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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Results of single nucleotide polymorphisms (SNPs) linear regression analysis
**Table S2.** Results of haplotype linear regression analysis

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

# A New Microdeletion Syndrome of 5q31.3 Characterized by Severe Developmental Delays, Distinctive Facial Features, and Delayed Myelination

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Chromosomal deletion including 5q31 is rare and only a few patients have been reported to date. We report here on the first two patients with a submicroscopic deletion of 5q31.3 identified by microarray-based comparative genomic hybridization. The common clinical features of both patients were marked hypotonia, feeding difficulty in infancy, severe developmental delay, and epileptic/nonepileptic encephalopathy associated with delayed myelination. Both patients also shared characteristic facial features, including narrow forehead, low-set and abnormal auricles, bilateral ptosis, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate. The deleted region contains clustered PCDHs, including and *PCDHG*, which are highly expressed in the brain where they function to guide neurons during brain development, neuronal differentiation, and synaptogenesis. The common deletion also contains neuregulin 2 (*NRG2*), a major gene for neurodevelopment. We suggest that 5q31.3 deletion is responsible for severe brain developmental delay and distinctive facial features, and that the common findings in these two patients representing a new microdeletion syndrome. We need further investigations to determine which genes are responsible for the patients' characteristic features. © 2011 Wiley-Liss, Inc.

**Key words:** microdeletion; 5q31.3; array-based comparative genomic hybridization (aCGH); developmental delay; protocadherin (PCDH); neuregulin 2 (*NRG2*)

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## INTRODUCTION

Interstitial deletions of the long arm of chromosome 5 are rare, except in the 5q35.2q35.2 region that includes the 2-Mb *NSD1* locus which is associated with Sotos syndrome [Visser and Matsumoto, 2003]. Although patients with proximal deletions that encompass the 5q15 to q22 region experience mild developmental delays, those with distal deletions that encompass the 5q22 to q31 region are more severely handicapped, fail to thrive, and present with significant craniofacial dysmorphism and joint dislocations or contractures [Garcia-Minaur et al., 2005]. Furthermore, there are only a few reports of patients with deletions encompassing the 5q31.3 region [Felding and Kristoffersson, 1980; Kramer et al., 1999; Arens et al., 2004].

Recently, we encountered 2 patients with severe developmental delay and distinctive facial features. Microarray-based comparative genomic hybridization (aCGH) analyses identified a common microdeletion of 5q31 in both patients. Radiological examination yielded characteristic finding with delayed myelination in both patients. The details of these cases are discussed in this report. Data on the patients were deposited in the DECIPHER database (Database of Chromosomal Imbalances and Phenotype in Humans using Ensembl Resources, https://decipher.sanger.ac.uk), and the corresponding DECIPHER number is given.

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## CLINICAL REPORTS

### Patient 1 (DECIPHER #TWM253734)

A Japanese boy was born at 40 weeks 5 days gestation by caesarean when labor had failed to begin. He is the first child of a 30-year-old father and a 26-year-old mother at the time of his birth. His birth weight was 2,925 g (−0.3 SD), length 50 cm (+0.5 SD), and head circumference 35 cm (+1.3 SD). Postaxial polydactyly of the right hand was noted. Patent ductus arteriosus (PDA) and a small ventricular septal defect (VSD) were revealed by echocardiography; PDA was surgically treated when he was 52 days old, and the small VSD was observed but not treated. He showed failure to thrive due to severe hypotonia and feeding difficulty, and aspiration was suspected because of recurrent pneumonia. Tube feeding was initiated at 6 months of age. Although he had no epileptic episodes, his electroencephalography showed spike waves on the right side of the posterior and occipital regions during natural sleep. Auditory brainstem response revealed obscure III waves in both sides, and the threshold was 40 dB. His median nerve conductive velocity (NCV) showed a delay with 32.5 m/s (−2.1 SD) on the left and 30.5 m/s (−2.5 SD) on the right. His posterior tibial NCV was also revealed to be delayed with 27.6 m/s (−3.2 SD) on both sides. These findings indicated peripheral neuropathy.

At 18 months of age, he showed delayed growth and microcephaly with height 76.4 cm (−1.6 SD), weight 8.7 kg (−1.7 SD), and head circumference 42.8 cm (−3.0 SD). He showed distinctive features including narrow forehead, low-set and abnormal auricles, bilateral ptosis, epicanthic folds, depressed nasal bridge, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate (Fig. 1A). His developmental milestones were markedly delayed with no contact eye movements, no smile response, and no head control. Brain magnetic resonance imaging (MRI) revealed reduced volume of the cerebrum and severely delayed myelination (brain appearance was that of an 8-month-old child) in T2-weighted imaging (Fig. 2A). Chromosomal G-banding showed a normal male karyotype.

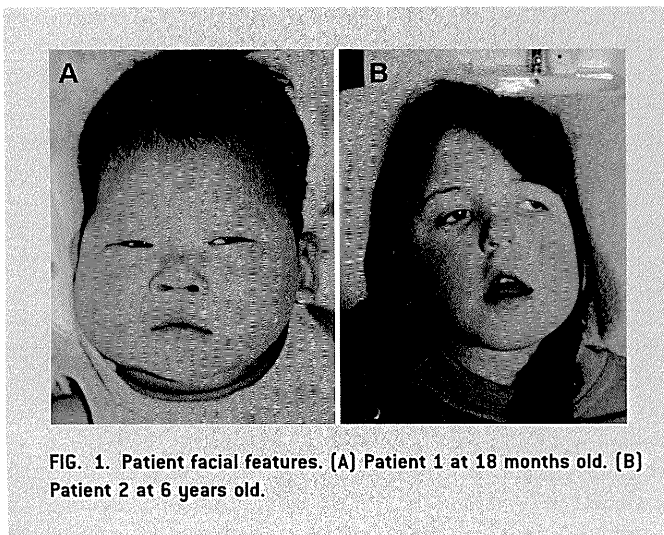


FIG. 1. Patient facial features. (A) Patient 1 at 18 months old. (B) Patient 2 at 6 years old.

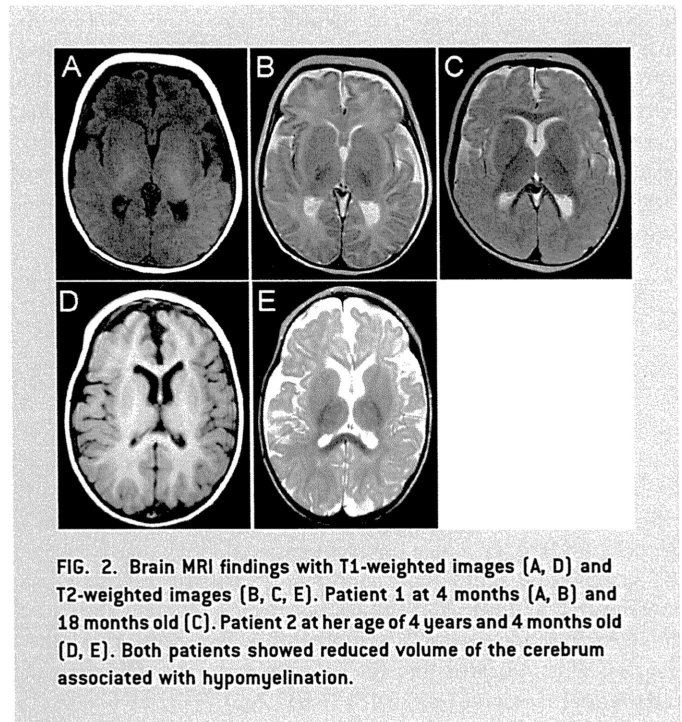


FIG. 2. Brain MRI findings with T1-weighted images (A, D) and T2-weighted images (B, C, E). Patient 1 at 4 months (A, B) and 18 months old (C). Patient 2 at her age of 4 years and 4 months old (D, E). Both patients showed reduced volume of the cerebrum associated with hypomyelination.

### Patient 2 (DECIPHER #4681)

An 8-year-old French girl had no family history and no consanguinity in her parents. There was no complication during pregnancy. She was born with a birth weight of 3,700 g (+0.5 SD), a length of 52 cm (+1.0 SD), and a head circumference of 36 cm (+1.0 SD). Since early infancy, she showed feeding difficulties due to severe hypotonia. She had severe developmental delay with sitting at 11 months. Since the age of 12 months, she suffered epileptic seizures which were drug-resistant (hydrocortisone, clonazepam, topiramate, lamotrigine). Her epileptic status was diagnosed as Lennox–Gastaut syndrome.

She was of relatively small stature at a height of 121 cm (−1.5 SD), had a weight of 16.7 kg (−2.5 SD), and head circumference of 50 cm (−1.5 SD). She was not able to walk unassisted and was apraxic for speech. Her features were distinctive with narrow forehead, low-set ears, bilateral ptosis, downslanting palpebral fissures, anteverted nares, long philtrum, tented upper vermilion, edematous cheeks, and high palate (Fig. 1B). Strabismus was also noted. Brain MRI examination showed ventriculomegaly with reduced volume of the cerebrum, particularly in the frontoparietal regions, and marked hypomyelination (Fig. 2B). Conventional chromosome analysis showed a normal female karyotype.

## MATERIALS AND METHODS

For further evaluation, microarray-based comparative genomic hybridization (aCGH) analyses, using Human Genome CGH Microarray 105A for Patient 1 and 44A for Patient 2 (Agilent Technologies, Santa Clara, CA), were performed according to the manufacturer's protocol, with genomic DNAs extracted from

peripheral blood samples. The identified aberrations were confirmed by fluorescence in situ hybridization (FISH) analyses, and both patients were also analyzed by FISH. Parental origin of the deletion in Patient 1 was determined using the microsatellite marker D5S1979 according to methods described elsewhere [Komoike et al., 2010]. Information regarding the primers used for the marker was obtained from the in-silico library (http://genome.ucsc.edu/).

## RESULTS

Losses of genomic copies of 5q31.3 were identified in both patients. Patient 1 showed a 5.0-Mb deletion with molecular karyotyping as arr chr5q31.2q31.3(137,538,788–142,574,719)(hg18)x1 and Patient 2 showed a 2.6-Mb deletion with molecular karyotyping as arr chr5q31.3q31.3(139,117,448–141,682,547)(hg18)x1 (Fig. 3). FISH analyses with only one signal for the targeted probe confirmed the deletion (Fig. 4), and subsequent parental FISH analyses using the same probe showed no abnormality in their parents (data not shown), indicating de novo occurrence. Patient 1 shared the D5S1979 allele with his mother but not with his father (Fig. 4). This indicated that the deletion was paternally derived, and the final karyotype was ish del(5)(q31.2q31.3)(RP11-678N8x1) dn pat.

## DISCUSSION

Both the patients in the present study showed an overlapping deletion of the region that included 5q31.3. The clinical features that were common for both patients were marked hypotonia, feeding difficulties in infancy, severe developmental delay, and epileptic/nonepileptic encephalopathy. Both patients also showed similar characteristic facial features, including a narrow forehead, low-set and abnormal auricles, bilateral ptosis, anteverted nares, long philtrum, tented vermilion of the upper lip, edematous cheeks, and high palate. Another characteristic finding was delayed myelination of the white matter, as identified by MRI examination. Thus, these findings are consistent, recognizable, and clinical features of 5q31.3 deletion.

To the best of our knowledge, five reports on patients with chromosome 5q31 deletions are available in the literature (Fig. 5). The first patient reported by Felding and Kristoffersson had manifestations similar to those of our patients [Felding and Kristoffersson, 1980]. Kramer et al. [1999] reported on a patient with 5q31q33 deletion whose condition was severely impaired; this patient showed congenital anomalies and died in the neonatal period. Arens et al. [2004] reported a patient with 5q22.1q31.3 deletion whose clinical findings included growth retardation, moderate psychomotor retardation, and mild facial dysmorphisms were similar to those of our patients. However, the severity of the developmental delay was milder than that of our patients, because she could walk without support and could speak a few words. These three patients were suspected to carry deletions of 5q31.3, but the deletion regions were ambiguous in conventional G-banding examination, and no neuroimaging test was available. We were thus unable to compare these patients with ours.

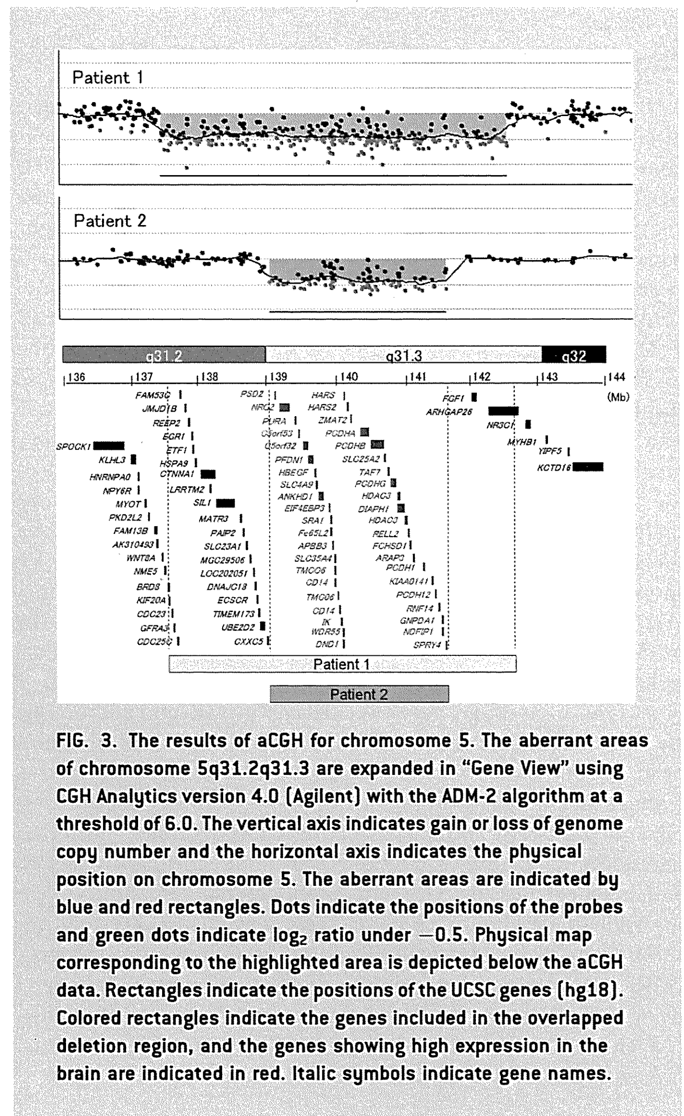


FIG. 3. The results of aCGH for chromosome 5. The aberrant areas of chromosome 5q31.2q31.3 are expanded in "Gene View" using CGH Analytics version 4.0 (Agilent) with the ADM-2 algorithm at a threshold of 6.0. The vertical axis indicates gain or loss of genome copy number and the horizontal axis indicates the physical position on chromosome 5. The aberrant areas are indicated by blue and red rectangles. Dots indicate the positions of the probes and green dots indicate $\log_2$ ratio under −0.5. Physical map corresponding to the highlighted area is depicted below the aCGH data. Rectangles indicate the positions of the UCSC genes (hg18). Colored rectangles indicate the genes included in the overlapped deletion region, and the genes showing high expression in the brain are indicated in red. Italic symbols indicate gene names.

Tzschach et al. [2006] reported on a patient with failure to thrive, psychomotor retardation, and mild facial dysmorphic features who carried a de novo deletion of 5q23.3q31.2, which did not overlap with those of our patients [Tzschach et al., 2006]. Mosca et al. [2007] reported a girl presenting with an abnormal cry, upslanting palpebral fissures, hypertelorism, anteverted nostrils, microretrognathia, growth retardation, and an adenoid cyst at the base of the tongue [Mosca et al., 2007]; the chromosomal deletion in this girl partially overlapped with that in Patient 1 of the present study, but the deletion did not involve the 5q31.3 band (Fig. 5).

In the present study, the common 2.6-Mb deletion region within the chromosomal band 5q31.3 is gene rich, containing 40 genes (UCSC Human genome browser, March 2006; http://genome.ucsc.edu/). The most intriguing finding is that the deleted region contained 5 genes classified as the protocadherin (PCDH) family which can be further divided into two main categories including clustered and non-clustered [Morishita and Yagi, 2007]. The clustered PCDHs including *PCDHA*, *PCDHB*, and *PCDHG*, which

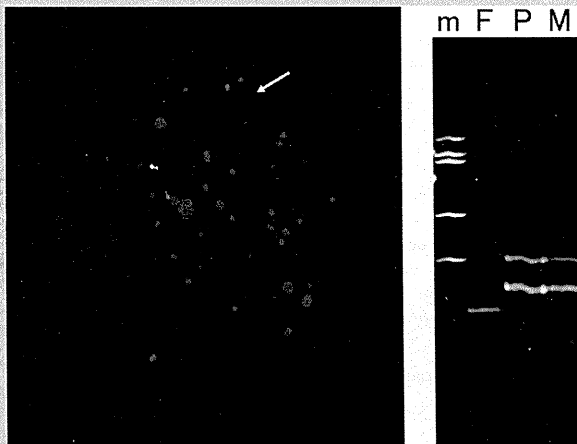


FIG. 4. Molecular cytogenetic validations for Patient 1. Two-color FISH analysis for patient 1 (left) using a combination of the BAC clones RP11-94J21 (red) and RP11-678N8 (green) as probes according to methods described elsewhere. A single green signal indicates the deletion of this region on the chromosome 5 (arrow). Microsatellite marker analysis of D5S1979 (right) separated on a polyacrylamide gel and visualized by staining with ethidium bromide. The bands for this patient are common only with those of the mother. m, molecular size marker; P, patient; F, father; M, mother. BAC clones RP11-94J21 and RP11-678N8 were selected from UCSC Human genome browser build 2006 (http://genome.ucsc.edu/cgi-bin/hgGateway).

are sequentially organized on the 5q31.3 region (Fig. 3) [Yagi, 2008]. The other non-clustered PCDHs, *PCDH1* and *PCDH12*, were also included in this region. PCDHs are highly expressed in the brain where they play roles in directing neurons during brain development, neuronal differentiation, and synaptogenesis [Akins and Biederer, 2006]. Although clustered PCDHs are suspected to have more important roles than non-clustered PCDHs in neuronal development, human diseases that are associated with clustered PCDHs have not yet been reported. The genomic organization of *PCDHA* and *PCDHG* includes multiple variable exons and a set of constant exons, similar to the gene encoding immunoglobulins and T-cell receptors [Morishita and Yagi, 2007; Takeichi, 2007]. These exons are combined by cis-splicing of the mRNA, leading to the production of a large number of isoforms and generating more than 50 transcripts from each gene, with various extracellular domain sequences [Morishita and Yagi, 2007; Takeichi, 2007]. Because of these characteristics, *PCDHA* and *PCDHG* are classified as clustered PCDHs. The expression mechanism of clustered PCDHs is also unique; different mouse neurons were found to express different sets of *Pcdha* and *Pcdhg*, indicating monoallelic gene expression that is unique to the clustered PCDHs [Esumi et al., 2005; Hirayama and Yagi, 2006].

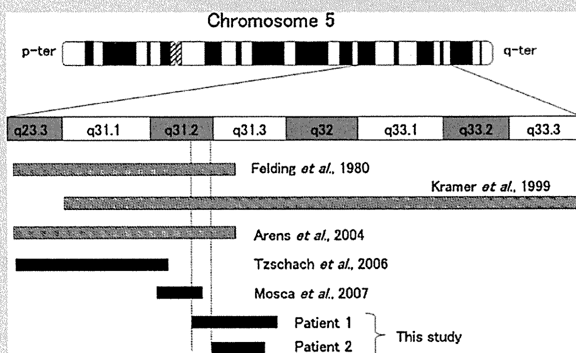


FIG. 5. Physical maps of the 5q31.3 region depict the regions deleted in the previously reported patients. Black rectangles indicate the range of the deletion, and the rectangles with diagonal lines indicate the suspected deletion region based on G-banded karyotyping. The reference author names are also indicated.

Although mutations of human *PCDHA*, *PCDHB*, and *PCDHG* have not been reported, hypomorphic *Pcdha* mutant mice exhibit enhanced contextual fear conditioning and abnormal spatial learning [Fukuda et al., 2008]. Morpholino-based reduction in levels of full-length Pcdh1a protein results in a dramatic increase in the extent of neuronal programmed cell death [Emond and Jontes, 2008]. These observations are similar to those in *Pcdhg−/−* mice that exhibit a loss of spinal interneurons [Wang et al., 2002]. Heterozygous mice of both *Pcdha* and *Pcdhg* have not been reported to show any neurological pathologies [Wang et al., 2002; Fukuda et al., 2008]; however, functional relevance of both *PCDHA* and *PCDHG* to human disorders cannot be denied, because mice heterozygous for the knockout alleles such as *Nsd1* and *Foxc1* show no manifestations [Rayasam et al., 2003; Aldinger et al., 2009]. Hemi-allelic deletions of the human homogues, *NSD1* and *FOXC1*, are associated with human disorders, i.e., Sotos syndrome and Dandy–Walker malformation, respectively. These findings suggest the biological difference between mice and human.

Another study showed that myelination functions as a trigger for the decline in Pcdha expression [Morishita et al., 2004]. Delayed myelination was another characteristic of our patients and may be associated with the deletions of *PCDHA*. Furthermore, *PCDHA* and *PCDHG* exhibit monoallelic expression [Esumi et al., 2005]. Thus, partial monosomy of 5q31.3 may affect the function of *PCDHA* and/or *PCDHG*.

By use of the UCSC genome browser, 6 genes other than *PCDHA* and *PCDHG* were found to be highly expressed in the brain among the 40 genes included in the common deletion region (Supplemental Table SI online). Neuregulin 2 gene (*NRG2*) was one of the 6 genes. *NRG2* is a member of the neuregulin family of signaling proteins that mediate cell–cell interactions in the nervous system and other organs [Rimer, 2007]. Recent genetic, transgenic, and postmortem brain studies support a potential contribution of NRG1-erbB4 signaling in schizophrenia [Banerjee et al., 2010]. Furthermore, *NRG2* is predominantly expressed by neurons in the central nervous system and exerts its effects on the perisynaptic Schwann cells at the neuromuscular junction [Longart et al., 2004; Rimer, 2007], suggesting a possible association of *NRG2* with neurological diseases. The findings of histological examinations of

the brain of *Nrg2* transgenic mice did not differ from those of the wild-type or heterozygous mice; however, homozygous knockout mice showed severe growth retardation, increased morbidity, and reduced reproductive capacity [Britto et al., 2004]. Thus, the peripheral neuropathy in Patient 1 may be attributable to *NRG2* deletion.

In this study, we reported the first two patients with deletions of the 5q31.3 region. We suggest that the deletion of 5q31.3, including clustered PCDHs and *NRG2*, lead to severe developmental delays, distinctive facial features, and delayed myelination. These characteristic manifestations comprise a new recognizable microdeletion syndrome. Although many genes in this region are highly expressed in the brain, the genes that specifically contributed to the unique characteristics of our patients could not be determined, because the crucial functions of the genes involved in the deletion region remain to be elucidated. Further studies need to be conducted to identify the genes that were associated with the characteristic features of our patients. Microcephaly was observed in Patient 1, but the head circumference of Patient 2 was within normal limit. Therefore, the gene associated with microcephaly in Patient 1 might be excluded from the deletion region that was common to both patients.

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NEUROPATHOLOGY

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Original Article

# Immunohistochemical expression of fibroblast growth factor-2 in developing human cerebrum and epilepsy-associated malformations of cortical development

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**To elucidate the biological significance of fibroblast growth factor-2 (FGF-2) expression in epilepsy-associated malformations of cortical development, immunohistochemical expression of FGF-2 was investigated in the developing human cerebral mantles obtained from 30 autopsy cases of fetuses, stillborn infants and children ranging from 12 weeks gestation to 15 years old, and 70 surgically-resected corticectomy specimens from patients with medically intractable epilepsy, including: group I, 12 tubers of tuberous sclerosis; group II, 24 cases of focal cortical dysplasia (FCD) with balloon cells (BC); group III, 11 FCD without BC; group IV, 23 histologically normal-appearing neocortices from patients with Rasmussen encephalitis, cystic-gliotic encephalopathy, temporal lobe epilepsy; and group V, 14 normal-appearing neocortices adjacent to dysplastic lesions from groups I and II. FGF-2 expression was detected in a population of matrix cells and/or neuroblasts within the ventricular zone in fetuses younger than 19 weeks gestation. Nuclei of glioblasts and immature astrocytes were also positive for FGF-2 in cases older than 18 weeks gestation. FGF-2 expression was not detected in immature cortical plate neurons. Astrocytes and ependymal cells were positive for FGF-2 in the postnatal brains. Choroid plexus epithelium was strongly positive for FGF-2 in all cases examined. Among the corticectomy specimens, the cytoplasms and/or nuclei of dysmorphic neurons (DNs) and BCs in groups I and II were variably positive for FGF-2. The proportions of FGF-2 immunoreactive cells (FGF-2-IR%) was significantly higher in groups I (36.9 ± 9.6) and II (45.1 ± 7.0) than in groups III (21.0 ± 5.7), IV (14.4 ± 4.7) and V (24.3 ± 10.3), and that in group V was higher than in group IV ($P < 0.01$). These results indicate that FGF-2 upregulation in DNs and BCs is an important feature common to groups I and II, and suggest that BCs and DNs in these groups represent disturbed gliogenesis from matrix cells and disturbed maturation of cortical neurons from migrating neuroblasts, respectively.**

**Key words:** astrocyte, epilepsy, FGF-2, immunohistochemistry, malformation of cortical development (MCD).

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## INTRODUCTION

Malformations of cortical development (MCDs) constitute a family of disorders characterized by an abnormal cytoarchitecture of the cerebral cortex, presumably resulting from deranged migration of neuroblasts from ventricular and subventricular zones (VZ and SVZ, respectively) to

the cortical plate during early stages of intrauterine life.[1] Focal cortical dysplasia (FCD),[2] a subset of MCDs, is characterized by cortical laminar disorganization, the presence of dysmorphic neurons (DNs) and characteristic large gemistocytic astrocyte-like "balloon cells" (BCs), histologically classified as FCD type IIB[3] or FCD type IIb[4] in recent proposals. These histological features are very similar to those seen in cortical tubers of tuberous sclerosis complex (TSC-tubers),[5,6] despite different clinical presentations. TSC-tubers and FCD type IIb are presumed to be disorders of cell differentiation in early stages of the developing brain.[7] Recent evidence has suggested several factors significant in morphogenesis of BCs, including aberrant expression of cytoskeletal proteins,[8,9] stem cell markers such as nestin,[10] and CD34 class II,[11] and altered signaling pathways.[12,13] However, the origin of BCs is largely unknown.

The fibroblast growth factor (FGF) family consists of at least 23 different members, having two highly conserved core-domain regions.[14,15] Ten of 23 classes of the FGF family along with four receptors are expressed in the developing brain in animals.[14] Among them FGF-2 has been suggested to play several important roles not only in neuroprotection following brain insults such as ischemia,[16–18] traumatic injury,[19,20] and epilepsy,[21,22] but also in neurogenesis[23–25] and neuronal and glial differentiation[26,27] in the developing CNS. Our previous study using a relatively small number of cases has demonstrated that FGF-2 is expressed in the nuclei of astrocytes in normal-appearing neocortex, and DNs and BCs in TSC-tubers and FCD with BC but not in FCD without BC, and that higher proportions of FGF-2 immunoreactive cells (FGF-2-IR%) in TSC-tubers and FCD with BC than FCD without BC may reflect the likely timing of insults underlying the pathogenesis of each disorder.[28] However, the spatial and temporal alterations of FGF-2 expression in the developing human brain have not yet been fully described in the literature. One study has demonstrated FGF-2 expression in both neuroblasts and glioblasts in the cortical plate using human fetal brains of 12 to 16 weeks gestation.[29] In rat brain, FGF-2 expression has been shown in VZ, SVZ and cerebral cortex at the embryonic day (E18),[30] and subsequently in astrocytes but not neurons, except those in the hippocampus and cingulate cortex in the postnatal period.[31,32]

In the present study, immunohistochemical expression of FGF-2 was investigated in 30 autopsy cases of the developing human cerebrum and 70 surgically resected corticectomy specimens from patients with medically intractable epilepsy using tissue microarray for the quantitative evaluation of FGF-2-IR% to elucidate the biological significance of FGF-2 expression in epilepsy-associated MCDs.

## MATERIALS AND METHODS

### Population characteristics

*Autopsied human developing brains*

Archival paraffin blocks from 30 autopsy brains of human fetuses, stillborn infants and children ranging from 12 weeks gestation to 15 years old were retrospectively chosen for this study. Histologically normal areas of the cerebral mantle were selected for immunohistochemical assessment (Table 1). The term "histologically normal" refers to histologically normal-appearing tissue, regardless of its functional state, in which there are no histological changes observed by HE staining in a given case.

*Epilepsy-associated brain lesions*

Seventy surgically-resected specimens from patients (M : F = 35:35; age at time of surgery ranging from 10 weeks to 49 years; mean, 14.9 ± 14.3 years) with medically intractable epilepsy or infantile spasms were retrospectively chosen for this study from archival paraffin blocks. These include tissue in the following group categories: (I) cortical tuber of tuberous sclerosis complex (TSC-tuber) ($n = 12$; M : F = 5:7; age range, 1–27 years; mean, 10.3 ± 9.7 years); (II) focal cortical dysplasia (FCD) with balloon cell (BC) ($n = 24$; M : F = 12:12; age range, 11 weeks to 45 years; mean, 12.2 ± 14.5 years); (III) FCD without BC ($n = 11$; M : F = 6:5; age range, 10 weeks to 25 years; mean, 6.9 ± 9.6 years); (IV) 23 cases of histologically normal neocortex (N-CTX) obtained from pathologically confirmed (IV-1) Rasmussen encephalitis ($n = 5$; M : F = 2:3; age range, 3–9 years; mean, 6.0 ± 2.5 years), (IV-2) cystic-gliotic encephalopathy ($n = 2$, M : F = 0:2; age range, 8–10 years; mean, 9 ± 1.4 years), (IV-3) mesial temporal lobe epilepsy with or without hippocampal sclerosis ($n = 16$; M : F = 10:6; age range, 9–49 years; mean, 31.3 ± 12.1 years); and (V) 14 cases of normal-appearing neocortex adjacent to dysplastic lesions (Ad-CTX) obtained from eight and six specimens in categories I and II, respectively ($n = 14$; M : F = 8:6; age range, 9 months to 42 years; mean, 14.8 ± 12.4 years). All patients in group I fulfilled the diagnostic criteria for clinically definite TSC.[33] The pathological diagnosis of FCD with BC was made on specimens from patients who had no signs or systemic manifestations of TSC (so-called isolated CD), and represent "severe" FCD in the previously described classification and proposed grading system,[34] equivalent to FCD type IIB[3] and FCD type IIb.[4] The diagnosis of CD without BC also represents isolated CD, but no BCs were observed even by extensive sampling of resected tissue, that include FCD type IB ($n = 3$) and IIA ($n = 8$) in this study.[3] The term "histologically normal" cortex refers to histologically normal-appearing cortex, regardless of its

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**Table 1** Clinicopathological summary of the 30 autopsy cases

| Case no. | Age | Sex | Clinical diagnosis | Pathological diagnosis |
|---|---|---|---|---|
| 1 | 12 w | U | Thoracoomphalopagus | Complete agenesis of the corpus callosum |
| 2 | 12 w | U | Thoracoomphalopagus | Complete agenesis of the corpus callosum |
| 3 | 13 w | F | Not described | Normal brain for age |
| 4 | 16 w | F | Trisomy 18 syndrome | Trisomy 18 syndrome |
| 5 | 18 w | M | Meckel-Gruber syndrome | Meckel-Gruber syndrome |
| 6 | 19 w | M | Trisomy 18 syndrome | Trisomy 18 syndrome |
| 7 | 20 w | F | Abortion due to uterus bicornis | Normal brain for age |
| 8 | 20 w | F | Abortion due to uterus bicornis | Normal brain for age |
| 9 | 20 w | F | Hunter syndrome | Hunter syndrome |
| 10 | 21 w | U | Defect of lower limbs | Focal cortical dysplasia |
| 11 | 21 w | F | Cardiac abnormality | Normal brain for age |
| 12 | 24 w | F | Cystic hygroma colli | Dysplasia of the cerebral and cerebellar cortices |
| 13 | 24 w | M | Hypophosphatasia | Normal brain for age |
| 14 | 30 w | M | Fetal hydrops | Fresh multiple periventricular hemorrhages |
| 15 | 33 w | F | 69XXX | Dysgenesis of the central nervous system |
| 16 | 34 w | M | Trisomy 18 syndrome | Trisomy 18 syndrome |
| 17 | 38 w | U | Asphyxia due to rotation abnormality | Congestive brain |
| 18 | 38 w | F | Congenital cardiac disease | Congestive brain, dysplasia of the hippocampus |
| 19 | 7 m | F | Sudden infant death syndrome | Congenital cytomegalovirus infection |
| 20 | 10 m | F | Hemophagocytic syndrome | Infiltration of histiocytes in the subarachnoid space |
| 21 | 12 m | M | Sudden infant death syndrome | Dysplasia of the brain |
| 22 | 15 m | F | Hydrencephalus after brain hemorrhage | Hydrencephalus, old subependymal hemorrhage |
| 23 | 4 y | F | Developmental disorder | Developmental disorder |
| 24 | 5 y | F | Developmental disorder, sudden death | Dysplasia of the brain |
| 25 | 6 y | F | Ependymoma in the fourth ventricle | Anaplastic ependymoma, Grade III |
| 26 | 6 y | M | Menkes kinky hair disease | Menkes kinky hair disease |
| 27 | 9 y | F | Holoprosencephaly | Holoprosencephaly |
| 28 | 10 y | F | Medulloblastoma | Medulloblastoma |
| 29 | 12 y | F | Death by drowning | Dysplastic brain |
| 30 | 15 y | M | Status epilepticus | Hepatic encephalopathy |

F, female; M, male; m, month(s) old; U, unknown; w, weeks gestation; y, year(s) old.

functional state, in which there are no histological changes observed by HE staining that presented adjacent to the specific lesion. No two specimens came from the same individual except those in Group V. Tissues of 62 cases are already included in a tissue microarray paraffin block originally containing 63 cases used in the previous study,[13] with one case of cystic-gliotic encephalopathy excluded due to insufficient amount of tissue remaining within the block. Paraffin blocks of eight cases (4 TSC-tubers, 2 FCD with BC, 2 FCD without BC) not allowed to be incorporated into the tissue microarray were also included in this study by defining a region of interest in each case throughout the study.

## Histological and immunohistochemical procedures

Paraffin blocks were cut at 5 μm thickness, subjected to HE and KB staining as routine procedures. Adjacent serial sections were subjected to immunohistochemistry for FGF-2 and GFAP. For FGF-2 immunostaining, deparaffinized sections were subjected to autoclave boiling in 0.015 mol sodium citrate buffer solution (pH 6.0) for 10 min at 121°C as an antigen retrieval procedure before incubation with 3% $H_2O_2$ diluted in distilled water for 30 min followed by blocking with 5% normal goat serum. Sections were incubated with rabbit polyclonal antibodies for FGF-2 (dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal antibodies for GFAP (dilution 1:300, Dako, Glostrup, Denmark) overnight at 4°C, followed by incubation with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer (EnVision+ System-HRP, Dako, Carpinteria, CA, USA) for 45 min at 37°C. Immunoreaction was visualized by 3–3'diaminobenzidine tetrahydrochloride (DAB, Dako, Carpinteria, CA, USA). Sections were counterstained with hematoxylin. Immunostaining with omission of primary antibodies was used as a negative control. Fibroblasts in human leptomeninges were used as built-in positive control for FGF-2.

## Semi-quantitative analysis of FGF-2 immunoreactivity in MCDs

The histology of all cores in tissue microarray was verified on HE-stained sections to confirm that a region of interest from a donor block appeared in a given core (round tissue with 600 μm diameter, approximately 0.28 $mm^2$). Cores lost or severely damaged during staining were excluded from the study. Immunoreactivity was judged as positive regard-

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less of staining intensity, when DAB signal in a given cell was higher than the background. Any cells within blood vessels or their walls, for example, vascular endothelial and smooth muscle cells, were excluded from the study. Only cells with the nucleus in the plane of the section were counted. The computer-assisted semi-quantitative analysis of FGF-2 immunoreactivity on tissue microarray was performed according to the previously described protocol.[13] For another eight cases that are not included in the tissue microarray, seven consecutive areas of interest (AOIs) were determined using a 40X objective lens (approximately 1.58 $mm^2$) based on the HE stained sections in each case for the semi-quantitative evaluation of FGF-2 expression.[28] The proportion of FGF-2 immunoreactive cells (FGF-IR%) in a given specimen was measured by counting all FGF-2 immunoreactive neuroglial cells divided by total number of cells in each core and AOI. The mean values of the positive ratio from each group were statistically compared by analysis of variance (ANOVA) followed by a post hoc Scheffe's test for multiple comparisons. The statistical difference was considered to be significant when $P < 0.01$.

## RESULTS

### FGF-2 expression in developing human brain

Weak or faint FGF-2 immunoreaction was observed and almost confined to a population of what appeared to be matrix cells and/or neuroblasts within the VZ in all five fetuses from 12 to 18 weeks gestation (Fig. 1A). In fetuses of 19 weeks gestation or older, nuclei of what seemed to be glioblasts and immature astrocytes within SVZ and intermediate zone (IMZ) as well as immature cells within the VZ and ependymal layer were variably immunoreactive for FGF-2 (Fig. 1B,C). Fetal immature ependymal cells were strongly immunoreactive for FGF-2 in their cytoplasm (Fig. 1C), while mature ependymal cells with ciliated cuboidal morphology, particularly in the postnatal brains, showed FGF-2 expression more localized in the apical brush border (Fig. 1D). The spatial and temporal alterations of FGF-2 expression in these developing human brains were almost parallel with those of GFAP expression, except the cortical plate and choroid plexus; that is, no FGF-2 immunoreactivity was observed in astrocytes and immature neurons in the cortical plate in all fetal cases (Fig. 1E), despite few GFAP-positive astrocytes scattered in the cortical plate in fetal brains of 19 weeks gestation or older. However, in 8/12 postnatal brains from infants and children, faint immunoreactivity of FGF-2 was observed in a population of cortical astrocytes, and nuclei and/or cytoplasm of GFAP-positive astrocytes in the subependymal layer and white matter were variably immunoreactive for FGF-2 (Fig. 1F). FGF-2 immunoreactivity was not detected in the neocortical neurons in any of the autopsy cases examined in the present study. Cytoplasm of choroid plexus epithelium was strongly positive for FGF-2 in all cases examined (Fig. 1D) (Table 2).

### FGF-2 immunoreactivity in MCDs and the proportions of FGF-2 immunoreactive cells

In cases from groups I (TSC-tubers) and II (FCD with BC), abnormal neuroglial cells including DNs and BCs were variably immunoreactive for FGF-2 (Fig. 2A,B). In contrast, no or very subtle, if any, FGF-2 immunoreactivity was detected in the neuronal component including, DNs in cases from group III (FCD without BC) (Fig. 2C). The cytoplasm and/or nuclei of both normal-appearing and reactive astrocytes was positive for FGF-2 in all cases (Fig. 2C,D) from all groups including group V (Ad-CTX). All of these findings were consistent with our previous study.[28] FGF-2 immunoreactivity was not detected in normal and normal-appearing neurons in cases from groups IV and V. The proportions of FGF-2-IR% were significantly higher in groups I (mean $\pm$ SD: $36.9 \pm 9.6$) and II ($45.1 \pm 7.0$) than in groups III ($21.0 \pm 5.7$), IV ($14.4 \pm 4.7$) and V ($24.3 \pm 10.3$) with statistical significance ($P \leq 0.0017$) (Fig. 3), and that in group V was higher than in group IV with statistical significance ($P = 0.0062$) (Fig. 3). There was no significant difference in the FGF-2-IR% between groups I and II ($P = 0.0534$), III and IV ($P = 0.2181$) and III and V ($P = 0.8726$).

## DISCUSSION

### FGF-2 expression is associated with gliogenesis from matrix cells to astrocytes

The observations in the present study indicate the transition of FGF-2 expression from matrix cells to glioblasts and astrocytes during human brain development. FGF-2 immunoreactive cells in VZ and SVZ before 19 weeks gestation may represent matrix cells and/or neuroblasts when glioblasts are not yet generated, although there are no specific immunohistochemical markers applicable to formalin-fixed paraffin-embedded human autopsy brain tissue to identify matrix cells, neuroblasts and glioblasts. However, FGF-2 immunoreactivity was not observed in neuroblasts and/or immature neurons in the cortical plate migrated from VZ/SVZ in the present study, although FGF-2 expression has also been demonstrated in both neuroblasts and glioblasts in the cortical plate using frozen sections from human fetal brains of 12–16 weeks gestation.[29] On the other hand, FGF-2 expression was observed in nuclei of GFAP-expressing astrocytes in IMZ or white

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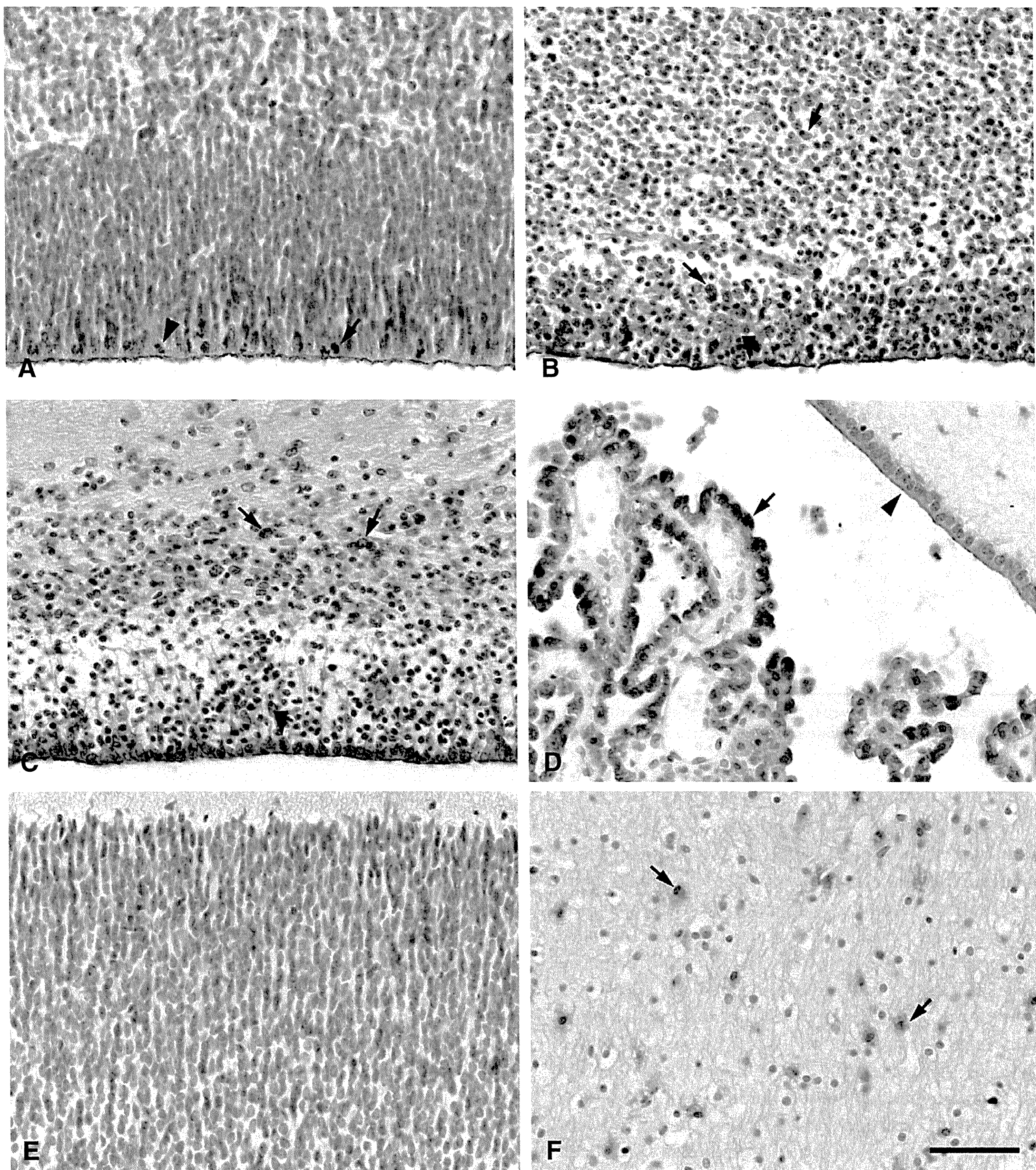


**Fig. 1** Fibroblast growth factor (FGF)-2 expression in developing human cerebrum. (A) Weak or faint FGF-2 immunoreactivity almost confined to a population of what appeared to be matrix cells and/or neuroblasts within the ventricular zone (VZ: arrow) in a fetal brain of 12 weeks gestation. Note scattered mitotic figures within the VZ (arrowhead). (B) Nuclei of what seem to be glioblasts and immature astrocytes (arrows) within the subventricular zone (SVZ) as well as immature cells within the VZ and ependymal layer (arrowhead) are variably immunoreactive for FGF-2. A fetal brain of 21 weeks gestation. (C) In addition to the positive reaction for FGF-2 in glioblasts (arrows), immature ependymal cells are strongly immunoreactive for FGF-2 in their cytoplasm (arrowhead). A 21 weeks gestation fetus. (D) Cytoplasm of the choroid plexus epithelium is strongly positive for FGF-2 in all cases examined (arrow). Mature ependymal cells with ciliated cuboidal morphology, particularly in the postnatal brains showed FGF-2 expression more localized in the apical brush border (arrowhead). A 7-month-old infant. (E) No FGF-2 immunoreactivity was observed in the cortical plate in all fetal cases. A 12 weeks gestation fetus. (F) Nuclei and/or cytoplasm of reactive astrocytes in the white matter are positive for FGF-2 (arrows). A 7-month-old infant. All panels were photographed at the same magnification. Bar = 50 μm.

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**Table 2** Summary of FGF-2 immunoreactivity in developing human cerebra

| Case No. | Age | VZ/EL | SVZ/SEL | IMZ/WM | CP/NCTX | CPE |
|---|---|---|---|---|---|---|
| 1 | 12 w | + | – | – | – | +++ |
| 2 | 12 w | + | – | – | – | +++ |
| 3 | 13 w | + | – | – | – | +++ |
| 4 | 16 w | ++ | – | – | – | +++ |
| 5 | 18 w | + | – | – | – | +++ |
| 6 | 19 w | ++ | + | + | – | +++ |
| 7 | 20 w | ++ | – | – | – | +++ |
| 8 | 20 w | ++ | ++ | + | – | +++ |
| 9 | 20 w | ++ | ++ | – | – | +++ |
| 10 | 21 w | ++ | ++ | + | – | +++ |
| 11 | 21 w | +++ | ++ | + | – | +++ |
| 12 | 24 w | + | – | – | – | ++ |
| 13 | 24 w | + | + | + | – | ++ |
| 14 | 30 w | + | + | + | – | +++ |
| 15 | 33 w | + | + | + | – | +++ |
| 16 | 34 w | + | + | + | – | +++ |
| 17 | 38 w | + | ++ | ++ | – | +++ |
| 18 | 38 w | + | ++ | ++ | – | +++ |
| 19 | 7 m | ++ | ++ | ++ | – | +++ |
| 20 | 10 m | ++ | ++ | ++ | + | +++ |
| 21 | 12 m | ++ | ++ | ++ | + | +++ |
| 22 | 15 m | ++ | ++ | ++ | – | +++ |
| 23 | 4 y | +++ | ++ | ++ | + | +++ |
| 24 | 5 y | +++ | ++ | ++ | – | +++ |
| 25 | 6 y | ++ | ++ | ++ | – | +++ |
| 26 | 6 y | ++ | ++ | ++ | + | +++ |
| 27 | 9 y | ++ | ++ | ++ | + | +++ |
| 28 | 10 y | ++ | ++ | ++ | + | +++ |
| 29 | 12 y | + | ++ | ++ | – | +++ |
| 30 | 15 y | ++ | ++ | ++ | + | ++ |

Immunoreactivity: –, negative; +, faintly positive; ++, positive; +++, strongly positive. CP, cortical plate; CPE, choroid plexus epithelium; EL, ependymal layer; IMZ, intermediate zone; NCTX, neocortex; SEL, subependymal layer; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter.

matter as well as VZ/SVZ in fetal brains of 19 weeks gestation or older. In postnatal brains, FGF-2 expression was mainly observed in the astrocytes in the white matter and faint FGF-2 expression was also observed in a population of neocortical astrocytes. These observations, together with the previous study showing FGF-2 expression mainly in astrocytes in histologically normal cerebral neocortex and white matter in adults,[28] suggest that FGF-2 is a developmentally regulated protein and its expression is associated with gliogenesis from matrix cells in human brain development. Although human autopsy brains younger than 9 weeks gestation consisting entirely of matrix cells were not available in this study, FGF-2 mRNA expression has been observed in cells in neural tubes at embryonic day E10 in mice, when neural crest precursors proliferate.[35] In fact, FGF-2 has been detected in astrocytes but not neurons, except those in CA2 of the hippocampus in normal postnatal and adult rat brains.[31,32] Hence, FGF-2 expression in abnormal neuroglial cells, particularly DNs in cases of MCD with BC (groups I and II), in the present study appears to represent one immature feature of these cells,[1] and reflects the putative perturbations of developmental events in the early fetal period underlying the pathogenesis of these dysplastic lesions.[28] Accordingly MCD with BC may differ from FCD without BC (group III) in the likely timing of insults underlying the pathogenesis of each disorder, despite the presence of morphologically identical DNs in these lesions. Although the presence of BCs is one histological hallmark of groups I and II, BCs may not always intermingle with DNs but also can exist as isolated small aggregates within the adjacent normal-appearing cortex and white matter.[4–6,13] In such situation, the presence of FGF-2 immunoreactive DNs may therefore even indicate the presence of potentially "hidden" BCs in a given specimen, and could be one supplemental immunohistochemical feature for accurate and differential diagnosis of FCD type IIb from FCD type IIa.

## Possible role of FGF-2 in the pathogenesis of BCs

The present study has updated our preliminary report[28] on FGF-2-IR% in epilepsy-associated MCDs, demonstrating that FGF-2-IR% in BC-containing cortical dysplasia, that is, TSC-tubers and FCD with BC, is significantly higher than that in FCD without BC, and that FGF-2 upregulation

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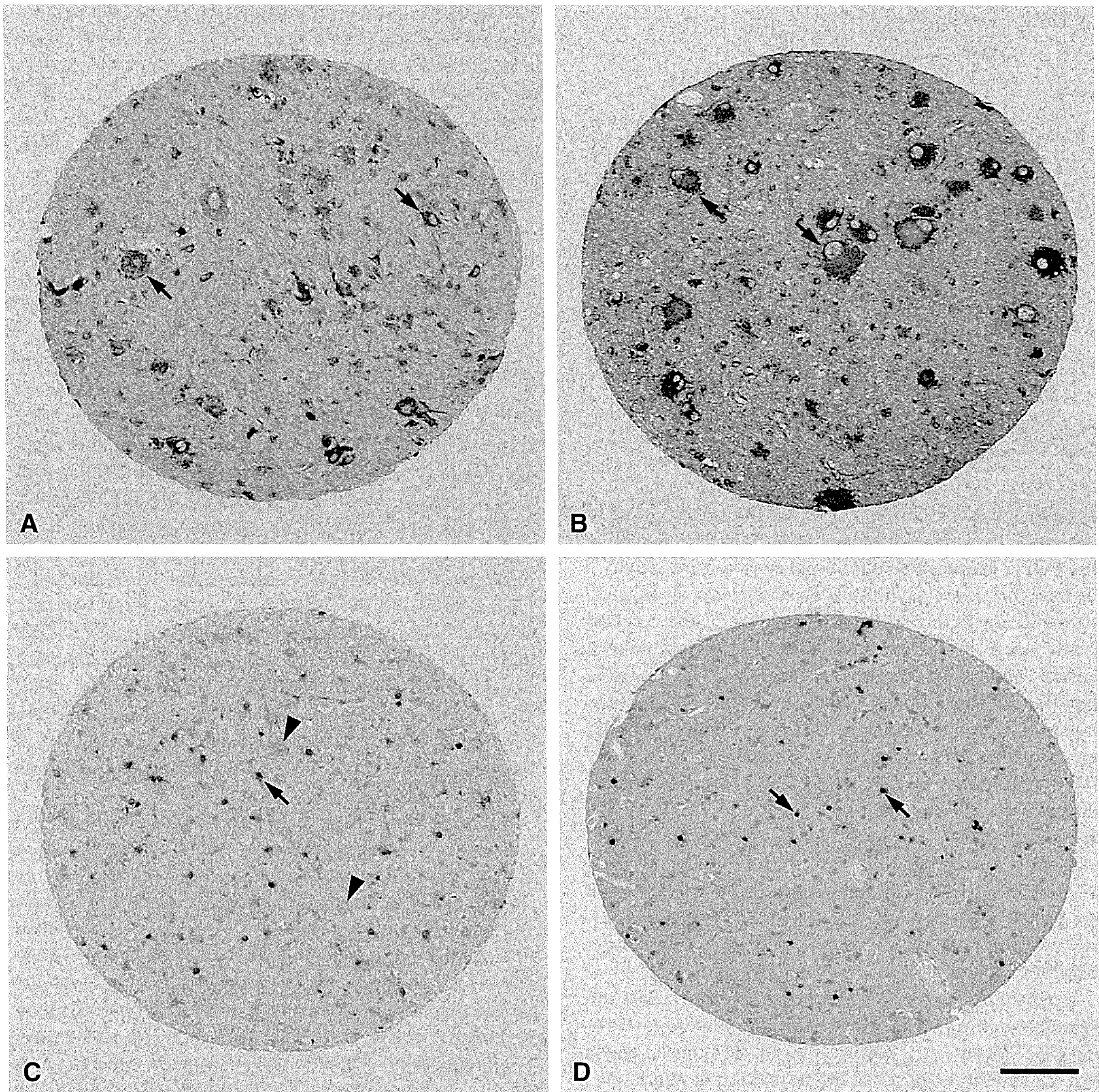


**Fig. 2** Tissue microarray immunohistochemistry for fibroblast growth factor (FGF)-2. Abnormal neuroglial cells are immunoreactive for FGF-2 in tuberous sclerosis complex (TSC)-tuber (panel A, arrows) and focal cortical dysplasia (FCD) with balloon cells (BC) (panel B, arrows). FGF-2 expression is observed in reactive astrocytes (arrow in panel C) but not in neuronal component including dysmorphic neurons in FCD without BC (arrowheads in panel C). In normal cerebral neocortex, nuclei and/or cytoplasm of astrocytes are positive for FGF-2 (panel D, arrows). All panels were photographed at the same magnification. Bar = 100 μm.

appears to be an important feature common to TSC-tubers and FCD with BC. In addition, FGF-2-IR% in group V (Ad-CTX) was significantly higher than that in group IV (N-CTX), indicating that Ad-CTX is also abnormal in terms of FGF-2 expression. This difference appears to be mainly due to the difference in the proportion of FGF-2 immunoreactive normal and reactive astrocytes, since there were no DNs or neuronal expression of FGF-2 observed in these groups.

Previous studies have demonstrated the upregulation of FGF-2 mRNA following bicuculline- or kainic acid-induced seizure mainly in the hippocampus,[21,22] and the

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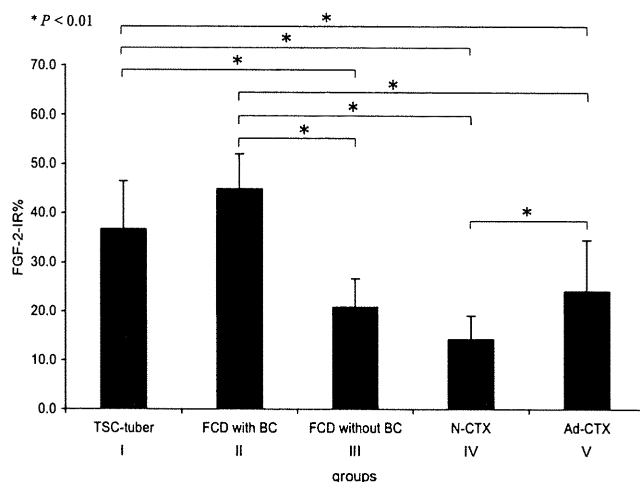


**Fig. 3** The ratios of fibroblast growth factor-2 (FGF-2) immunoreactive cells (FGF-2-IR%). *$P < 0.01$.

upregulation of both FGF-2 mRNA and FGF-2 protein in astrocytes by kainate-mediated excitotoxicity, indicating that FGF-2 is upregulated in response to seizure activity.[36] Furthermore, there have also been several reports suggesting a role for FGF-2 in the development of the cerebral cortex using FGF-2 knockout mice showing deranged cortical laminar structures.[23–25] Accordingly, a possible hypothesis is that FGF-2 protein is upregulated in the disorganized cerebral cortex of MCDs secondary to the upregulation of FGF receptors in response to possible FGF-2 deficiency during an early stage of intrauterine life. However, our results indicated that the FGF-2-IR% was significantly higher in groups I (TSC-tubers) and II (FCD with BC) than groups III (FCD without BC) and IV (N-CTX), suggesting a strong relationship between FGF-2 and BC. BCs show morphological and immunohistochemical features of both neurons and astrocytes, suggesting a failure of commitment in neuroglial differentiation.[5,10,37]

Extrinsic FGF-2 has also been suggested to regulate the differentiation of cortical stem cells into mature neurons and glia.[27] Moreover, a higher concentration of exogenous FGF-2 can induce abnormal differentiation of neural precursor cells into balloon-like cells expressing both GFAP and neurofilament.[35] Hence studies on the expressions of FGFR and FGF-2 mRNA in BCs would be helpful to further elucidate the histogenesis of BCs.

## Biological significance of FGF-2 immunoreactivity in choroid plexus epithelium

We demonstrated constant and strong immunoreactivity of FGF-2 in the choroid plexus epithelium (CPE) and moderate immunoreactivity in ependymal cells in all cases examined in the present study. The choroid plexuses (CPs) are specialized secretory tissues within the ventricle of the brain involved in the production of CSF and the maintenance of the blood–CSF barrier. For these reasons, some have hypothesized that CPE is involved in the synthesis and secretion of FGF-2 into the CSF,[38] and that FGF-2 immunoreactive ependymal cells function to transport FGF-2 from the VZ into brain parenchyma.[39] However, there have been no reports to date demonstrating the expression of FGF-2 mRNA in CPs. Some previous studies have shown expression of FGFR1 mRNA and FGFR2 mRNA but no expression of FGF-2 mRNA in CPE.[40,41] In addition, expression of FGFR mRNA has been shown in the CPE of developing murine and adult rat brains,[42,43] and FGFR2 mRNA is highly expressed in the CPs in rats.[44] These results imply that strong immunoreactivity of FGF-2 in CPE represents the reception but not production of FGF-2 through FGFR on CPE. However, the functional role and effect of FGF-2 on CPE is yet to be elucidated. Parallel analysis of FGF-2 expression and cell proliferation have suggested that FGF-2 is not involved in CPE proliferation, and that transthyretin, a marker of secretory activity, is not affected by FGF-2 treatment using murine CPE, indicating that FGF-2 is not involved in CSF production.[45] Furthermore, infusion of FGF-2 into the lateral ventricle can induce hydrocephalus by increased resistance to CSF absorption, conceivably due, at least in part, to enhanced fibrosis and collagen deposits in the arachnoid villi.[46] Hence CPE may absorb excess amounts of FGF-2 within CSF via FGFRs. Further study is needed to elucidate a biological significance for FGF-2 in CNS development and MCDs as well as in CSF.

In conclusion, the present study demonstrates FGF-2 expression in the developing human cerebrum and epilepsy-associated MCDs. Upregulation of FGF-2 is an important feature common to TSC-tubers and FCD with BC, and the transition of FGF-2 expression in our developmental study suggests that BC and DN in these MCDs represent disturbed gliogenesis from matrix cells and disturbed maturation of cortical neurons from migrating neuroblasts, respectively. FGF-2-IR% is associated with histological subtypes of MCD, particularly depending on whether a given lesion is accompanied by BC pathology or not, reflecting the likely timing of insults underlying the pathogenesis of each disorder.

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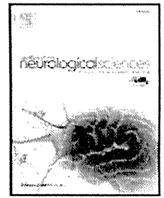

# Mild parkinsonian signs in a community-dwelling elderly population sample in Japan

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ABSTRACT

Mild parkinsonian signs (MPS) may represent the mild end of a disease spectrum that spans from normal aging to neurodegenerative diseases. We conducted a population-based study in a rural island town in western Japan, Ama-cho. Participants included 1129 subjects, aged 60 years and older, residing in the town. Participants were classified according to a modified Unified Parkinson's Disease Rating Scale (mUPDRS) score. MPS was determined to be present if any of the following conditions were met: (1) two or more mUPDRS ratings = 1 [MPS-mild]; (2) one mUPDRS rating ≥ 2; or (3) mUPDRS rest tremor rating ≥ 1; [(2) and (3): MPS-severe]. Subjects wore a uniaxial accelerometer (Actiwatch), resulting in the measurement of actigraphic activity counts (AC).

Of the 804 participants with complete data, 178 subjects (22.1%) were classified as demonstrating MPS. AC was significantly lower in the MPS-severe group compared with both the CTL and the MPS-mild groups. Diagnostic sensitivity for MPS-severe became 100% when we adopted a cutoff point of low physical activity, as measured by actigraphy, combined with the presence of subjective depression.

We established the prevalence of MPS in a community-dwelling elderly population sample in Japan. Actigraphy may be a useful objective tool for screening MPS-severe.

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## 1. Introduction

Mild parkinsonian signs (MPS), including bradykinesia, rigidity, gait disturbance and resting tremor, may represent the mild end of a disease spectrum that spans from normal aging [1] to neurodegenerative diseases [2], including Parkinson's disease (PD). MPS has also been reported to be the result of nigrostriatal Alzheimer's disease (AD)-type pathology [3], associated with increased risk of dementia [4], associated with vascular lesions of basal ganglia and white matter [5,6], and a significant predictor of mortality [7]. However, the clinical significance of MPS is not yet fully understood. The prevalence of MPS in sample populations in East Boston, England [8], New York, USA [9] and Jiangsu, China [7] has been reported, but inconsistencies exist across reports because of differences in MPS definition, study methodology, age structure, and cognitive status [10]. The prevalence of MPS in Japan has hitherto not been reported.

We have conducted the first epidemiological study to suggest the prevalence of MPS in Japan. Furthermore, we examined the usefulness of actigraphy as an objective indicator for MPS through a population-based study in order to establish screening methods for MPS in association with questionnaires about motor and nonmotor symptoms of Parkinson's disease (PD).

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## 2. Methods

### 2.1. Subjects

This study was conducted in the municipality of Ama-cho, a rural island town located 70 km from Yonago city, in the northwestern part of Japan [11]. To be included in the study, subjects were required to be living and to be legally residing in the town on March 31, 2008. The total population of Ama-cho on this day was 2402 (1124 men). The number of elderly people aged 60 years and older was 1129 (479 men, mean age ± SD 74.6 ± 9.1 years old). Board certified neurologists of the Japanese Society of Neurology (neurologists) belonging to our department have visited this town twice a year since 1980, and diagnosed patients having neurological disorders. Before this study, 11 patients with PD were recognized through these visits.

The study was approved by the committee for medical research ethics at Tottori University following the principles outlined in the "Declaration of Helsinki", and all participants provided written informed consent to participate in the study.

### 2.2. Questionnaire survey

We administered a questionnaire survey in May 2008. First, we mailed the questionnaires to residents aged 60 years or older. To assess motor symptoms, we included the Tanner questionnaire, [12], which is validated as a PD patient screening form. To evaluate depressive symptoms, we included the Japanese version of the

Geriatric Depression Scale with 15 questions (GDS-15). [13]. It has been validated for the diagnosis of depression, and the recommended cutoff points are ≥6 as mild depression and ≥10 as severe depression [13,14]. We included the Pittsburgh Sleep Quality Index (PSQI) [15] and the REM Sleep Behavior Disorder Screening Questionnaire (RBDSQ) [16] to assess sleep disturbances. The cutoff value of the PSQI for a poor sleeper was 5/6 points, [15], and the RBDSQ to detect REM sleep behavior disorder (RBD) was 5/6 points. [17]. Demographic data, including age, gender, duration of education, and present smoking and drinking habits, were collected using the questionnaire. In order to evaluate nonmotor symptoms, we assessed the presence of constipation, hallucinations, hyposmia, and orthostatic hypotension with the questionnaire.

### 2.3. Neurological examination

Each participant underwent a structured medical interview including a past history of hypertension, diabetes mellitus, and hyperlipidemia. A standardized neurological examination was conducted by one of four neurologists, including an abbreviated (10-item) version of the motor portion of the Unified PD Rating Scale (UPDRS) in 2008–2009. The 10 items screened for speech, facial expression, tremor at rest, rigidity (rated separately in the neck, right arm, left arm, right leg, and left leg), posture, and body (axial) bradykinesia, with each item rated from 0 to 4. A rating of 1 indicated a mild abnormality and a rating of ≥2 indicated an abnormality of moderate or greater severity [9]. Subjects with a total UPDRS score of 0 were classified as being normal controls (CTL). We assigned a diagnosis of PD based on research criteria [18] and participants were considered to have PD if (1) they had previously received a diagnosis of PD by neurologists and responded to L-dopa or (2) their symptoms fulfilled the UK PD brain bank criteria, [19], or both. Those who had two or more cardinal signs (UPDRS rating≥2) on the standardized neurologic examination were classified as having parkinsonism. These cardinal signs include bradykinesia, rigidity, postural instability, and resting tremor.

MPS were defined as present when any one of the following conditions was met: (1) two or more UPDRS ratings = 1; (2) one UPDRS rating≥2; or (3) a UPDRS resting tremor rating≥1 [10]. MPS was further stratified into subtypes according to symptom types and severity as shown in Table 1 [4, 20, 21].

### 2.4. Measurement of physical activity using actigraphy

In order to obtain participants for the actigraphy study, we gathered subjects in five districts, randomly selected from the fourteen districts in the town (participation rate: 65.0%).

Physical activity was quantified using wrist-worn uniaxial accelerometers (Actiwatch-16, Mini Mitter-Philips Respironics, Bend, OR) [22]. Physical activity was monitored in the participant's own homes, and participants were instructed to continue their normal daily routine. Participants wore Actiwatches on their nondominant wrist for 1 week collecting data in 1-minute epochs. Those subjects with unilateral PD wore monitors on their least affected side. This placement has been shown to better represent whole-body movement [25] and was intended to reduce artifacts such as low level constant activity when writing with the dominant hand or dyskinesias in the most affected arm. At the same time, all participants completed a sleep log for 7 days. All actigraphic data were validated in accordance with entries in sleep logs. Automatic activity analysis using dedicated software (Actiware, Mini Mitter-Philips Respironics) was conducted. The measures analyzed were Total AC (the sum of all valid physical activity counts for all awake epochs), Avg AC (the average of all valid physical activity counts for all awake epochs divided by the epoch length in minutes), and Max AC (the largest of any valid physical activity count for all awake epochs).

**Table 1**
Classification of mild parkinsonian signs.

| | |
|---|---|
| *Classification according to symptoms* | |
| Axial dysfunction | (1) UPDRS ratings = 1 in two or more of the four items of axial function (changes in speech, facial expression, posture, and axial bradykinesia), or (2) one UPDRS rating≥2 in one of the four items |
| Abnormality in rigidity | Either (1) UPDRS ratings = 1 in two or more of the five items of rigidity, or (2) one UPDRS rating≥2 in one of the five items |
| Tremor | A UPDRS resting tremor rating≥1 |
| Unclassified | Could not be classified into any of the above-mentioned categories |
| *Classification according to severity of UPDRS score* | |
| MPS-mild | A UPDRS rating of 1 |
| MPS-severe | A UPDRS rating of 2 or higher, or presence of resting tremor |

MPS: mild parkinsonian signs, UPDRS: Unified PD Rating Scale.

### 2.5. Statistical analyses

The adjusted prevalence was calculated for all types of MPS and PD using the Japanese population on March 1, 2008. Paired $t$ tests and analysis of variance (ANOVA) were used for comparison of medians for continuous variables, and categorical variables were analyzed using a chi-square test. Pearson's test was used for correlation analyses. Differences in the total physical activities between groups were evaluated with an analysis of covariance (ANCOVA), adjusting for age. Analyses of the relationship between the background of the nonmotor symptoms and MPS-severe were performed by multivariate logistic regression analysis. Significance was defined as $p<0.05$, and all analyses were conducted using the Statistical Package for the Social Sciences version 17.0 software (SPSS17.0, 2008, Tokyo, Japan).

## 3. Results

### 3.1. Questionnaire survey

Nine hundred sixty-eight (85.7%) of 1129 residents returned their questionnaire. As compared to survey nonrespondents, respondents were similar in age (mean = 74.7 years vs. 75.1 years) and gender (47.1% male vs. 43.5% male).

### 3.2. Prevalence of PD and MPS in a community-dwelling elderly population sample

Eight hundred four of 1129 subjects received a neurological examination (71.2%). We diagnosed 69 subjects as having parkinsonism (24 men, 82.9 ± 7.1 years). Of the parkinsonism patients, 14 were diagnosed as having PD (4 men, 79.6 ± 7.6 years). The crude prevalence of PD and the age-adjusted prevalence when calculated using the Japanese population in 2008 were 1.5% and 1.3% for PD in those over the age of 65.

Of the examined subjects, 178 were diagnosed as having MPS (62 men, 78.1 ± 8.1 years). The crude prevalence of MPS was 22.1% (95% CI: 19.3–25.0) in participants over 60 years of age, and 23.7% (95% CI: 20.6–26.9) in participants over 65 years of age. The age-adjusted prevalence of MPS was 13.8% in the over 60 population, and 16.8% in the over 65 population. We showed the classification of MPS according to its type and severity in Table 2.

### 3.3. Physical activity measured by actigraphy

Using actigraphy, we evaluated 265 subjects (121 men; age: 74.2 ± 7.9 years), including 174 control (CTL) subjects (75 men; 72.2 ± 7.2 years), 53 subjects with MPS-mild (22 men; 78.3 ± 7.2 years), 19 subjects with MPS-severe (5 men; 78.4 ± 6.6 years), and 19 subjects with parkinsonism (7 men; 81.0 ± 7.5 years) including 7 PD patients

**Table 2**
Age- and sex-specific prevalence of MPS.

| Age (years) | Residents | Population at risk | MPS | | | | | | | | | | | | | | | | Parkinsonism | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | | Type | | | | | | | | | | Severity | | | | | |
| | | | | | Axial dysfunction | | Rigidity | | Mixed | | Tremor | | Unclassified | | MPS-mild | | MPS-severe | | | |
| | | | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence | Cases | Prevalence |
| *Both sexes* | | | | | | | | | | | | | | | | | | | | |
| 60–64 | 183 | 88 | 8 | 9.1% | 1 | 1.1% | 7 | 8.0% | – | – | – | – | – | – | 7 | 8.0% | 1 | 1.1% | – | – |
| 65–69 | 180 | 135 | 18 | 13.3% | 2 | 1.5% | 12 | 8.9% | 2 | 1.5% | 2 | 1.5% | – | – | 15 | 11.1% | 3 | 2.2% | 1 | 0.7% |
| 70–74 | 198 | 164 | 28 | 17.1% | 5 | 3.0% | 18 | 11.0% | 3 | 1.8% | 2 | 1.2% | – | – | 23 | 14.0% | 5 | 3.0% | 9 | 5.5% |
| 75–79 | 227 | 183 | 49 | 26.8% | 12 | 6.6% | 25 | 13.7% | 11 | 6.0% | 1 | 0.5% | – | – | 34 | 18.6% | 15 | 8.2% | 13 | 7.1% |
| 80–84 | 158 | 121 | 43 | 35.5% | 14 | 11.6% | 16 | 13.2% | 7 | 5.8% | 2 | 1.7% | 4 | 3.3% | 26 | 21.5% | 17 | 14.0% | 11 | 9.1% |
| 85– | 183 | 113 | 32 | 28.3% | 9 | 8.0% | 18 | 15.9% | 4 | 3.5% | – | – | 1 | 0.9% | 23 | 20.4% | 9 | 8.0% | 35 | 31.0% |
| Total | 1129 | 804 | 178 | 22.1% | 43 | 5.3% | 96 | 11.9% | 27 | 3.4% | 7 | 0.9% | 5 | 0.6% | 128 | 15.9% | 50 | 6.2% | 69 | 8.6% |
| *Men* | | | | | | | | | | | | | | | | | | | – | – |
| 60–64 | 94 | 38 | 3 | 7.9% | 1 | 2.6% | 2 | 5.3% | – | – | – | – | – | – | 2 | 5.3% | 1 | 2.6% | – | – |
| 65–69 | 84 | 63 | 8 | 12.7% | 1 | 1.6% | 5 | 7.9% | 1 | 1.6% | 1 | 1.6% | – | – | 8 | 12.7% | – | – | 1 | 1.6% |
| 70–74 | 89 | 67 | 11 | 16.4% | 1 | 1.5% | 7 | 10.4% | 2 | 3.0% | 1 | 1.5% | – | – | 9 | 13.4% | 2 | 3.0% | 5 | 7.5% |
| 75–79 | 97 | 72 | 17 | 23.6% | 4 | 5.6% | 7 | 9.7% | 6 | 8.3% | – | – | – | – | 11 | 15.3% | 6 | 8.3% | 3 | 4.2% |
| 80–84 | 53 | 38 | 10 | 26.3% | 2 | 5.3% | 3 | 7.9% | 2 | 5.3% | 1 | 2.6% | 2 | 5.3% | 6 | 15.8% | 4 | 10.5% | 2 | 5.3% |
| 85– | 62 | 44 | 13 | 29.5% | 2 | 4.5% | 10 | 22.7% | – | – | – | – | 1 | 2.3% | 11 | 25.0% | 2 | 4.5% | 13 | 29.5% |
| Total | 479 | 322 | 62 | 19.3% | 11 | 3.4% | 34 | 10.6% | 11 | 3.4% | 3 | 0.9% | 3 | 0.9% | 47 | 14.6% | 15 | 4.7% | 24 | 7.5% |
| *Women* | | | | | | | | | | | | | | | | | | | – | – |
| 60–64 | 89 | 50 | 5 | 10.0% | – | – | 5 | 10.0% | – | – | – | – | – | – | 5 | 10.0% | – | – | – | – |
| 65–69 | 96 | 72 | 10 | 13.9% | 1 | 1.4% | 7 | 9.7% | 1 | 1.4% | 1 | 1.4% | – | – | 7 | 9.7% | 3 | 4.2% | – | – |
| 70–74 | 109 | 97 | 17 | 17.5% | 4 | 4.1% | 11 | 11.3% | 1 | 1.0% | 1 | 1.0% | – | – | 14 | 14.4% | 3 | 3.1% | 4 | 4.1% |
| 75–79 | 130 | 111 | 32 | 28.8% | 8 | 7.2% | 18 | 16.2% | 5 | 4.5% | 1 | 0.9% | – | – | 23 | 20.7% | 9 | 8.1% | 10 | 9.0% |
| 80–84 | 105 | 83 | 33 | 39.8% | 12 | 14.5% | 13 | 15.7% | 5 | 6.0% | 1 | 1.2% | 2 | 2.4% | 20 | 24.1% | 13 | 15.7% | 9 | 10.8% |
| 85– | 121 | 69 | 19 | 27.5% | 7 | 10.1% | 8 | 11.6% | 4 | 5.8% | – | – | – | – | 12 | 17.4% | 7 | 10.1% | 22 | 31.9% |
| Total | 650 | 482 | 116 | 24.1% | 32 | 6.6% | 62 | 12.9% | 16 | 3.3% | 4 | 0.8% | 2 | 0.4% | 81 | 16.8% | 35 | 7.3% | 45 | 9.3% |

(2 men; 77.8 ± 7.2 years). Ruling out a selection bias, there were no significant differences between activity measurement participants and non-participants with regard to age (74.3 ± 8.0 vs. 75.0 ± 9.4 years, respectively, p = 0.253), gender (43.3% male vs. 42.1% male, respectively, p = 0.390), or UPDRS score (1.4 ± 2.3 vs. 1.2 ± 2.4, respectively, p = 0.239).

While there was no significant difference in Total AC between the CTL and MPS-mild groups, Total AC in the MPS-severe group was significantly lower than that in the CTL and MPS-mild groups (Fig. 1). Our measure of Avg AC showed the same tendency as Total AC. However, our measure of Max AC was not significantly different among the groups. These three indices of physical activity were significantly associated with age (Total AC: $r = -0.358$, $p<0.001$, Avg AC: $r = -0.330$, $p<0.001$, Max AC: $r = -0.258$, $p<0.001$). ANCOVA analysis, adjusted for the age of subjects, revealed that Total AC in the MPS-severe group was significantly lower than that in the CTL group.

We divided the MPS group according to axial dysfunction scores into three subgroups: non-axial dysfunction (axial dysfunction score = 0, n = 34), mild axial dysfunction (axial dysfunction score = 1 or 2, n = 28), and moderate/severe axial dysfunction (axial dysfunction score = 3 or more, n = 10). Total AC, Avg AC and Max AC in the non-axial dysfunction group were 323,834.6 ± 21,927.8, 383.9 ± 25.0, and 2507.9 ± 151.5, those in the mild axial dysfunction group were 240,077.7 ± 22,175.5, 300.8 ± 25.9, and 2149.2 ± 124.9, and those in the moderate/severe axial dysfunction group were 193,873.6 ± 20,551.1, 245.7 ± 25.6, and 1755.9 ± 174.4, respectively. Total AC and Avg AC of the moderate/severe axial dysfunction group were significantly lower than those of the non-axial dysfunction group. In addition, Total AC, Avg AC, and Max AC of the mild axial dysfunction group were significantly lower than those of the non-axial dysfunction group. However, there were no significant differences in the three activity parameters between the mild axial dysfunction group and the moderate/severe axial dysfunction group.

We also divided the MPS group according to rigidity scores into three subgroups: non-rigidity (maximum rigidity score = 0, n = 17), mild rigidity (maximum rigidity score = 1, n = 53), and moderate/severe rigidity (maximum rigidity score = 2, n = 2). There were no significant differences in the three activity parameters among these groups.

Finally, we also divided the MPS group according to tremor scores into three subgroups: non-tremor (tremor score = 0, n = 67), mild tremor (tremor score = 1, n = 5), and moderate/severe tremor (tremor score = 2, n = 0). There were no significant differences in activity between these groups.

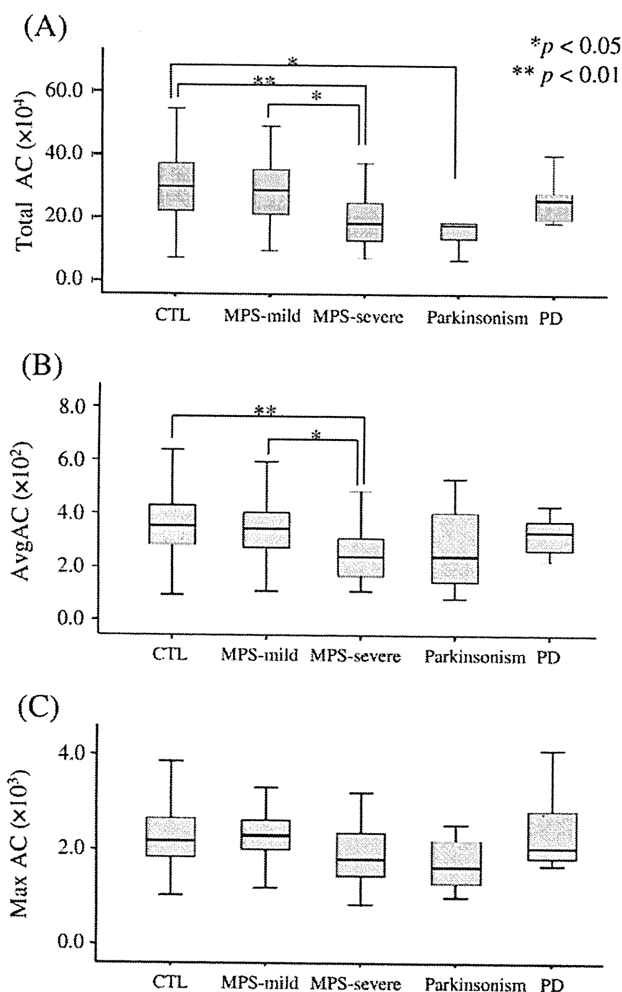


**Fig. 1.** Comparison of physical activity. The box plots show the median values (thick lines), 25th percentile (lower line of box), and 75th percentile (upper line of box). T bars indicate the 10th and 90th percentiles. Statistical differences were calculated using an ANOVA followed by Tukey tests. CTL: normal controls, MPS: mild parkinsonian signs. PD: Parkinson's disease. (A) Total AC: the sum of all valid physical activity counts for all epochs from the start time to the end time of the given awake interval, (B) Avg AC: the average of all valid physical activity counts for all awake epochs divided by the epoch length in minutes, (C) Max AC: the largest of any valid physical activity count for all awake epochs. *$p<0.05$, **$p<0.01$.

### 3.4. Association of nonmotor PD symptoms with MPS

There were no significant differences between the CTL group and both the MPS-mild and MPS-severe groups for habitual history, past history, nonmotor PD symptoms, or RBDSQ scores (Table 3). There was a significantly lower proportion of 'sleep disturbance' on the PSQI in the MPS-mild group, but not in the MPS-severe group, as compared with the CTL group.

The GDS scores of the MPS group were significantly higher than those of the CTL group (4.3 ± 3.4 vs. 3.2 ± 3.1, $p = 0.01$) and there was a significantly higher proportion of subjects with 'mild depression' on the GDS in the MPS group as compared with the CTL group (41.3% vs. 27.0%, $p<0.001$), indicating a strong association of subjective depression with MPS.

The proportion of subjects with 'mild depression' on the GDS was significantly higher in the MPS-mild group than in the CTL group. The proportion of subjects with 'severe depression' was significantly higher in the MPS-severe group than in the CTL group.

### 3.5. Screening for MPS

In the present study, when one point was assumed to be a cutoff in the Tanner questionnaire, the sensitivity for detecting PD was 100%. However, it was only 71.9% for detecting MPS (both MPS-mild and MPS-severe) and 73.3% for detecting MPS-severe. When predictors of MPS-severe were examined by multivariate logistic analysis, GDS and

**Table 3**
Demographic characteristics of participants stratified by MPS.

| | CTL | MPS-mild | MPS-severe |
|---|---|---|---|
| Present smoking, n (%) | 33 (7.5%) | 4 (3.9%) | 3 (7.1%) |
| Present drinking, n (%) | 116 (26.6%) | 19 (18.4%) | 4 (9.3%) |
| Constipation, n (%) | 97 (22.9%) | 37 (37.0%) | 15 (38.5%) |
| Hallucination, n (%) | 30 (7.0%) | 8 (8.3%) | 7 (17.1%) |
| Hyposmia, n (%) | 49 (11.4%) | 17 (17.0%) | 7 (17.1%) |
| Orthostatic hypertension, n (%) | 79 (18.3%) | 24 (24.2%) | 14 (32.6%) |
| GDS ≥ 6, n (%) | 123 (27.4%) | 42 (40.4%)** | 17 (39.5%) |
| GDS ≥ 10, n (%) | 23 (5.1%) | 7 (6.7%) | 7 (17.1%)** |
| RBDSQ ≥ 5, n (%) | 37 (8.2%) | 17 (16.3%) | 5 (11.6%) |
| PSQI ≥ 6, n (%) | 107 (23.8%) | 18 (17.3%)* | 8 (18.6%) |

GDS: Geriatric Depression Scale, PSQI: Pittsburgh Sleep Quality Index, RBDSQ: REM Sleep Behavior Disorder Screening Questionnaire. *$p<0.05$, **$p<0.01$ vs. CTL.