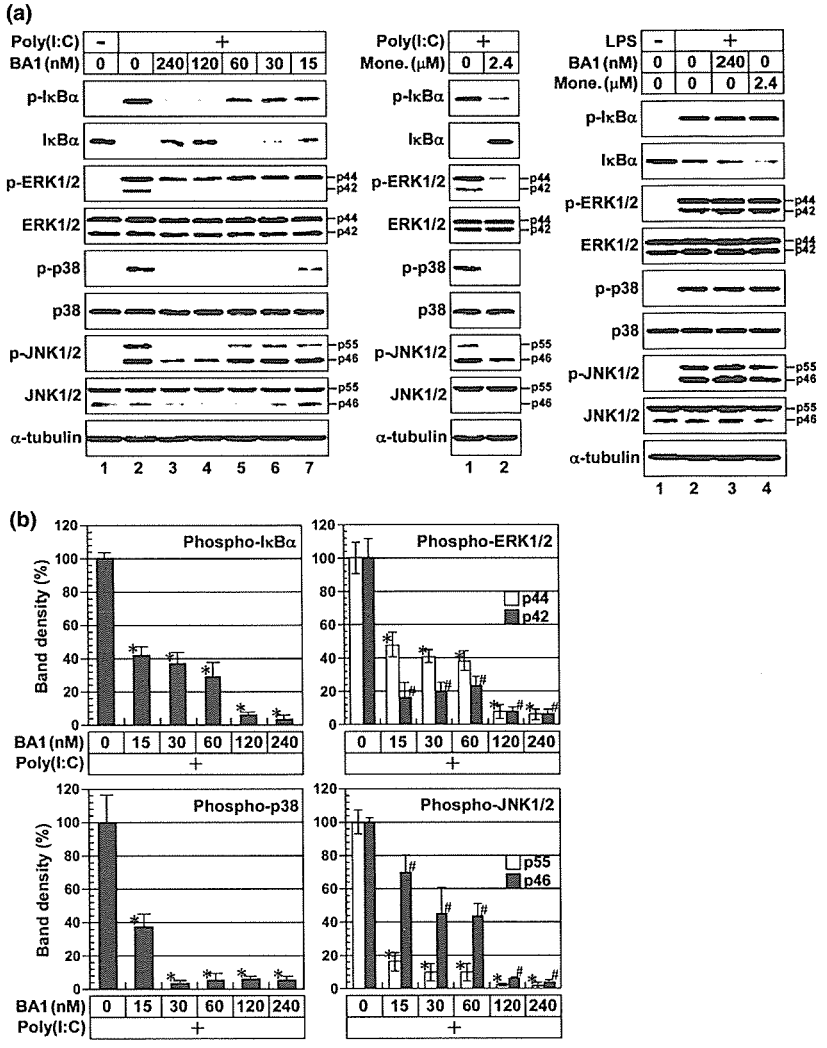

Fig. 6 Effect of endosomal pH-neutralizing agents on the activation of signaling pathways in the double-stranded RNA (dsRNA)-stimulated microglia. (a) Microglial cells were pretreated with the indicated doses of bafilomycin A1 (BA1) and monensin (Mone.) and then stimulated with polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL] or lipopolysaccharide (LPS: 50 ng/mL) for 1 h. The protein extracts from each culture were subjected to Western blot analyses as described in the text. The data are one from three independent experiments with similar results. (b) The digital images of each blot shown in (a) were prepared, and the density of each band was quantified by image analysis. The percentages of band densities of phosphorylated proteins were calculated with reference to the values for the drug-untreated control. Mean values and standard deviations from results of three separate experiments are shown. Statistically significant differences ($p < 0.05$) are indicated by asterisks and hashes (comparison to the values for drug-untreated cells).

Requirement of vacuolar acidification for double-stranded RNA-induced activation of signaling pathways in microglia

Recent studies using culture of dendritic cells suggested that TLR3 molecules, which recognize dsRNA pattern, are localized in intracellular vesicles (Matsumoto *et al.* 2003; Funami *et al.* 2004). In the experiments shown in Fig. 6, we examined whether the acidic environment of these vesicles is required for the dsRNA-induced activation of signaling pathways in microglia. To prevent endosomal acidification, microglia are pretreated with the indicated concentrations of BA1, a selective and potent inhibitor of vacuolar proton pump (H^+-ATPase) (Gagliardi *et al.* 1999) or monensin, an ion carrier that increases vesicular pH (Prabhananda and Kombrail 1992), and were then stimulated with poly(I:C). We also examined the effects of BA1 and monensin on the LPS-induced activation of signal-transducing molecules, which is known to have low sensitivity to vacuolar pH neutralization in macrophages (Weber and Levitz 2000; He and Kogut 2003). As shown in Fig. 6(a), the dsRNA-evoked phosphorylation of IκBα, ERK1/2, p38, and JNK1/2 in microglia was dramatically decreased in the presence of BA1 and monensin, whereas the treatment with these compounds had little or no effect on the LPS-induced phosphorylation of signal-transducing molecules. To assess the effect of endosomal neutralization on the activation of signal-transducing molecules more quantitatively, Western blotting was independently repeated three times, and the relative amounts of phosphorylated IκBα and MAPK proteins were measured by using image analyses as described above. As shown in Fig. 6(b), the dsRNA-induced phosphorylation of IκBα, ERK1/2, and JNK1/2 was significantly decreased in the presence of BA1 ranging from 15 to 60 nM, and a more dramatic diminution was seen when high doses of this compound were added (120 and 240 nM). The levels of p38 phosphorylation were readily abrogated in the presence of BA1 at doses between 30 and 240 nM. Therefore, these results demonstrate that the endosomal acidification is

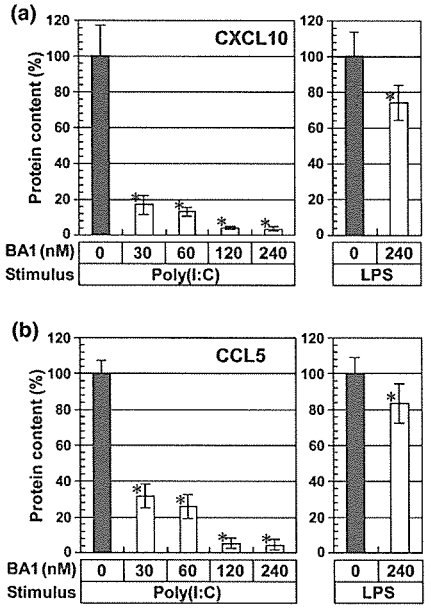

Fig. 7 Effect of endosomal pH-neutralizing agents on the double-stranded RNA-induced production of CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) in microglia. The cells were pretreated with the indicated concentrations of bafilomycin A1 (BA1) and then stimulated with polyinosinic-polycytidylic acid [poly(I:C), 50 μg/mL] or lipopolysaccharide (LPS: 50 ng/mL) for 15 h. The protein contents of CXCL10 (a) and CCL5 (b) were determined as described in the text. The percentages of chemokine contents were calculated with reference to the values for drug-untreated control. Mean values and standard deviations from results of three individual experiments are shown. Statistically significant differences ($p < 0.05$) are indicated by asterisks.

required for the activation of signaling pathways in microglia through recognition of dsRNA structure. If the dsRNA-induced production of CXCL10 and CCL5 was attributable to the activation of NF-κB and MAPK pathways, then the neutralization of vesicular pH should interfere with the production of these chemokines. As shown in Figs 7(a) and (b), the production of CXCL10 and CCL5 in the poly(I:C)-stimulated microglia were dose-dependently decreased in the presence of BA1, and the treatment with maximal concentration of this compound (240 nM) nearly completely diminished the dsRNA-induced chemokine production in microglia. In contrast, the LPS-induced production of either chemokine had low sensitivity to vacuolar pH neutralization. These data show that the dsRNA-induced expression of CXCL10 and CCL5 in microglia is mediated by the vacuolar pH-dependent activation of intracellular signaling pathways.

Discussion

The blood–brain barrier serves as a protective mechanism for the brain by preventing entry of most pathogens from free access to the CNS. However, neurotropic viruses enter the CNS in infected migratory leukocytes, by intraneuronal transfer from peripheral nerves, or by transcytosis across blood–brain barrier via direct infection of cerebral endothelial cells (Zhang and Tuomanen 1999). Microglia are resident immune effector cells within the CNS and are hence likely to encounter infectious agents at very early stages of infection, as well as at later stages, when peripheral leukocytes are recruited into the brain parenchyma (Kreutzberg 1996). It is well known that CXCL10 and CCL5 exert potent chemotactic effects on peripheral leukocytes such as T cells and monocytes (Schall *et al.* 1988; Neville *et al.* 1997). Recent extensive studies have indicated that these chemokines are produced by microglia upon infection with a variety of neurotropic viruses (Lokensgard *et al.* 2001; Palma and Kim 2001; Si *et al.* 2002; Cheeran *et al.* 2003; Chen *et al.* 2004; Olson and Miller 2004).

In this report, we present a comprehensive study on the induction of chemokines by measuring and correlating events from cellular signaling to chemokine responses in microglia upon stimulation with dsRNA, a conserved molecular pattern of virus infection. On the basis of our data, we suggest that the recognition of dsRNA structure strongly induces the expression of CXCL10 and CCL5. To the best of our knowledge, we believe this to be the first report showing the dsRNA-induced expression of these chemokines in microglia. Previous study suggested that the poly(I:C) stimulation of microglia has no effect on the gene expression levels of CCL5 and other chemokines (Olson and Miller 2004), and these observations are contrasted by our current data. However, these differing results can be explained by the kinetics of CCL5 gene expression. In the previous investigation, the level of CCL5 transcription was investigated at 24 h post-stimulation with poly(I:C), whereas its expression appeared to be triggered at earlier time points, as demonstrated in the present study.

The data obtained in the current study also demonstrate that the microglial CXCL10 response to dsRNA was primarily achieved by NF-κB pathway, and was partly mediated through p38 and JNK activation. As the dsRNA-induced IκBα phosphorylation was not diminished by p38 and JNK inhibitors, it is postulated that these MAPK pathways mediate CXCL10 expression without affecting the NF-κB activity. Previous studies suggested that several stimuli, such as lipopolysaccharide, CD40 ligand, human immunodeficiency virus Tat protein, IFN-γ, and cytomegalovirus infection, activate CXCL10 expression in microglia via cellular signaling mediated by NF-κB, signal transducer and activator of transcription-1, p38, ERK1/2, phosphatidylinositol 3-kinase (D'Aversa *et al.* 2002; Si *et al.* 2002; Cheeran *et al.* 2003; Delgado 2003; D'Aversa *et al.* 2004). In addition to these signal-transducing molecules, our current data constitute the first evidence that the JNK-mediated pathway contributes to microglial CXCL10 response.

Recent studies suggested that the dsRNA-induced CCL5 expression in peripheral tissue-derived cell types, such as epithelial cells, fibroblasts, and macrophages, are mediated through activation of NF-κB, IRF3, dsRNA-activated protein kinase, and phosphatidylinositol 3-kinase (Gern *et al.* 2003; Guillot *et al.* 2004; Ieki *et al.* 2004; McWhirter *et al.* 2004). Although the cellular signaling, which regulates microglial CCL5 response to dsRNA, has not been reported, the accumulating evidences suggest that the microglial activation evoked by several stimuli results in CCL5 expression via signaling pathways that involve NF-κB, ERK1/2, and nuclear factor interleukin-6 (Hu *et al.* 1999; Delgado *et al.* 2002; Jang *et al.* 2002; Kim *et al.* 2002; Chen *et al.* 2004; D'Aversa *et al.* 2004). In the present study, we demonstrated that the JNK activation is crucial for the dsRNA-induced CCL5 response in microglia. To our knowledge, the present study is the first report demonstrating a JNK-dependent CCL5 production during microglial activation. It is probable that the dsRNA-induced stimulation of JNK pathways leads to downstream activation of transcription factors, culminating in transcription of CCL5 gene (Melchjorsen *et al.* 2003).

Our results obtained in this study seem to be unique in that the CXCL10 and CCL5 responses in microglia are differentially regulated by intracellular signaling pathways. It is known that the promoter regions of CXCL10 and CCL5 genes contain recognition sites for NF-κB and activator protein-1, a downstream target of JNK pathways (Melchjorsen *et al.* 2003). However, the dsRNA-induced CCL5 production in microglia was not accompanied by NF-κB activation, despite the fact that the inhibition of this signaling markedly diminished CXCL10 response. We also observed that the dsRNA-induced production of CCL5 in microglia was notably impaired in the absence of JNK activation when compared to that of CXCL10. Much remains to be understood about this differential regulation of CXCL10 and CCL5 responses in microglia upon dsRNA recognition. However, considering the strong chemotactic effects of either chemokine on inflammatory cells, it seems reasonable that the gene expression of CXCL10 and CCL5 in microglia is intrinsically up-regulated by a specific set of transcription factors, thereby controlling the excessive leukocyte trafficking into the brain parenchyma.

The poly(I:C)-induced production of CXCL10 and CCL5 was slightly decreased by treatment with JAK inhibitor. These results imply that JAK signaling partly mediates chemokine production in dsRNA-stimulated microglia. In preliminary experiments, the poly(I:C) treatment did not lead to the activation of JAK signaling within the same time frame as the activation of NF-κB and MAPK pathways (data not shown). Thus, it seems possible that cytokines, such as type 1 IFNs, secreted from dsRNA-treated microglia autocrinely stimulates JAK signaling, which subsequently facilitates chemokine expression at later time points.

The striking finding in this study is that the acidic environment within intracellular vesicles is crucial for the dsRNA-induced activation of cellular signaling pathways and the consequential production of CXCL10 and CCL5 in microglia. Although a previous report indicated that endosomal/lysosomal compartments are required for the maturation of cellular proteases (Sastradipura *et al.* 1998), the roles of these vesicles in microglial function, especially that in cellular activation upon pathogen recognition, remains to be elucidated. The results from two recent studies using peripheral tissue-derived cells imply that the NF-κB signaling is triggered by the poly(I:C)/TLR3 association in intracellular vesicles and that the endosomal acidification is required for the dsRNA-induced activation of IFN-β promoter and interleukin-12 production (Matsumoto *et al.* 2003; Funami *et al.* 2004). Our data obtained in the current study provide evidence that the dsRNA-evoked activation of MAPK, as well as that of NF-κB, depends on acidic milieu of intracellular vesicles.

In conclusion, the results obtained in the present study demonstrate that the recognition of dsRNA structure triggers the activation of NF-κB and MAPK signaling pathways, leading to the strong induction of CXCL10 and CCL5 production in microglia. Our study provides an insight into the mechanism involved in microglial responses to the conserved molecular pattern of virus infection.

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References

Aloisi F. (2001) Immune function of microglia. *Glia* **36**, 165–179.

Baeuerle P. A. and Henkel T. (1994) Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* **12**, 141–179.

Barker C. F. and Billingham R. E. (1977) Immunologically privileged sites. *Adv. Immunol.* **25**, 1–54.

Cheeran M. C., Hu S., Sheng W. S., Peterson P. K. and Lokensgard J. R. (2003) CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10. *J. Virol.* **77**, 4502–4515.

Chen C. J., Chen J. H., Chen S. Y., Liao S. L. and Raung S. L. (2004) Upregulation of RANTES gene expression in neuroglia by Japanese encephalitis virus infection. *J. Virol.* **78**, 12 107–12 119.

D'Aversa T. G., Weidenheim K. M. and Berman J. W. (2002) CD40–CD40L interactions induce chemokine expression by human microglia: implications for human immunodeficiency virus encephalitis and multiple sclerosis. *Am. J. Pathol.* **160**, 559–567.

D'Aversa T. G., Yu K. O. and Berman J. W. (2004) Expression of chemokines by human fetal microglia after treatment with the

human immunodeficiency virus type 1 protein Tat. *J. Neurovirol.* **10**, 86–97.

Delgado M. (2003) Inhibition of interferon (IFN) gamma-induced Jak-STAT1 activation in microglia by vasoactive intestinal peptide: inhibitory effect on CD40, IFN-induced protein-10, and inducible nitric-oxide synthase expression. *J. Biol. Chem.* **278**, 27 620–27 629.

Delgado M., Jonakait G. M. and Ganea D. (2002) Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit chemokine production in activated microglia. *Glia* **39**, 148–161.

Dixon D. W., Mattiace L. A., Kure K., Hutchins K., Lyman X. and Brosnan C. F. (1991) Microglia in human disease, with an emphasis on acquired immune deficiency syndrome. *Lab. Invest.* **64**, 135–156.

Eugenin E. A. and Berman J. W. (2003) Chemokine-dependent mechanisms of leukocyte trafficking across a model of the blood–brain barrier. *Methods* **29**, 351–361.

Funami K., Matsumoto M., Oshiumi H., Akazawa T., Yamamoto A. and Seya T. (2004) The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *Int. Immunol.* **16**, 1143–1154.

Gagliardi S., Rees M. and Farina C. (1999) Chemistry and structure activity relationships of bafilomycin A1, a potent and selective inhibitor of the vacuolar H+-ATPase. *Curr. Med. Chem.* **6**, 1197–1212.

Gern J. E., French D. A., Grindle K. A., Brockman-Schneider R. A., Konno S. and Busse W. W. (2003) Double-stranded RNA induces the synthesis of specific chemokines by bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **28**, 731–737.

Guillot L., Le Goffic R., Bloch S., Escriou N., Akira S., Chignard M. and Si-Tahar M. (2004) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* **280**, 5571–5580.

He H. and Kogut M. H. (2003) CpG-ODN-induced nitric oxide production is mediated through clathrin-dependent endocytosis, endosomal maturation, and activation of PKC, MEK1/2 and p38 MAPK, and NF-kappaB pathways in avian macrophage cells (HD11). *Cell. Signal.* **15**, 911–917.

Hu S., Chao C. C., Ehrlich L. C., Sheng W. S., Sutton R. L., Rockswold G. L. and Peterson P. K. (1999) Inhibition of microglial cell RANTES production by IL-10 and TGF-beta. *J. Leukoc. Biol.* **65**, 815–821.

Ieki K., Matsukura S., Kokubu F., Kimura T., Kuga H., Kawaguchi M., Odaka M., Suzuki S., Watanabe S., Takeuchi H., Schleimer R. P. and Adachi M. (2004) Double-stranded RNA activates RANTES gene transcription through co-operation of nuclear factor-kappaB and interferon regulatory factors in human airway epithelial cells. *Clin. Exp. Allergy.* **34**, 745–752.

Inoue H., Sawada M., Ryo A. *et al.* (1999) Serial analysis of gene expression in a microglial cell line. *Glia* **28**, 265–271.

Jang S. B., Won J., Kim H., Kim J., Lee K. H., Han H., Rha H. K. and Choi C. R. (2002) TAK1 mediates lipopolysaccharide-induced RANTES promoter activation in BV-2 microglial cells. *Mol. Cells* **14**, 35–42.

Jaramillo M., Gowda D. C., Radzioch D. and Olivier M. (2003) Hemozoin increases IFN-gamma-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways. *J. Immunol.* **171**, 4243–4253.

Kanazawa T., Sawada M., Kato K., Yamamoto K., Mori H. and Tanaka R. (2000) Differentiated regulation of allo-antigen presentation by different types of murine microglial cell lines. *J. Neurosci. Res.* **62**, 383–388.

Karin M. and Ben Neriah Y. (2000) Phosphorylation meets ubiquitination: the control of NF-kB activity. *Annu. Rev. Immunol.* **18**, 621–663.

Kim M. O., Si Q., Zhou J. N., Pestell R. G., Brosnan C. F., Locker J. and Lee S. C. (2002) Interferon-beta activates multiple signaling cascades in primary human microglia. *J. Neurochem.* **81**, 1361–1371.

Kreutzberg G. W. (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **19**, 312–318.

Lacroix-Lamande S., Mancassola R., Naciri M. and Laurent F. (2002) Role of gamma interferon in chemokine expression in the ileum of mice and in a murine intestinal epithelial cell line after Cryptosporidium parvum infection. *Infect. Immun.* **70**, 2090–2099.

Lokensgard J. R., Hu S., Sheng W., vanOijen M., Cox D., Cheeran M. C. and Peterson P. K. (2001) Robust expression of TNF-alpha, IL-1beta, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus. *J. Neurovirol.* **7**, 208–219.

Matsumoto M., Funami K., Tanabe M., Oshiumi H., Shingai M., Seto Y., Yamamoto A. and Seya T. (2003) Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* **171**, 3154–3162.

McWhirter S. M., Fitzgerald K. A., Rosains J., Rowe D. C., Golenbock D. T. and Maniatis T. (2004) IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc. Natl Acad. Sci. USA* **101**, 233–238.

Melchjorsen J., Sorensen L. N. and Paludan S. R. (2003) Expression and function of chemokines during viral infections: from molecular mechanisms to *in vivo* function. *J. Leukoc. Biol.* **74**, 331–343.

Mogensen T. H. and Paludan S. R. (2001) Molecular pathways in virus-induced cytokine production. *Microbiol. Mol. Biol. Rev.* **65**, 131–150.

Nakamichi K., Kuroki D., Matsumoto Y. and Otsuka H. (2001) Bovine herpesvirus 1 glycoprotein G is required for prevention of apoptosis and efficient viral growth in rabbit kidney cells. *Virology* **279**, 488–498.

Nakamichi K., Inoue S., Takasaki T., Morimoto K. and Kurane I. (2004) Rabies virus stimulates nitric oxide production and CXC chemokine ligand 10 expression in macrophages through activation of extracellular signal-regulated kinases 1 and 2. *J. Virol.* **78**, 9376–9388.

Natarajan K., Singh S., Burke T. R. Jr, Grunberger D. and Aggarwal B. B. (1996) Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc. Natl Acad. Sci. USA* **93**, 9090–9095.

Neumann B., Zantl N., Veihelmann A., Emmanuilidis K., Pfeffer K., Heidecke C. D. and Holzmann B. (1999) Mechanisms of acute inflammatory lung injury induced by abdominal sepsis. *Int. Immunol.* **11**, 217–227.

Neville L. F., Mathiak G. and Bagasra O. (1997) The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev.* **8**, 207–219.

Olson J. K. and Miller S. D. (2004) Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J. Immunol.* **173**, 3916–3924.

Palma J. P. and Kim B. S. (2001) Induction of selected chemokines in glial cells infected with Theiler's virus. *J. Neuroimmunol.* **117**, 166–170.

Pierce J. W., Schoenleber R., Jesmok G., Best J., Moore S. A., Collins T. and Gerritsen M. E. (1997) Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *J. Biol. Chem.* **272**, 21 096–21 103.

Prabhananda B. S. and Kombrail M. H. (1992) Monensin-mediated transports of H+, Na+, K+ and Li+ ions across vesicular membranes: T-jump studies. *Biochim. Biophys. Acta* **1106**, 171–177.

Rock R. B., Gekker G., Hu S., Sheng W. S., Cheeran M., Lokensgard J. R. and Peterson P. K. (2004) Role of microglia in central nervous system infections. *Clin. Microbiol. Rev.* **17**, 942–964.

Sastradipura D. F., Nakanishi H., Tsukuba T., Nishishita K., Sakai H., Kato Y., Gotow T., Uchiyama Y. and Yamamoto K. (1998) Identification of cellular compartments involved in processing of cathepsin E in primary cultures of rat microglia. *J. Neurochem.* **70**, 2045–2056.

Saunders L. R. and Barber G. N. (2003) The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J.* **17**, 961–983.

Sawada M., Imai F., Suzuki H., Hayakawa M., Kanno T. and Nagatsu T. (1998) Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain. *FEBS Lett.* **433**, 37–40.

Schall T. J., Jongstra J. B., Dyer J., Jorgensen J., Clayberger C., Davis M. M. and Krensky A. M. (1988) A human T cell-specific molecule is a member of a new gene family. *J. Immunol.* **141**, 1018–1025.

Si Q., Kim M. O., Zhao M. L., Landau N. R., Goldstein H. and Lee S. (2002) Vpr- and Nef-dependent induction of RANTES/CCL5 in microglial cells. *Virology* **301**, 342–353.

Suzumura A., Sawada M. and Takayanagi T. (1998) Production of interleukin-12 and expression of its receptors by murine microglia. *Brain Res.* **787**, 139–142.

Thompson J. E., Cubbon R. M., Cummings R. T. *et al.* (2002) Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor. *Bioorg. Med. Chem. Lett.* **12**, 1219–1223.

Tibbles L. A., Spurrell J. C., Bowen G. P., Liu Q., Lam M., Zaiss A. K., Robbins S. M., Hollenberg M. D., Wickham T. J. and Muruve D. A. (2002) Activation of p38 and ERK signaling during adenovirus vector cell entry lead to expression of the C-X-C chemokine IP-10. *J. Virol.* **76**, 1559–1568.

Weber S. M. and Levitz S. M. (2000) Chloroquine interferes with lipopolysaccharide- induced TNF-alpha gene expression by a nonlysosomotropic mechanism. *J. Immunol.* **165**, 1534–1540.

Wu D., Marko M., Claycombe K., Paulson K. E. and Meydani S. N. (2003) Ceramide-induced and age-associated increase in macrophage COX-2 expression is mediated through up-regulation of NF-kappa B activity. *J. Biol. Chem.* **278**, 10 983–10 992.

Zhang J. R. and Tuomanen E. (1999) Molecular and cellular mechanisms for microbial entry into the CNS. *J. Neurovirol.* **5**, 591–603.

Zlotnik A. and Yoshie O. (2000) Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121–127.

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パーキン蛋白の機能解析と黒質変性とその防御に関する研究

平成１８年度　総括・分担研究報告書

主任研究者　服部　信孝

分担研究者　田中　啓二
高橋　良輔
澤田　誠

平成１９（２００７）年　３月

Clinical Heterogeneity of α-Synuclein Gene Duplication in Parkinson's Disease

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Objective: **Recently, genomic multiplications of α-synuclein gene (*SNCA*) have been reported to cause hereditary early-onset parkinsonism. The objective of this study was to assess the frequency of SNCA multiplications among autosomal dominant hereditary Parkinson's disease (ADPD).** ***Methods:*** **We screened 113 ADPD probands and 200 sporadic PD cases by quantitative polymerase chain reaction and confirmed *SNCA* multiplications by flurorescence in situ hybridization (FISH) and comparative genomic hybridization array.** ***Results:*** **Two families (two patients from Family A and one from Family B) with SNCA duplication were identified among ADPD patients. Even though they had the same *SNCA* duplication, one patient had dementia. Because there was exactly the same difference between the regions originated from each patient, the finding suggests that the phenotype of *SNCA* multiplication may be also influenced by the range of duplication region. We also detected asymptomatic carriers in the families of both patients. Interestingly, the penetrance ratio was 33.3% (2/6) in one kindred, indicating that the ratio was very much lower than expected.** ***Interpretation:*** **These two newly identified Japanese patients with *SNCA* duplication and the five previously identified American and European families with *SNCA* triplication or duplication mutations indicate that the incidence of *SNCA* multiplication may be more frequent than previously estimated.**

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Parkinson's disease (PD) is the second most common neurodegenerative disorder next to Alzheimer's disease (AD). Although the exact cause for PD remains to be elucidated, genetic factors could contribute to the pathogenesis of PD. Indeed, six causative genes and four chromosomal loci for familial PD (FPD) have been identified.[1–13] *α-Synuclein*, *UCH-L1*, and *LRRK2* have been identified as causative genes for autosomal dominant forms of FPD (ADPD), whereas *parkin*, *PINK1*, and *DJ-1* have been identified as causative genes for autosomal recessive forms of FPD (ARPD).[1,10,14] The presence of several causative genes and loci for FPD indicates that the pathogenic mechanisms of sporadic PD are also multifactorial. Studies of FPD are important as they enhance our understanding of nigral neuronal death. Furthermore, it has been proposed that the gene products for FPD are components of common pathways in sporadic PD. As testament, missense mutations such as A30P,[15] E46K,[16] and A53T,[9] in the N-terminal of α-synuclein gene (*SNCA*) have been linked to a rare form of FPD, and α-synuclein subsequently was confirmed to be a major component of Lewy bodies (LBs) and Lewy neurites, the pathological hallmark of sporadic PD and dementia with LBs (DLB).[17] Based on large population-based studies, missense mutations of *SNCA* are infrequent.[18] In particular, the SNCA A53T mutations identified in patients with FPD originate from a single founder. To date, SNCA A30P and E46K mutations have been found in only one family each, suggesting that missense mutations are a very rare cause of parkinsonism. Recently, *SNCA* multiplications in FPD haves been re-

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ported in two families with genomic triplication and in three families with duplications.[11–13,19] These findings suggest that overproduction of α-synuclein is one of the most important factors in FPD. In general, unequal intrachromosomal crossovers that result from misalignment of two homologous flanking sequences may account for genomic multiplications as well as deletions. The *SNCA* multiplications mutations, triplications, and duplications found in five unrelated patients probands with FPD are de novo within each kindred.[11–13,19] Affected individuals within the Iowa kindred, with *SNCA* genomic triplication, have fulminant, early-onset disease with a phenotype ranging clinically and pathologically from PD to diffuse LB disease (DLBD).[20] In contrast, *SNCA* duplication families have later onset disease and a longer duration to death, and neither cognitive decline nor dementia are prominent. Therefore, overproduction of wild-type α-synuclein (*SNCA*) may result in phenotypes of PD, PD with dementia (PDD), and DLBD, suggesting that regulation of α-synuclein protein levels is central to the cause of these phenotypes. In summary, the phenotype may be dependent on copy numbers of *SNCA*.

In this study, to gain further insight into the role of this multiplication, we assessed a series of 113 PD patients with autosomal dominant mode of inheritance and 200 sporadic PD patients for multiplication at this locus.

Subjects and Methods

Patients

This study consisted of 113 patients with ADPD and 200 patients with sporadic PD. Diagnosis of PD was adopted by the participating neurologists and the diagnosis was established based on the United Kingdom Parkinson's Disease Society Brain Bank criteria.[21] The mean age at onset of the 56 male and 57 female index patients with ADPD was 66.0 ± 9.5 (±SD), and that of the 81 male and 119 female patients with sporadic PD was 64.7 ± 10.0 (±SD). All patients were of Japanese origin. The study was approved by the ethics review committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining informed consent from each patient and 17 unaffected relatives. None had mutations in *parkin*, *PINK1*, or *DJ-1*. We could not detect heterozygous exon deletions of such recessive genes by quantitative analysis in the patients studied. In addition, none had mutations in exon 41 in *LRRK2*.

Gene Dosage Analysis for SNCA

DNA was prepared using standard methods. The mutation screening was performed as described previously.[22] Semiquantitative multiplex polymerase chain reaction (PCR) of genomic DNA samples was performed using a real-time PCR method to detect the dosage of *SNCA* (ABI Prism 7700 sequence detector; Applied Biosystems, Foster City, CA). As the first step, we targeted exon 3 of *SNCA* to screen the gene dosage of *SNCA*. β-*Globin* gene was amplified as an endogenous reference. In addition, we used a DNA sample from the Iowa family (patients had triplication of *SNCA*) as a positive control. The primer and TaqMan MGB probe sequences used in this study are described in Table 1. PCR was conformed with PCR universal master mix using 25ng of genomic DNA, 900nM primers, and 250nM probes (β-globin is 50–200nM) in a total reaction volume of 50μL. PCR cycling conditions were 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute (40 cycles). Values between 0.4 and 0.6 were considered as heterozygous deletion, between 0.8 and 1.2 as normal, between 1.3 and 1.7 as heterozygous duplication, and greater than 1.8 as triplication.

In the second step, we performed semiquantitative analysis on exons 1/2, 4, 6, and 7 for the patients found to carry multiplication of this gene in the first step. All the sequences of this gene are shown in Table 1.

Fluorescence In Situ Hybridization Analysis

We used two-color standard fluorescence in situ hybridization (FISH) and prophase FISH for metaphase and interphase. FISH analyses were performed as described previously,[23] using a BAC located around the region of interest. The location of each bacterial artificial chromosome (BAC) was archived by the database of UCSC (http://genome.ucsc.edu) or NCBI (http://www.ncbi.nlm.nih.gov). Two BAC contigs representing the region at 4q21-22. BACs RP11-17p8 and RP11-61407 were used as probes. BAC RP11-17p8 locates at site of centromere of chromosome 4, and BAC RP11-61407 locates at site of telomere of the same chromosome. PR11-61407 contains *SNCA*, suggesting that the signal of this clone shows the copy numbers of *SNCA*. The distance between the two BAC clones was approximately 1.4Mb. Probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP by nick-translation (Roche Diagnostics, Tokyo, Japan). The copy number of the region was assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes. We established Epstein–Barr virus (EBV)–transformed lymphoblastoid cell line as described previously.[24]

Multiplication (duplication) Region Using Comparative Genomic Hybridization Array and Gene Dosage Technique

The triplication region in Iowa family is between 1.61 and 2.04Mb and contains 17 annotated or putative genes. A recently constructed high-density comparative genomic hybridization (CGH) array, designated MCG Whole Genome Array-4500,[25] which contains 4532 BAC/P1-artificial chromosome (PAC) clones covering the entire genome at intervals of approximately 0.7Mb, was used for CGH array analysis. This array is suitable for detecting the size of the multiplication if the size is greater than 0.7Mb. Hybridizations were performed as described previously with minor modifications.[26,27] In brief, test and reference genomic DNAs from the patient's lymphoblastoid cells and normal lymphocytes, respectively, were labeled with Cy3- and Cy5-dCTP (Amersham Biosciences, Tokyo), respectively, precipitated together with ethanol in the presence of Cot-1 DNA, redissolved in a hybridization mix (50% formamide, 10% dextran sulfate, 2 × standard saline citrate [SSC], and 4% sodium dodecyl sulfate [SDS], pH 7.0),

Table 1. Sequences of Primer and TaqMan Probes Used in the This Study

		Forward Primer	Reverse Primer
β-globin	—	5′-TGGGCAACCCTAAGGTGAAG-3′	5′-GTGAGCCAGGCCATCACTAAA-3′
ABCG2	Exon 1	5′-GGAAGGTCCGGGTGACTCA-3′	5′-GGAGGCAGCGCTTTAACAAT-3′
—	Exon 2	5′-GTGTCACAAGGAAACACCAATGG-3′	5′-AGCTCCTTCAGTAAATGCCTTCAG-3′
DFKZ	Exon 7	5′-CTGGACCACTTACTGGTGAAAGC-3′	5′-CACTGTGCCTGGCCAAATT-3′
FAM13A1	Exon 12	5′-GAAGAGGACCTAACTCCCAGGAT-3′	5′-TTCTCAAGTTGGGAACCAAAACTCT-3′
LOC345278	Exon 8	5′-GTTGGCTGGGCCAATCTCT-3′	5′-TGGTCTTAGCTGAAGGCCAGTT-3′
SNCA	Exon s1/2	5′-CCTTCAAGCCTTCTGCCTTTC-3′	5′-CGAATGGCCACTCCCAGTT-3′
—	Exon 3	5′-TTCCAGTGTGGTGTAAAGAAATTCAT-3′	5′-CCTTGGCCTTTGAAAGTCCTT-3′
—	Exon 4	5′-CAGCAATTTAAGGCTAGCTTGAGACT-3′	5′-CCACTCCCTCCTTGGTTTTG-3′
—	Exon 6	5′-TATGCCTGTGGATCCTGACAAT-3′	5′-TCAGCTTGGACTCCTACCTCAGA-3′
—	Exon 7	5′-TCTTTGCTCCCAGTTTCTTGAGA-3′	5′-TGGAACTGAGCACTTGTACAGGAT-3′
MMRN1	Exon 1	5′-ATCAAACTCTCACATCCAC-3′	5′-CACCTGCTGAGGGTGTGAGA-3′
—	Exon 5	5′-CAGGCAATGAAACTGACTCTTCTG-3′	5′-CTTCTAGGGAGGAGTAAGTGTTCCT-3′
—	Exon 6	5′-GTTTCAATAGCAGCCCAGCAAAA-3′	5′-CAGTCAAAGTGGGCCGATTCT-3′
—	Exon 8	5′-GCTTCATATACCCCAAGAACTGGAA-3′	5′-GCACTAAATGACTCGATGGTGTACT-3′
KIAA1680	Exon 1	5′-TTAAATAACGCAGCTGGACTCTGT-3′	5′-TTAAATAACGCAGCTGGACTCTGT-3′
—	Exon 2	5′-GGCCACAATGATTCTACCTCTCA-3′	5′-CCGTAAGTTCTGTTGTTGTCTTTGT-3′
—	Exon 3	5′-AGCTCAGGTAGCACAGGTAAACG-3′	5′-TGGTGGAAGCTAATGGAAGGA-3′
—	Exon 4	5′-CCATTTCGTGAAGGAAGATTTATAGAG-3′	5′-TCCCTGCAGTGCCTTCTGA-3′
—		MGB probe	
β-globin	—	5′-CTCATGGCAAGAAAGTGCTCGGTGC-3′	
ABCG2	Exon 1	5′-CCCAACATTTACATCCTT-3′	
—	Exon 2	5′-CCGCGACAGCTTCCAA-3′	
DFKZ	Exon 7	5′-ACCATGCAAAAGAAAT-3′	
FAM13A1	Exon 12	5′-AAGCAACACACTCCCC-3′	
LOC345278	Exon 8	5′-CAGAAGCTGACTCTCA -3′	
SNCA	Exons 1/2	5′-ACCCTCGTGAGCGGA-3′	
—	Exon 3	5′-AGCCATGGATGTATTC-3′	
—	Exon 4	5′-TGTCTTGAATTTGTTTTTGTAGGC-3′	
—	Exon 6	5′-AGGCTTATGAAATGCC-3′	
—	Exon 7	5′-TGCTGACAGATGTTC-3′	
MMRN1	Exon 1	5′-ACTTGACCACTCCTTCTGCTTTCT-3′	
—	Exon 5	5′-CACAGTCAAAGAAATATTG-3′	
—	Exon 6	5′-CTTGCACCAAAACAAAC-3′	
—	Exon 8	5′-TCCAAGATACGGAATTCTA-3′	
KIAA1680	Exon 1	5′-TCCCCTTCTCGGCTGTTG-3′	
—	Exon 2	5′-ATGTCCCTCAATTCTG-3′	
—	Exon 3	5′-AGGAGCATATTCCG-3′	
—	Exon 4	5′-AGACTGCGATCCTC-3′	

and denatured at 75°C for 8 minutes. After 40-minute preincubation at 42°C, the mixture was applied to array slides and incubated at 50°C for 10 minutes, 46°C for 10 minutes, and 43°C for 60 hours in a hybridization machine, GeneTAC (Harvard Bioscience, Holliston, MA). After hybridization, the slides were washed once in a solution of 50% formamide, 2 × SSC (pH 7.0) for 10 minutes at 50°C and 1 × SSC for 10 minutes at 42°C, respectively, and then scanned with a GenePix 4000B (Axon Instruments, Foster City, CA). The acquired images were analyzed with GenePix Pro 4.1 imaging software (Axon Instruments). Fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratios across the array was zero. The average values for each clone were within the thresholds of 0.2 and −0.2 (log2ratio), and the mean ± 2 SD values of all clones were within the range of 0.4 and −0.4 (log2ratio). The thresholds for copy number gain and loss were set at log2 ratios of 0.4 and −0.4, respectively.

We picked up the locus region between *ABCG* and *KIAA1680* of approximately 1.6 to 2.0 Mb. To identify the region of duplication spanning *SNCA*, we performed semiquantitative PCR on target genes including *ABCG*, *DFKZ*, *FAM13A1*, *LOC345278*, *MMRN*, and *KIAA1680* using the same methods. The sequences of all primer and probe sets are shown in Table 1.

Haplotype Analysis

To determine whether the same haplotype was shared between our probands with *SNCA* multiplication, we performed haplotype analysis in patients with *SNCA* duplication from unrelated families. We used four microsatellite markers including *D4S2361*, *D4S2505E* (located within *SNCA*), *D4S2380*, *D4S1647*, and *D4S421*.

Results

Gene Dosage Analysis for α-Synuclein

Using semiquantitative PCR to detect gene dosage, we did not find patients harboring *SNCA* multiplication

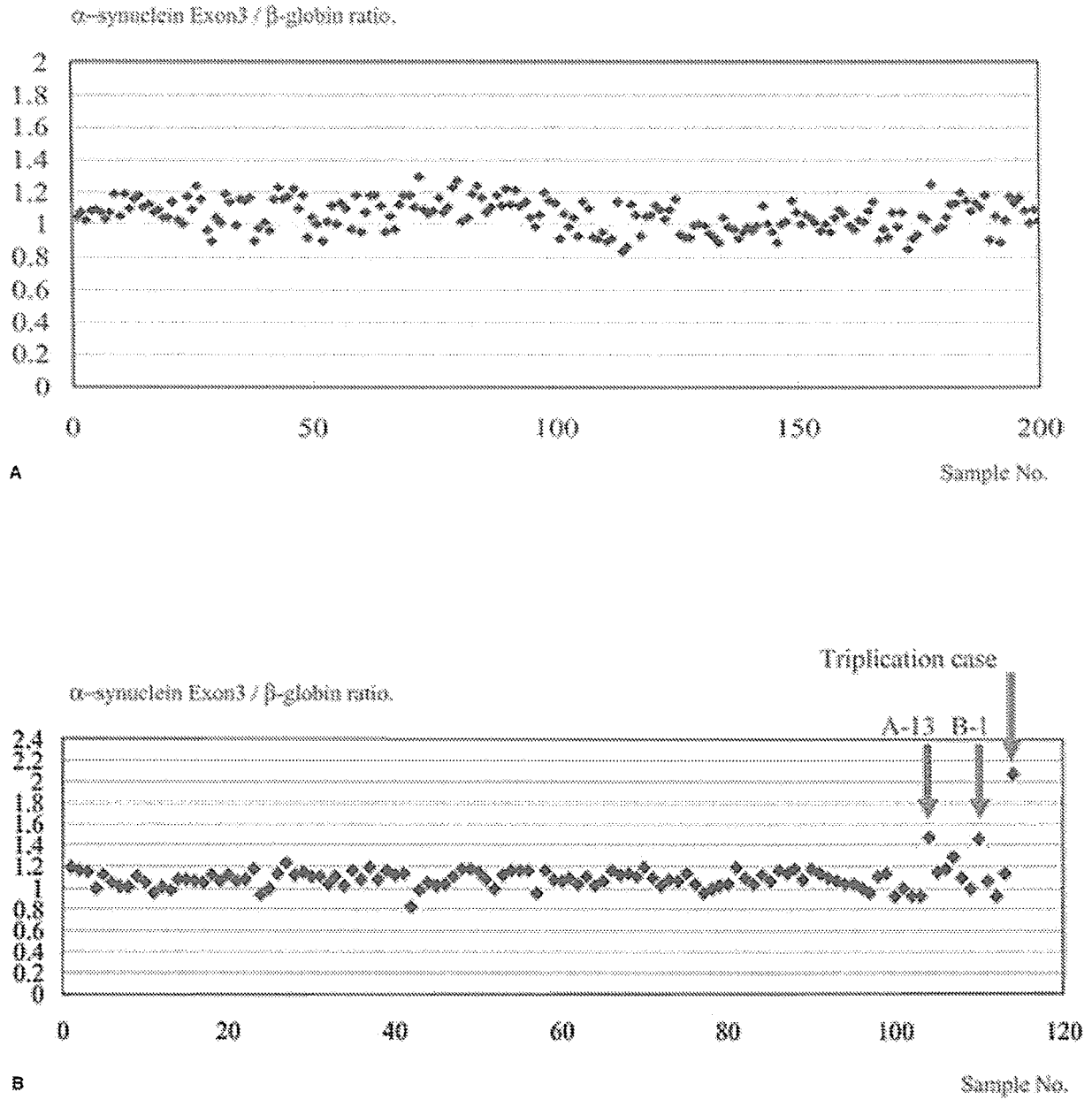
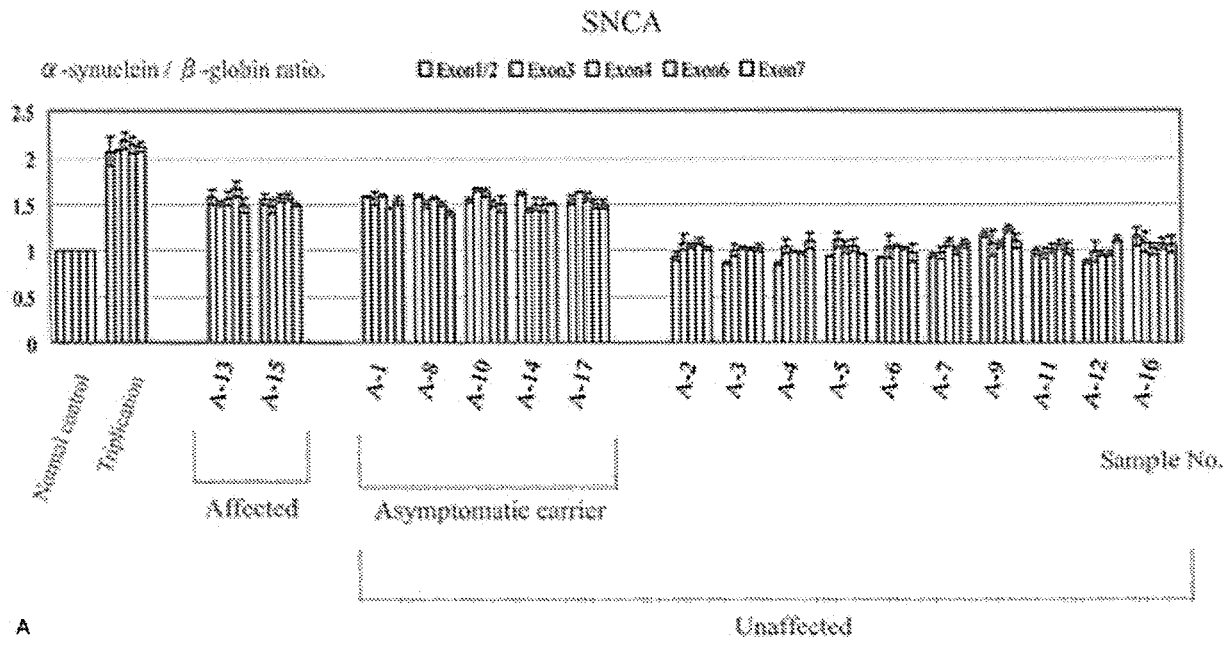


Fig 1. The ratio of α-synuclein exon 3, used as a target gene, to β-globin, used as a reference gene, as determined by semiquantitative real-time polymerase chain reaction in: (A) 200 patients with sporadic PD (the ratio ranged from 0.8 to 1.3, suggesting that single SNCA *copy exists in one allele, and (B) 113 patients with autosomal dominant hereditary Parkinson's disease. Note the two cases of duplication ratio (the ratio is 1.46 in one patient and 1.48 in the other), and the single Iowa family triplication case with a ratio of 2.07.*

among 200 sporadic cases (Fig 1A) but detected two index patients (A-13 and B-1) with potential *SNCA* duplications among 113 autosomal dominant pedigrees using exon 3 of *SNCA* (Fig 1B). To confirm the entire region of the α-synuclein gene was multiplied, we performed the exon dosage analysis including exons 1/2, 4, 6, and 7. We confirmed duplication of this gene in two patients. Thus, we were able to confirm that two families (Families A and B) were ADPD with *SNCA* duplication. In Family A, two patients with duplication had typical PD whereas five carriers were asymptomatic (Fig 2A). In Family B, one patient had duplication of the *SNCA* gene; two members were carriers (see Fig 2B).

FISH analysis also confirmed the *SNCA* duplication in the two index patients (Fig 3A, B). Figure 3 shows



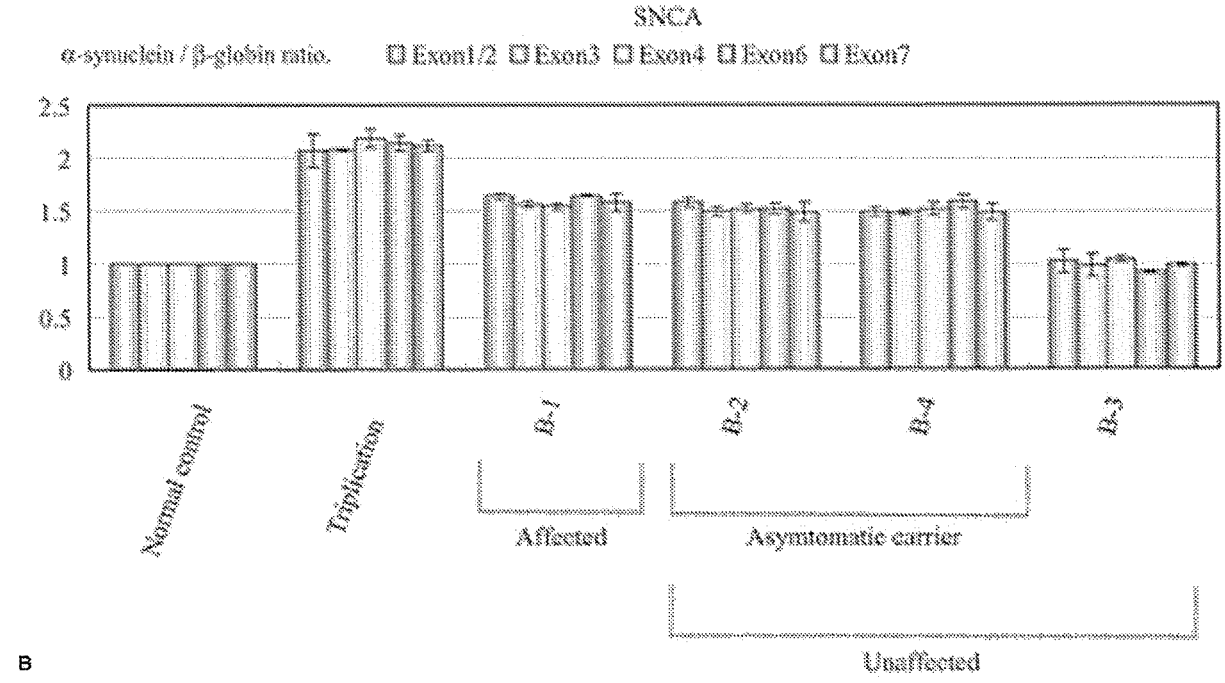


Fig 2. Results of screening for SNCA *multiplications for exons 1 to 7 in Family A (A). We detected two patients with* SNCA *duplication and five asymptomatic carriers in this family (a penetrance ratio of 33.3%) and (B) Family B. We detected three patients with* SNCA *duplication in four family members.*

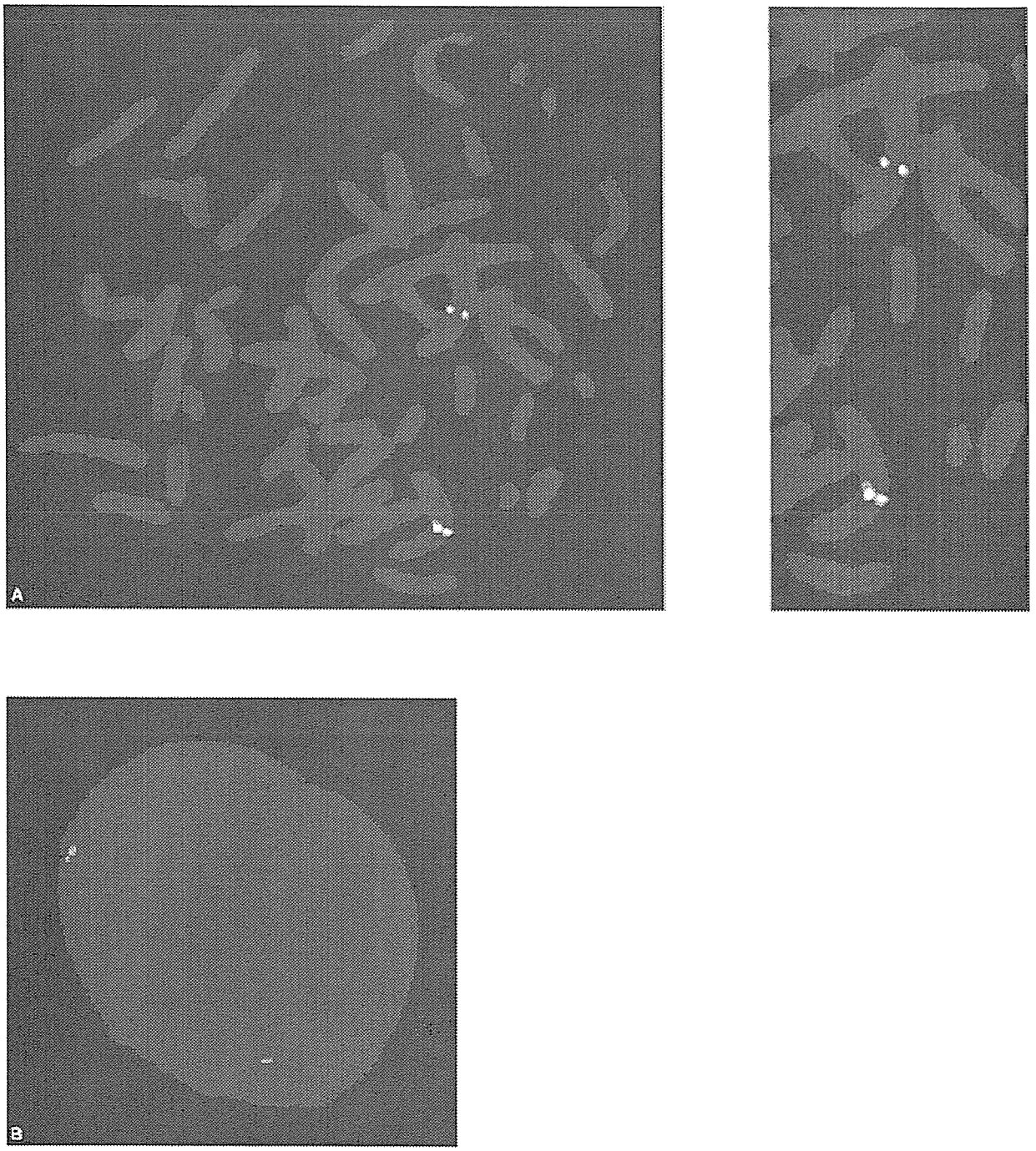


Fig 3. (A) Schematic representation of fluorescence in situ hybridization assay of metaphase chromosomes from Epstein–Barr virus (EBV)–transformed lymphocytes derived from Patients A-13 and B-1. We used BACs RP11-17p8 for normal control sample (shown in green and located 1.4Mb centromeric to SNCA, *left panel) and RP11-61407, which included the* SNCA *shown in red on chromosome region 4q21-22 (right panel). These pictures show clearly disproportional segregations compared with the normal control. (B) Standard one-color FISH of the interphase, using BACs RP11-61407. Note the two disproportional signals.*

the representative results of FISH analysis of interphase and metaphase chromosomes from EBV-transformed lymphocytes derived from Patients A-13 and B-1. We detected tight apposition of the metaphase chromatids compared with signals of BAC RP11-17P8 located 1.4Mb centromeric to *SNCA*. The intensity of the sig-

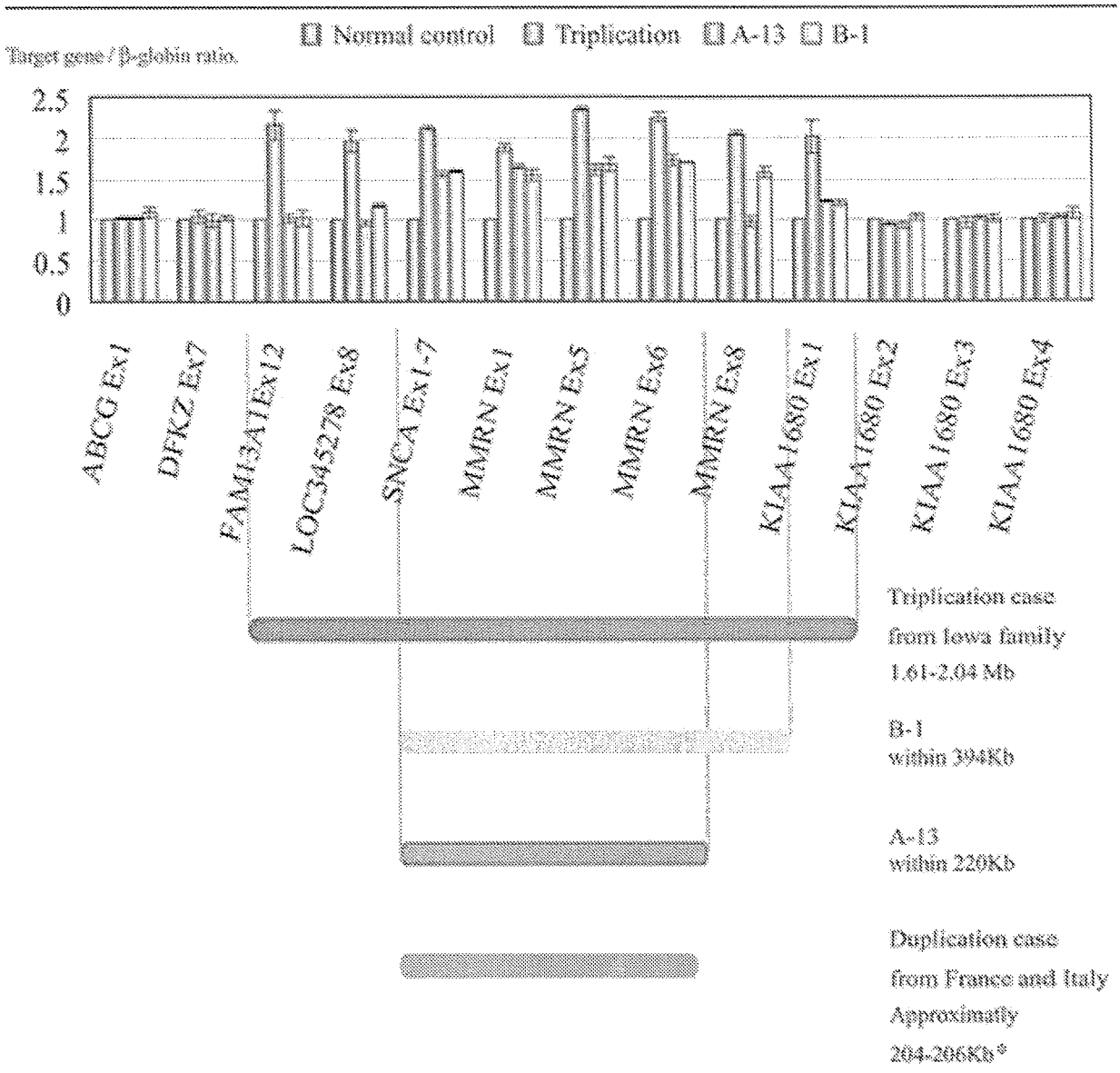


Fig 4. Identification of the region of SNCA *duplication between* ABCG *to* KIAA1680 *by using real-time semiquantitative polymerase chain reaction method. The different duplication region appears on* MMRN1 *Exon 8. *Duplication case as reported in Ibanez et al.*[13]

nal suggests *SNCA* duplication in these two patients. When considered together with the results of gene dosage analysis, we were able to confirm *SNCA* duplication. We did not observe two separate signals between BACs RP11-17P8 and PR11-61407, suggesting that the size of the duplication region is less than 1.4Mb. CGH array analysis showed that the specific elevation ratio could not be detected because the *SNCA* region could not be directly included in BAC probes used in MCG Whole Genome Array-4500. However, this BAC-based array contains BACs RP11-49M7 and RP11-17p8 that are close to 5′ or 3′ sites of *SNCA*, respectively. Alternatively, this finding indicates that the *SNCA* duplication region is less than 0.7Mb based on information archived by the database of UCSC (http://genome.ucsc.edu) and NCBI (http://www.ncbi.nlm.nih.gov). Although MCG Whole Genome Array-4500 covers the entire genome, no specific multiplication or deletions existed in other regions apart from 4q21-22. Identification of the *SNCA* duplication region was carefully assessed by gene dosage analysis for flanking genes around *SNCA* (Fig 4). The length of *SNCA* duplication of Patient A-13 spanned all of *SNCA* and part of *MMRN1* such as exons 1 to 6. In

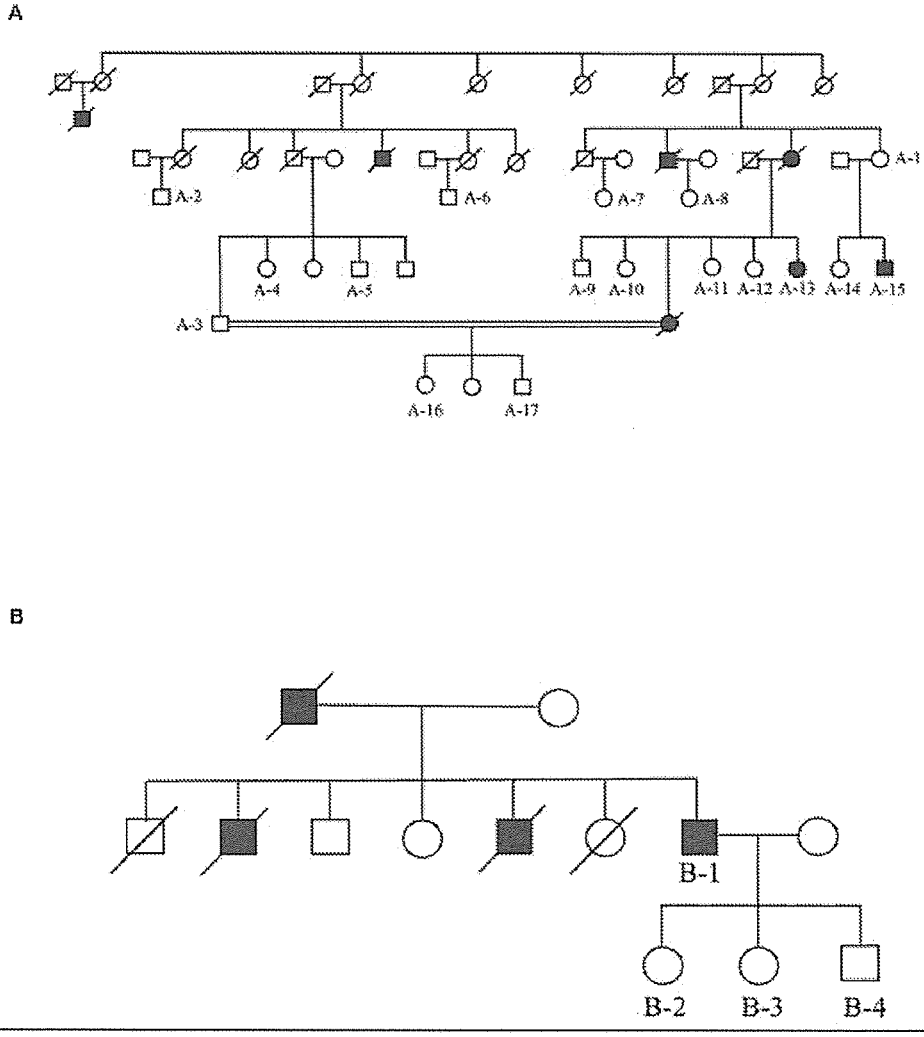


Fig 5. (A) Pedigrees of Patient A-1 with Parkinson's disease (PD) showing four generations. Black boxes represent affected patients. Symbols with numbers represent family members who were examined clinically by neurologists and from whom blood samples were collected. In 17 members, two patients were affected and five members (A-1, A-8, A-10, A-14, A-17) were carriers. Among seven carriers with SNCA *duplication, the ages of all carriers except for A-17 were beyond the mean age at onset of patients with* SNCA *duplication. Thus, the penetrance ratio was 33.3% (two patients/six asymptomatic carriers). (B) Pedigree of Patient B-1 with PD showing three generations. Symbols are as for Figure 6A. In four members, one patient was affected, and two members were carriers.*

contrast, the duplication region of Patient B-1 spanned all of *SNCA* and *MMRN1*. In addition, the regions of both patients did not span *LOC345278* and in Patient B-1, no duplication of *KIAA1680* was observed. Thus, the length of the duplication of Patient A-13 was shorter than that of Patient B-1, suggesting that the different lengths of the duplications differ by approximately 100 to 200kb. Furthermore, these two families have different allele sizes in microsatellite markers, suggesting that SNCA duplication is also de novo (data not shown). Clinical data, including the results of neuroimaging such as magnetic resonance imaging (MRI) and single-photon emission computed tomography (SPECT) and [^{123}I] meta-iodobenzylguanidine (MIBG) myocardial scintigraphy, are described below.

Family A

We collected DNA samples from 17 members of this family, including three affected and 14 unaffected members (Fig 5A). Among the three affected members, one patient (A-2) had no *SNCA* duplication. In addition, the age at onset of parkinsonism was 74 years. Moreover, L-dopa responsiveness was not excellent. Although MRI examination was not available, we considered that the cause of PD in this patient was not duplication but rather vascular parkinsonism based on

neurological findings. The mean age at onset of the disease was 43 years. The parents of A-16 and A-17 were close relatives. Five asymptomatic carriers were recognized by genomic analysis. No parkinsonism was observed in these asymptomatic carriers based on clinical neurological examination by two expert neurologists (K.N. and N.H.). The youngest age at onset was 38 years including the deceased patient (50 years old at onset). Thus, age 43 years was the cutoff age in this family. Considering this point, the penetrance ratio was 33.3% (2/6).

Patient A-13

The age of onset was 48 years. The initial symptom in Patient A-13 was rigidity and bradykinesia. She responded well to L-dopa. Six years after commencement of treatment with L-dopa, she developed drug-induced dyskinesia, which subsequently showed marked resolution. No tremor at rest has yet been noted. During the day, clinical assessment indicated Hohen and Yahr stage III. No dementia has developed yet and she has no symptoms related to autonomic nervous system dysfunction. Brain MRI study showed no abnormal mass or ischemic changes (Fig 6A) and ^{123}I-IMP SPECT study showed no evidence of hypoperfusion. However, the H/M ratio of MIBG myocardial scintigraphy was less than that of the normal control (A-13; early: 1.4, late: 1.24; see Fig 6D, E).

Patient A-15

The age at onset was 38 years. This patient was the cousin of Patient A-13. The initial symptom was gait disturbance with frequent falls. Tremor and autonomic nervous dysfunction were not seen. He was diagnosed with depression during the course of the disease, but neither dementia nor cognitive deterioration was prominent. The clinical course of this patient was similar to that of Patient A-13. Although this patient responded to L-dopa, he showed excellent response to anticholinergic agents such as trihexyphenidyl hydrochloride rather than L-dopa. In addition, the patient developed psychosis at 43 years of age.

Family B

DNA samples were collected from four members of Family B (see Fig 5B). Among the two generations, the number of affected member was four including three deceased members, and the unaffected members were three including two carriers with *SNCA* duplication. The age of asymptomatic carriers (B-2, B-3, and B-4) was younger than 35 years at the time of collection of DNA samples. Thus, it is difficult to speculate whether these carriers will develop PD in the future.

Patient B-1

The age at onset was 47 years. In the early stage, he responded to L-dopa; however, at 58 years of age, the disease was evaluated as stage III. Moreover, the gait disturbance and bradykinesia worsened and he suffered from cognitive dysfunction a few years later. Since 61 years of age, he has found it difficult to communicate with others and started gradually to develop abnormal behavior. Mini-Mental State Examination score was 17/30 at 61 years of age. At 62 years, his gait disturbance and hallucination worsened. At 64 years, he spent most of the day on the bed and required tracheostomy because of repeated episodes of aspiration pneumonia. Brain MRI showed moderate dilation of Sylvian fissure and atrophic changes in the temporal lobe on both sides. There was no evidence of ischemic changes or abnormal mass (see Fig 6B). A 99m-Tc-ECD SPECT study showed hypoperfusion predominantly on both frontotemporal lobes (see Fig 6C). The H/M ratio of MIBG myocardial scintigraphy was reduced (B-1; early: 1.40, late: 1.24).

Subject B-4

Subject B-4 was mentally retarded and had autism and generalized seizure. Since 1 year of age, he could not speak and was diagnosed with mental retardation by a pediatrician. At 12 years of age, he started to speak a few words and was sometimes observed to have sudden outburst of rage. At 15 years, he developed generalized seizure. EEG showed spiking waves predominantly localized to the right frontal lobe. Brain computed tomography scan showed no abnormal densities or other signs. No parkinsonism has been noted so far.

Table 2 summarizes the clinical features of these cases, including the results of neuroimaging and MIBG scintigraphy.

Discussion

Several recent studies suggest *SNCA* multiplications are a rare cause of PD, PDD, and DLBD.[22,28,29] In this study, we detected *SNCA* duplication in PD patients from 2 of 113 unrelated Japanese families with autosomal dominant parkinsonism. Thus, the incidence of *SNCA* multiplication may be more frequent than previously estimated. To our knowledge, the Iowa family and a single family of Swedish-American descent have been reported previously to have *SNCA* triplication.[11,19] In addition, two French families and one Italian family with *SNCA* duplication have been reported.[12,13] Taken together with this study, a total of seven families with *SNCA* multiplication, including triple and double *SNCA* copies, have been reported worldwide.

For all patients with *SNCA* duplication reported here, including patients of Family A, the phenotype was indistinguishable from idiopathic PD and no other

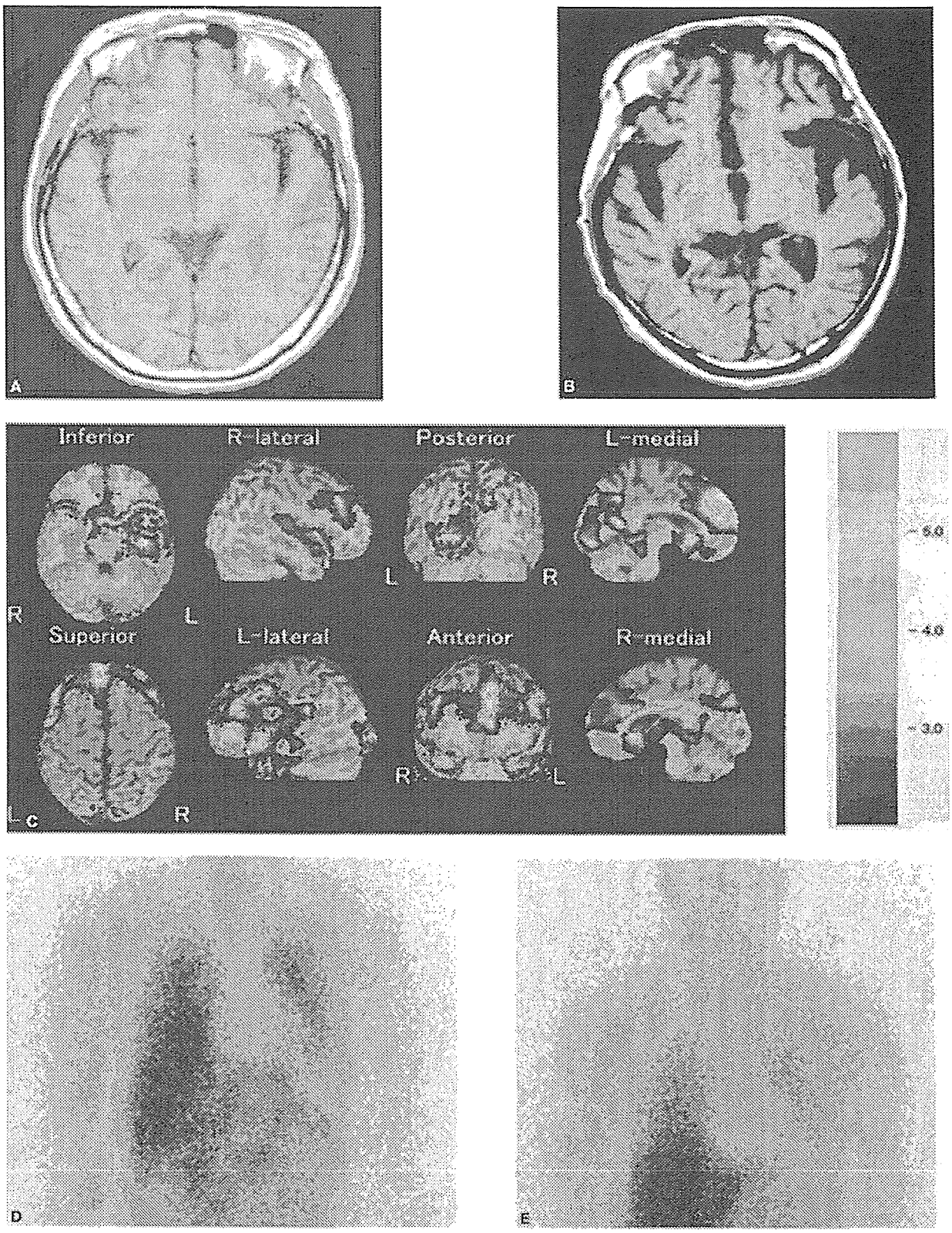


Fig 6. (A) Brain magnetic resonance imaging (MRI) T1 wedge study of Patient A-13. No abnormal masses or ischemic changes were evident. (B) Brain MRI T1 wedge study of Patient B-1. Note the dilation of Sylvian fissure and atrophic changes in both temporal lobes. (C) ^{123}I-IMP SPECT study of Patient B-1. Note the hypoperfusion of both frontotemporal lobes and medial-occipital lobes. (D, E) [^{123}I]meta-iodobenzylguanidine (MIBG) myocardial scintigraphy (D; early, E; late) of Patient A-13. The H/M ratio was reduced in this patient.

Table 2. Clinical Features of Four Affected Patients in Two Unrelated Pedigrees

	A Family			B Family
Feature	A-13	A-15	A-2	B-1
Age (yr)	57	43	77	65
Age at onset (yr)	48	38	74	47
Disease duration (yr)	10	6	4	19
Initial symptom	Rigidity	Rigidity	Bradykinesia	Bradykinesia
Bradykinesia	+	+++	++	+++
Rigidity	+++	+++	++	+++
Resting tremor	−	−	−	−
Postural instability	−	+	+	−
UPDRS	10/108	32/108	27/108	−
MMSE	30/30	30/30	17/30	17/30
L-Dopa response	+++	+	−	+
SNCA duplication	+	+	−	+

UPDRS = Unified Parkinson's disease rating scale; MMSE = Mini-Mental Status Examination.

clinical features such as dementia were present, in contrast with families with *SNCA* triplication. Notably, dementia was observed in one patient of Family B. Therefore, it is important to screen PDD or DLB for *SNCA* multiplications. However, the age of onset of PD in the patient with dementia was older than that of Iowa patients (36.0 ± 10.5 years) and the patient of Swedish-American family (31 years).[19] Moreover, the age at onset of Japanese patients was similar to those of other families with *SNCA* duplication (48.4 ± 15.0 years). In addition, the asymptomatic carrier, B-2, had epilepsy, which has been reported in one French PD patient.[13] In addition, autism was observed in the same patient, although no clear parkinsonism was evident. Patient B-1 had dementia, in contrast with previously reported cases with *SNCA* duplication, although the duration of the disease was longer (18 years) compared with reported cases of *SNCA* duplication. In addition, dementia only appeared after 14 years of diagnosis of parkinsonism. Therefore, *SNCA* duplication may be a risk factor for development of dementia.

Within each kindred the SNCA multiplication is a de novo mutation. The 4q21 genomic duplication in Patient B-1 included all of *SNCA* and *MMRN1*, whereas the duplicated region in Patient A-13 contained all of *SNCA* but only part of *MMRN1*. The *SNCA* triplication in the Iowa family also contains *MMRN1*, suggesting that overexpression of MMRN1 plays a role in cognitive deficit.

However, northern blotting analysis indicates a paucity of expression for *MMRN1* in neurons.[30] It therefore is unlikely that the effects of *MMNR1* are related to the development of dementia. *MMRN1* more likely plays a role in hemostasis and if vasogenic factors, including platelets and endothelial cells, are involved in dementia, *MMRN1* overexpression may still contribute to the dementia phenotype.

Previous studies reported the association of cardiac denervation and parkinsonism caused by *SNCA* gene triplication.[31] Low H/M ratios by [^{123}I]MIBG myocardial scintigraphy were reported in patients with sporadic PD.[32,33] In contrast, the H/M ratio was not decreased in patients with *parkin* mutations who lacked LBs in the autopsied brains.[34] In this regard, this finding is similar in patients with *SNCA* multiplication.

This study showed that the disease penetrance of Family A was 33.3%. The current ages of the asymptomatic carriers in this family are beyond the mean age at onset of patients. Thus, the difference may be caused by the *SNCA* expression levels between patients and asymptomatic carriers. Considering the multiple copies of *SNCA*, the expression level could be important. Indeed, double expression level of this protein compared with the normal brain was identified in Iowa family with *SNCA* triplication.[19] In addition, several haplotypes in the promoter region of SNCA including the sequence repeat element Rep1 were shown to associate with increased risk for sporadic PD.[35,36] However, whether the promoter alleles are risk factors for the development of PD is currently controversial.

Recently, Mueller and colleagues reported that single nucleotide polymorphisms located within the 3′side of exons 5 and 6, but not promoter polymorphism, correlated significantly with PD.[35] However, the functional association between PD and the associated region of SNCA remains unclear. In our study, the presence of asymptomatic carriers indicated that not only *SNCA* dosage but also another genetic variability in *SNCA* may be a risk factor for the development of PD.

References

1. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003;21:256–259.

2. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998;18:262–265.
3. Hicks AA, Petursson H, Jonsson T, et al. A susceptibility gene for late-onset idiopathic Parkinson's disease. Ann Neurol 2002; 52:549–555.
4. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451–452.
5. Kitada T, Asakawa S, Hattori N, et al., Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
6. Le WD, Xu P, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 2003;33: 85–89.
7. Paisan-Ruiz C, Jain S, Evans EW, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 2004;44:595–600.
8. Pankratz N, Nichols WC, Uniacke SK, et al. Significant linkage of Parkinson disease to chromosome 2q36–37. Am J Hum Genet 2003;72:1053–1057.
9. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 1997;276: 2045–2047.
10. Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004;21:1158–1160.
11. Singleton AB, Farrer M, Johnson J, et al. α-Synuclein locus triplication causes Parkinson's disease. Science 2003;302: 841.
12. Chartier-Harlin MC, Kachergus J, Roumier C, et al. α-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 2004;364:1167–1169.
13. Ibanez P, Bonnet AM, Debarges B, et al., Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet 2004;25:1169–1171.
14. Zimprich A, Biskup S, Leitner P, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 2004;44:601–607.
15. Kruger R, Kuhn W, Leenders KL, et al. Familial parkinsonism with synuclein pathology: clinical and PET studies of A30P mutation carriers. Neurology 2001;22:1355–1362.
16. Zarranz JJ, Alegre J, Gomez-Esteban JC, et al. The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 2004;55:164–173.
17. Spillantini MG, Schmidt ML, Lee VM, et al. α-Synuclein in Lewy bodies. Nature 1997;28:839–840.
18. Vaughan J, Durr A, Tassin J, et al. The alpha-synuclein Ala53Thr mutation is not a common cause of familial Parkinson's disease: a study of 230 European cases. European Consortium on Genetic Susceptibility in Parkinson's Disease. Ann Neurol 1998;44:270–273.
19. Farrer M, Kachergus J, Forno L, et al. Comparison of kindreds with parkinsonism and α-synuclein genomic multiplications. Ann Neurol 2004;55:174–179.
20. Muenter MD, Forno LS, Hornykiewicz O, et al. Hereditary form of parkinsonism-dementia. Ann Neurol 1998;43:768–781
21. Hughes AJ, Daniel SE, Kilford L, et al. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinicopathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55: 181–184.
22. Johnson J, Hague SM, Hanson M, et al. SNCA multiplication is not a common cause of Parkinson disease or dementia with Lewy bodies. Neurology 2004;63:554–556.
23. Ariyama T, Inazawa J, Uemura Y, et al. Clonal origin of Philadelphia chromosome negative cells with trisomy 8 appearing during the course of alpha-interferon therapy for Ph positive chronic myelocytic leukemia. Cancer Genet Cytogenet 1995; 81:2023.
24. Saito-Ohara F, Fukuda Y, Ito M, et al. The Xq22 inversion breakpoint interrupted a novel ras-like GTPase gene in a patient with Duchenne muscular dystrophy and profound mental retardation. Am J Hum Genet 2002;71:637–645.
25. Inazawa J, Inoue J, Imoto I. Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. Cancer Sci 2004;95:559–563.
26. Sonoda I, Imoto I, Inoue J, et al. Frequent silencing of low density lipoprotein receptor-related protein 1B (LRP1B) expression by genetic and epigenetic mechanisms in esophageal squamous cell carcinoma. Cancer Res 2004;64:3741–3747.
27. Takada H, Imoto I, Tsuda H, et al. Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization. Cancer Sci 2005;96: 100–110.
28. Gispert S, Trenkwalder C, Mota-Vieira L, et al. Failure to find alpha-synuclein gene dosage changes in 190 patients with familial Parkinson disease. Arch Neurol 2005;62:96–98.
29. Lockhart PJ, Kachergus J, Lincoln S, et al. Multiplication of the alpha-synuclein gene is not a common disease mechanism in Lewy body disease. J Mol Neurosci 2004;24:337–342.
30. Hayward CP, Hassell JA, Denomme GA, et al. The cDNA sequence of human endothelial cell multimerin. A unique protein with RGDS, coiled-coil, and epidermal growth factor-like domains and a carboxyl terminus similar to the globular domain of complement C1q and collagens type VIII and X. J Biol Chem 1995;270:18246–18251.
31. Singleton A, Gwinn-Hardy K, Sharabi Y, et al. Association between cardiac denervation and parkinsonism caused by alpha-synuclein gene triplication. Brain 2004;127:768–772.
32. Nagayama H, Hamamoto M, Ueda M, et al. Reliability of MIBG myocardial scintigraphy in the diagnosis of Parkinson's disease. J Neurol Neurosurg Psychiatry 2005;76:249–251.
33. Orimo S, Ozawa E, Nakade S, et al. [^{123}I] metaiodobenzylguanidine myocardial scintigraphy differentiates corticobasal degeneration from Parkinson's disease. Intern Med 2003;42:127–128.
34. Suzuki M, Hattori N, Orimo S, et al. Preserved myocardial [^{123}I]metaiodobenzylguanidine uptake in autosomal recessive juvenile parkinsonism: first case report. Mov Disord 2005;20: 634–636.
35. Mueller JC, Fuchs J, Hofer A, et al. Multiple regions of alpha-synuclein are associated with Parkinson's disease. Ann Neurol 2005;57:535–541.
36. Pals P, Lincoln S, Manning J, et al. α-Synuclein promoter confers susceptibility to Parkinson's disease. Ann Neurol 2004;56: 591–595.

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Clinicogenetic Study of Mutations in *LRRK2* Exon 41 in Parkinson's Disease Patients From 18 Countries

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Abstract: We screened *LRRK2* mutations in exon 41 in 904 *parkin*-negative Parkinson's disease (PD) patients (868 probands) from 18 countries across 5 continents. We found three heterozygous missense (novel I2012T, G2019S, and I2020T) mutations in *LRRK2* exon 41. We identified 11 (1.3%) among 868 PD probands, including 2 sporadic cases and 8 (6.2%) of 130 autosomal dominant PD families. The *LRRK2* mutations in exon 41 exhibited relatively common and worldwide distribution. Among the three mutations in exon 41, it has been reported that Caucasian patients with G2019S mutation have a single-founder effect. In the present study, Japanese patients with G2019S were unlikely to have a single founder from the Caucasian patients. In contrast, I2020T mutation has a single-founder effect in Japanese patients. Clinically, patients with *LRRK2* mutations had typical idiopathic PD. Notably, several patients developed dementia and psychosis, and one with I2020T had low cardiac ^{123}I-metaiodobenzylguanidine (MIBG) heart/mediastinum ratio, although the ratio was not low in other patients with I2020T or G2019S. Clinical phenotypes including psychosis, dementia, and MIBG ratios are also heterogeneous, similar to neuropathology, in PD associated with *LRRK2* mutations. © 2006 Movement Disorder Society

Key words: genetics; Parkinson's disease; *PARK8*; *leucin-rich repeat kinase 2* (*LRRK2*); cardiac ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy

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Parkinson's disease (PD) is the second most common neurodegenerative disorder with a prevalence of 3% in individuals older than 65 years.[1] Although the majority of PD cases are sporadic, it is now clear that genetic factors contribute to the pathogenesis of PD. Indeed, *alpha-synuclein*,[2] *UCH-L1*,[3] and *LRRK2*[4,5] have been reported as causative genes of autosomal dominant PD (ADPD); and *parkin*,[6] *DJ-1*,[7] and *PINK1*[8] as causative genes of autosomal recessive PD (ARPD). Recently, *LRRK2* was identified as the causative gene for *PARK8*,[4,5] and a common single *LRRK2* mutation in exon 41 (G2019S) has been found in mainly Caucasian patients with familial PD.[9] In the present study, we performed direct sequencing of *LRRK2* exon 41 in a large number of patients with familial and sporadic PD of different ethnic origins. We also analyzed the haplotype to determine whether a single haplotype was associated with *LRRK2* mutations. In addition, we investigated the clinicogenetic features, including cardiac ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy, of patients with *LRRK2* mutations.

PATIENTS AND METHODS

All blood samples and clinical information were obtained by the neurologists working in the respective countries after obtaining informed consent from their patients. The study was approved by the ethics review committee of Juntendo University. Diagnosis of PD was made by the participating neurologists based on the presence of Parkinsonism, good response to anti-PD treatment, and radiological findings. In all countries, the clinical evaluation was based on the Unified Parkinson's Disease Rating Scale. Controls were evaluated by neurologists to ensure none of them had PD. For control subjects, the mean age of the Japanese was 56.1 years (range, 23–98) and that of the Taiwanese was 34.4 years (range, 21–86). DNA was prepared using standard methods. The mutation screening was performed as described previously.[6,10] Dideoxy cycle sequencing was performed with Big Dye Terminater Chemistry (Applied Biosystems, Foster City, CA). This was followed by exon sequencing on ABI377 and 310 automated DNA sequence analyzers (Applied Biosystems). None of the participants had *parkin* mutations (including exonic deletions and multiplications), as confirmed by polymerase chain reaction (PCR), direct sequencing, and quantitative assays based on real-time PCR with Taq-Man probes (Applied Biosystems) of all exons.

Direct sequencing of *LRRK2* exon 41 was performed in 904 PD patients (male, 413; female, 437; not reported, 54; age, 7–100 years; mean, 47.5 years), including 868 probands from 18 countries (Japan, Korea, Taiwan, China, Philippines, Australia, Israel, Tunisia, Morocco, Turkey, Greece, Poland, Bulgaria, Yugoslavia, United Kingdom, United States, Canada, and Brazil). Asian probands (Japanese, Koreans, Taiwanese, and Chinese) formed the majority (763/868; 87.9%). The remainder included 72 Caucasian probands (Israelis, Australians, Turkish, Greek, Polish, Bulgarians, Yugoslavs, British, Americans, and Canadians) and 15 North African probands (Tunisians and Moroccans). The other 18 probands were Filipinos (n = 3), Brazilians (n = 1), or could not be specified (n = 14). In this study, families with more than two affected members or two members with a mutation at least in two generations were classified as ADPD, and families with at least two affected siblings in only one generation as (potential or pseudo-) ARPD. Among 904 PD patients, we considered 130 families as ADPD, 80 families as ARPD, and 417 patients as sporadic PD.

Haplotype analysis was performed as described previously.[10,11] Genotyping of patients with *LRRK2* mutations was performed using the microsatellite markers (D12S2514, D12S2515, D12S2516, D12S2518, D12S2519, D12S2520, D12S2521, D12S2522, and D12S2523) and the single nucleotide polymorphisms (rs1427263, rs11176013, and rs11564148).[10] Alleles were sized by GENESCAN (Applied Biosystems).

Cardiac MIBG scintigraphy was performed with an intravenous injection of 111 MBq of ^{123}I-MIBG (Daiichi Radioisotope Laboratories, Tokyo, Japan). Early images were obtained 15 minutes and delayed images were obtained 3 to 4 hours after injection. Whole cardiac MIBG uptake was measured on a planar image as the heart/mediastinum (H/M) activity ratio.

RESULTS

Genetic Studies

We found two heterozygous missense mutations (6059T>C, I2020T, and 6055G>A, G2019S), which had been reported previously,[5,9,12–14] and one novel heterozygous missense mutation (6035T>C; I2012T; Fig. 1). The novel I2012T mutation is also highly conserved across various species, similar to the other previously reported mutation sites of I2020T and G2019S.[5,12–14] We did not find any of these mutations in 200 chromosomes from normal Taiwanese and 200 chromosomes from normal Japanese populations. We found 3 Japanese patients (two probands) with the I2020T. Patient A1 with the novel I2012T mutation was of Taiwanese origin.

Including all ethnic groups, we found 11 (1.3%) of 868 PD probands and 8 (6.2%) of 130 ADPD families (1 Taiwanese, 2 Japanese, 2 Israelis, and 3 Tunisians) with