved amino acid. This *LYST* mutation was not found in 200 Japanese control DNAs. Microscopic observation of peripheral blood in the two patients disclosed large peroxidase-positive granules in both patients' granulocytes, although they had no symptoms of immune deficiency or bleeding tendency.

**Conclusions** We diagnosed these patients as having adult CHS presenting spastic paraplegia with cerebellar ataxia and neuropathy. The clinical spectrum of CHS is broader than previously recognised. Adult CHS must be considered in the differential diagnosis of AR-HSP.

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## INTRODUCTION

Hereditary spastic paraplegias (HSP) comprise a genetically diverse group of inherited neurological disorders mainly exhibiting increased tone of the lower limb muscles with various associated symptoms. HSP are classified into two subtypes, that is, pure and complicated forms. The pure HSP presents with symptoms of progressive bilateral leg spasticity and weakness, exaggerated tendon reflexes and positive pathological reflexes. Meanwhile, the complicated HSP has additional neurological symptoms, as follows: mental impairment, extrapyramidal signs, cerebellar ataxia, peripheral neuropathy, muscle atrophy and optic atrophy, as well as symptoms other than neurological ones.[1]

HSP can be inherited in an autosomal-dominant (AD), autosomal-recessive (AR) or X-linked recessive (XR) pattern. Fifty-seven spastic paraplegia gene (SPG) loci have been assigned and about 40 causative genes have been identified to date. The pure HSP is mainly transmitted as an AD trait, whereas the complicated HSP exhibits AR or XR transmission. SPG4, the most common pure AD-HSP, is accounting for about 50% of such cases.[2] The most frequent AR-HSP is SPG11, which shows a complex phenotype, including cognitive impairment, a thin corpus callosum and peripheral neuropathy.[3] However, the majority of causative genes for AR-HSPs remain to be identified.

A number of pathogenic mechanisms underlying HSPs have been suggested by studies on several implicated genes for HSP. HSP is thought to be involved in intracellular trafficking, resulting from disruption of the axonal transport of molecules, organelles and other cargos, which predominantly affects the distal parts of motor neurons.[4]

Here, we present an AR-HSP family with cerebellar ataxia and neuropathy with a novel homozygous missense mutation in the *lysosomal trafficking regulator* (*LYST*) gene. *LYST* is known as the causative gene for Chédiak–Higashi syndrome (CHS, OMIM #214500),[5] which is a rare AR syndrome characterised by hypopigmentation, severe immune deficiency, a bleeding tendency and progressive neurological dysfunction.

## METHODS

### Patients

This study included two patients from a family with spastic paraplegia, cerebellar ataxia and peripheral neuropathy. The family tree is shown in figure 1C. The parents were first cousins. Two affected (II-1 and II-2) and two unaffected members (II-3, III-1) of the family underwent neurological examinations and blood analyses. All of the genomic DNA samples were obtained with written informed consent.


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