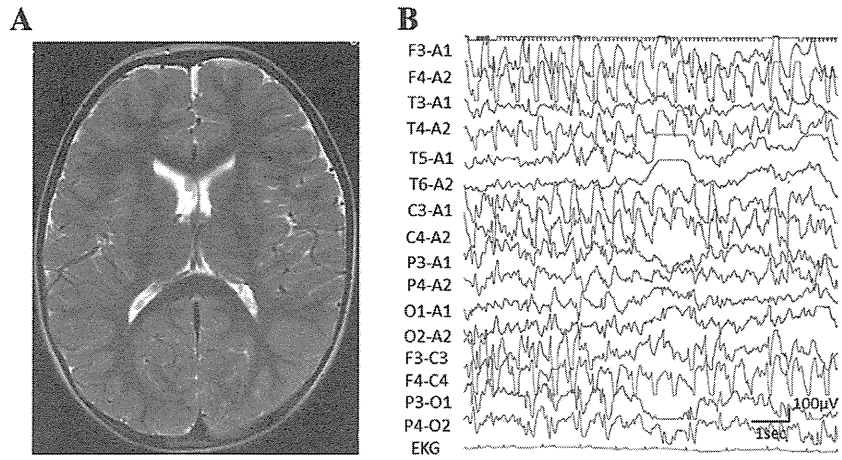

Fig. 2. Magnetic resonance imaging and electroencephalogram of the patient. (A) No significant abnormalities are observed in the T2-weighted image. (B) Multifocal spikes and spikes and waves are observed predominantly over the right frontal and bilateral occipital areas.

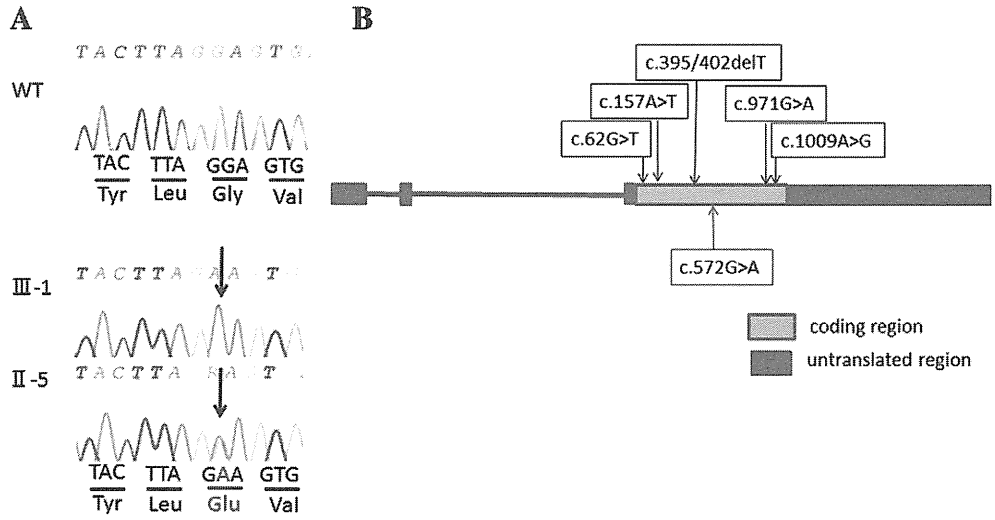

Fig. 3. Mutation analysis of *AGTR2*. (A) Sequences around the *AGTR2* mutation in the patient. The patient carries a 572G>A transversion, which leads to a G191E substitution. His mother was healthy and a carrier of the mutation. Mutated bases are indicated by arrows. (B) Genomic structure of *AGTR2*. Mutations reported previously are shown above and the mutation found in this study is shown below the region of interest.

between genetic and environmental factors. Previous reports suggested that polymorphisms of the angiotensin-converting enzyme gene are associated with Kawasaki disease [10]. No report has indicated a correlation between *AGTR2* mutations and Kawasaki disease.

This novel mutation was detected in this patient and his healthy mother. If his mother becomes pregnant with next child, the child will inherit mutant *AGTR2* gene with a half probability.

In conclusion, the pathogenic nature of the *AGTR2* mutation in the present case may require additional functional verification. Although *AGTR2* mutations are rare, we should suspect the possibility of these mutations as causes of MR with epilepsy, autistic behavior, hyperactivity, and restlessness. This report could facilitate further exploration of the role of *AGTR2* in human brain development and cognitive function.

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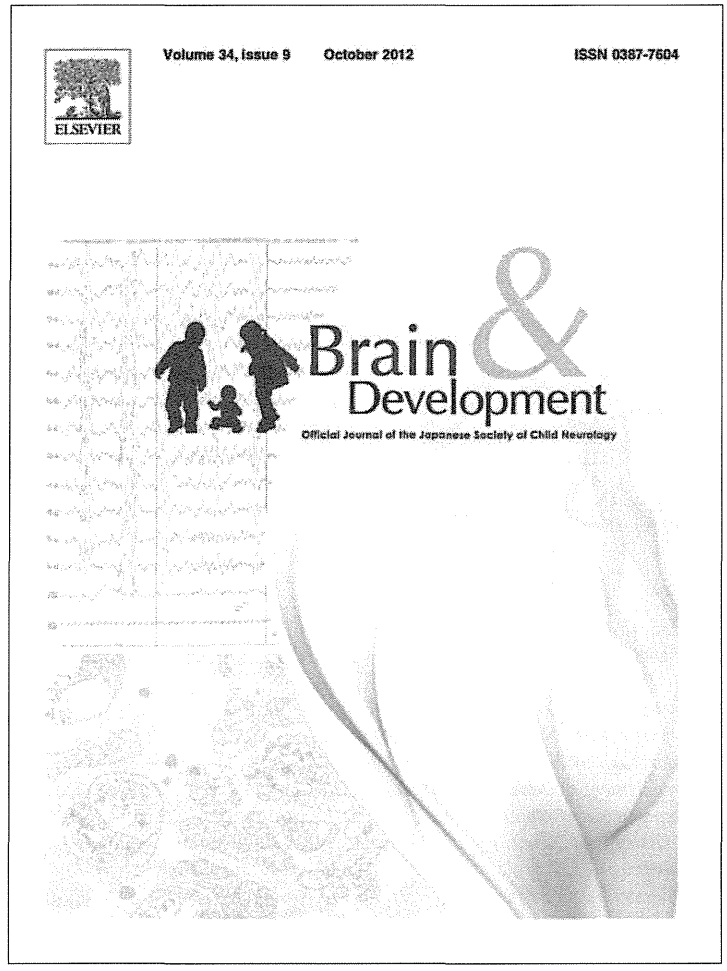

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Original article

Efficacy and tolerance of gastrostomy feeding in Japanese muscular dystrophy patients

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Abstract

Although muscular dystrophy patients often have feeding difficulty and need long-term enteral nutrition, only a few reports have described gastrostomy feeding in these patients. This study was designed to evaluate the efficacy and tolerance of gastrostomy feeding in patients with muscular dystrophy. We performed a retrospective, multicenter study on 144 patients with muscular dystrophy who received gastrostomy feeding between 2007 and 2009 in 25 neuromuscular centers in Japan. There were 77 Duchenne muscular dystrophy (median age at gastrostomy placement 26 years, range 13–47 years), 40 myotonic dystrophy (median age 54.5 years, range

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13–70 years), 11 Fukuyama congenital muscular dystrophy (median age 22 years, range 13–29 years), 5 limb girdle muscular dystrophy (median age 62 years, range 43–78 years), and 5 facioscapulohumeral muscular dystrophy (median age 52 years, range 28–67 years) patients. Many benefits including amelioration of malnutrition, swallowing difficulty and respiratory status were observed after the introduction of gastrostomy feeding. Especially in patients with Duchenne muscular dystrophy, mean body weight significantly increased after gastrostomy placement. Although most complications, which are commonly observed in other populations, were tolerable, respiratory failure and peritonitis were important concerns. These findings suggest that gastrostomy placement at an appropriate time is advisable in patients with muscular dystrophy.

Keywords: Muscular dystrophy; Duchenne muscular dystrophy; Myotonic dystrophy; Gastrostomy; Efficacy; Complications

1. Introduction

Patients with muscular dystrophy often have feeding difficulties and malnutrition, necessitating enteral nutrition. Since advances in the comprehensive management of complications, including respiratory failure, heart failure and orthopedic problems, have prolonged life expectancy in patients with muscular dystrophy, nutritional problems have become an important issue [1,2]. Malnutrition can aggravate respiratory dysfunction and subsequent respiratory or systemic infections. Although gastrostomy feeding is preferred for patients who require long-term enteral nutrition because of swallowing difficulty and malnutrition, only a few reports have described the efficacy and tolerance of gastrostomy feeding in muscular dystrophy patients [3–5]. We evaluated the efficacy and tolerance of gastrostomy feeding in muscular dystrophy patients.

2. Patients and methods

We performed a retrospective, multicenter study in 25 neuromuscular centers in Japan. We collected data from the medical files of patients treated at these 25 centers. Patients were eligible for the study if they had been diagnosed with muscular dystrophy and received gastrostomy feeding between 2007 and 2009. The diagnosis of muscular dystrophy was based on immunohistochemistry of muscle biopsy specimens, molecular biology, and/or clinical findings. The data included each hospital's system for performing and managing gastrostomy placement and patient profiles. Patient data included diagnosis, age at review, age at gastrostomy placement, indications for gastrostomy, operative method, respiratory status, scoliosis status, subjective evaluations of gastrostomy placement by patients, their families and their attending doctors, and complications during and after the perioperative period, which was defined as within one month after the operation. Malnutrition was subjectively evaluated based on body weight loss, skin condition and blood test results by attending doctors. Data on patients' body weights at the time of gastrostomy placement, 6 months before, and 6 months after were collected and analyzed using a paired t test. Statistical analyses were performed using SPSS software (12.0J, SPSS Japan Inc.), and the significance level was $p < 0.05$. We obtained institutional review board approval for this study.

3. Results

Of the 25 hospitals included in this study, 22 had managed patients receiving gastrostomy feeding between 2007 and 2009. Seventeen hospitals were able to perform in-hospital gastrostomy. Although the doctors who performed the gastrostomy were surgeons in 11 hospitals, those handling complications were attending physicians in most of the hospitals. Many hospitals felt the need to have guidelines for gastrostomy placement and the management of complications.

The number of patients who received gastrostomy feeding between 2007 and 2009 was 144, comprising 77 with Duchenne muscular dystrophy (DMD), 40 with myotonic dystrophy (MyD), 11 with Fukuyama congenital muscular dystrophy (FCMD), 5 with limb girdle muscular dystrophy (LGMD), 5 with facioscapulohumeral muscular dystrophy (FSHD), and 6 with other diagnoses including Becker muscular dystrophy and oculopharyngeal muscular dystrophy. Of these 144 patients, 101 received percutaneous endoscopic placement (PEG) with local anesthesia, 11 PEG with general anesthesia, 17 laparotomy, 3 placement by laparoscope, and other unknown procedures were performed in 12. The median age at the time of gastrostomy placement was 26 years (range 13–47 years) in DMD patients, 54.5 years (range 13–70 years) in MyD patients, 22 years (range 13–29 years) in FCMD patients, 62 years (range 43–78 years) in LGMD patients, and 52 years (range 28–67 years) in FSHD patients. DMD and MyD accounted for 81% of the entire patient population. Therefore, we performed a comparative review of DMD and MyD patients. The median ages at review in DMD and MyD patients were 28 years (range 16–48 years) and 55.5 years (range 15–72 years), respectively. Respiratory status at the time of gastrostomy placement is shown in Table 1. Seventy-two patients (94%) with DMD and 27 (67%) with MyD required noninvasive positive pressure ventilation (NPPV) or tracheal positive pressure ventilation (TPPV).

Table 1
Patient characteristics.

	DMD		MyD	
Median age at review (range)	28 years	(16–48 years)	55.5 years	(15–72 years)
Median age at GS placement (range)	26 years	(13–47 years)	54.5 years	(13–70 years)
Respiratory status	$n = 77$		$n = 40$	
None	5	(6%)	13	(33%)
NPPV	46	(60%)	7	(17%)
TPPV	26	(34%)	20	(50%)
Scoliosis	$n = 74$		$n = 39$	
No	4	(5%)	36	(92%)
Mild	18	(24%)	3	(8%)
Moderate	36	(49%)		
Severe	16	(22%)		
Feeding tube before GS	$n = 77$		$n = 40$	
Yes	34	(44%)	14	(35%)

DMD, Duchenne muscular dystrophy; MyD, myotonic dystrophy; GS, gastrostomy placement; NPPV, noninvasive positive pressure ventilation; TPPV, tracheal positive pressure ventilation. Scoliosis was defined as mild if Cobb's angle was under 25°, moderate if the angle was over 25° but under 60°, and severe if the angle was over 60°.

Scoliosis was defined as mild if Cobb's angle was under 25°, moderate if the angle was over 25°but under 60°, and severe if the angle was over 60°. Scoliosis was severer in DMD than in MyD patients, most of whom did not have scoliosis (Table 1). Thirty-four patients with DMD and 14 with MyD had received nutrition via feeding tubes before gastrostomy placement (Table 1).

The indications for gastrostomy are shown in Table 2. Swallowing difficulty was the most common reason in both DMD and MyD patients. In DMD patients, the rate of malnutrition was higher than that in MyD patients (36% versus 5%), while the rate of recurrent respiratory infection or respiratory disturbance was lower in DMD than in MyD patients (16% versus 23%). Trouble with tube feeding, digestive symptoms, such as gastroesophageal reflux and abdominal bloating, and patient choice, were the less frequent indications in both groups. The benefits suggested by their attending doctors are shown in Table 2. Amelioration of malnutrition was the most common benefit in both DMD and MyD patients. Improvements of swallowing and respiratory status were observed in about 20% of patients in each group. Almost all DMD patients and their families felt very good or rather good about the gastrostomy placement, while

Table 2
Indications for gastrostomy, and subjective evaluations of gastrostomy placement by attending doctors, patients and their families.

	DMD		MyD	
Indications (multiple answers allowed)	$n = 76$		$n = 39$	
Swallowing difficulty	68	(89%)	37	(95%)
Malnutrition	27	(36%)	2	(5%)
Recurrent respiratory infections orrespiratory disturbance	12	(16%)	9	(23%)
Trouble with tube feeding	10	(13%)	1	(3%)
Digestive symptoms*	9	(12%)	1	(3%)
Patient's choice	3	(4%)		
Benefits suggested by attending doctors (multiple answers allowed)	$n = 77$		$n = 36$	
Amelioration of malnutrition	56	(73%)	25	(69%)
Easy care	37	(48%)	10	(28%)
Stable general condition	28	(36%)	7	(19%)
Improvement of swallowing	17	(22%)	9	(25%)
Improvement of respiratory status	13	(17%)	7	(19%)
Improvement of digestive symptoms	7	(9%)		
Patients' evaluations	$n = 54$		$n = 21$	
Good	39	(72%)	6	(28%)
Rather good	13	(24%)	2	(10%)
Rather bad			1	(5%)
Bad	2	(4%)	12	(57%)
Neither				

* Gastroesophageal reflux and abdominal bloating.

MyD patients and their families were less satisfied (Table 2).

In both DMD and MyD patients, body weight data were collected from a subset of patients. Body weight 6 months before gastrostomy placement, at the time of placement, and 6 months after placement were 30.1 (±7.7) kg, 29.1 (±8.6) kg, and 30.4 (±7.2) kg, respectively, in the 68 DMD patients whose data were obtained, and 46.7 (±12.5) kg, 44.0 (±10.7) kg, and 44.7 (±10.3) kg, respectively, in the 33 MyD patients whose data were obtained (Fig. 1). In DMD patients, mean body weight increased significantly after placement ($p = 0.009$). Routes of nutritional intake after gastrostomy placement are shown in Fig. 2. More DMD patients received oral nutrition after placement.

Sixteen complications (incidence: 13.7%) occurred in 16 patients during the perioperative period (Table 3). The most common complications were cutaneous symptoms such as bleeding, wound infection (5 patients), and respiratory failure (5 patients). Thirty complications (incidence: 25.9%) occurred after the perioperative period in 28 patients (Table 3). Problems with the gastrostomy tube such as disruption, obstruction, difficulty with insertion, buried bumper syndrome, meaning that the bumper became lodged anywhere between the gastric wall and the skin along the gastrostomy tube tract, and accidental tube removal were observed in 15 patients; cutaneous symptoms, such as granulation, pain and inflammation of the skin, were observed in 8 patients; and digestive symptoms such as abdominal bloating, diarrhea, gastroesophageal reflux and hemorrhagic gastric ulcer were observed in 5 patients. With regard to serious complications, respiratory failure occurred in 5 patients (age range 26–65 years) during the perioperative period, and peritonitis in 3 (age range 20–26 years) during and after the perioperative period. Among those with respiratory failure, 4 patients received tracheal intubation and mechanical ventilation. Of these patients, only 1 was extubated, and 1 died a month after the surgery. Two patients needed NPPV for 24 h before gastrostomy placement, and they received the same respiratory management during the operation. Sedative drugs such as intravenous diazepam and midazolam were used in both patients. Among those with peritonitis, 1 patient developed peritonitis 12 days after the surgery, and this was managed conservatively. The cause was dissection of the abdominal and gastric walls in this patient. Another patient developed peritonitis 3 days after surgery that eventually resulted in death. At autopsy, dissection of the abdominal and gastric walls and *Candida* infection of the abdominal wall were observed. Both patients underwent gastropexy at gastrostomy placement. The third patient developed peritonitis 3 years postoperatively and required surgical treatment; the cause of peritonitis was unknown. The first 2 patients

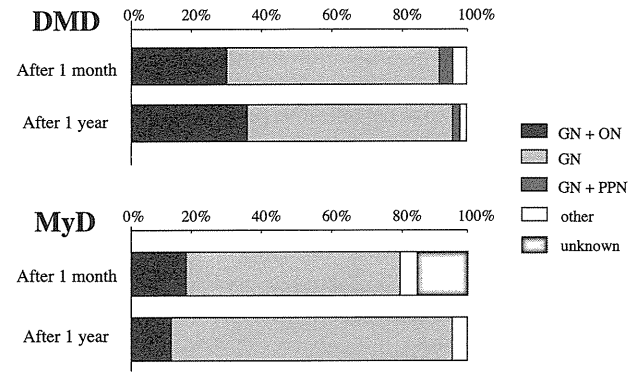

Fig. 2. Routes of nutritional intake after gastrostomy placement GN, gastrostomy nutrition; ON, oral nutrition; PPN, peripheral parenteral nutrition.

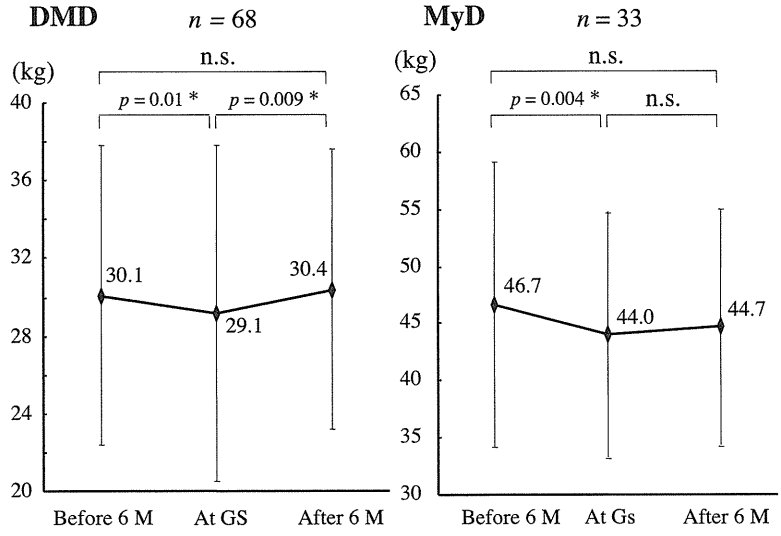

Fig. 1. Body weight changes before versus after gastrostomy placement 6 M, 6 months; GS, gastrostomy placement; *, statistically significant ($p < 0.05$); n.s., not significant.

Table 3
Complications during and after the perioperative period.

	DMD	MyD
Complications during the perioperative period	$n = 77$	$n = 40$
Cutaneous symptoms	3	2
Respiratory failure	3	2
Pneumonia	2	
Peritonitis	2	
Digestive symptoms	1	1
Complications after the perioperative period	$n = 76$	$n = 40$
Trouble with gastrostomy tube	11	4
Cutaneous symptoms	7	1
Digestive symptoms	3	2
Peritonitis		1
Peristomal leakage	1	

weighed only about 20 kg and presented with severe intestinal gaseous distention.

4. Discussion

Although gastrostomy feeding has been widely used in various conditions [6–10], few studies have focused on gastrostomy feeding in patients with muscular dystrophy [3–5]. Martigne et al., reporting gastrostomy feeding in 25 DMD patients, showed gastrostomy to be effective for improving nutritional status and to be well-tolerated by individuals with DMD.

In our study, although malnutrition was cited as the indication for gastrostomy in only 36% of DMD and 5% of MyD patients, amelioration of malnutrition was cited as the main benefit of gastrostomy in about 70% of both groups. This indicates that malnutrition had been unrecognized before gastrostomy placement in most patients. Improvements of swallowing and respiratory status were seen in some patients. Possible reasons include these patients becoming free of their feeding tube, decreased aspiration, and stability of their general conditions. There were differences in the indications for gastrostomy between DMD and MyD patients. Swallowing difficulty in DMD patients implies that they gradually become unable to eat, and malnutrition then becomes pronounced. On the other hand, MyD patients were able to eat, but they had repeated respiratory infections due to aspiration. After gastrostomy placement, mean body weight gain was significant, and the percentage capable of oral ingestion increased among DMD patients, while mean weight gain was not significant and the percentage capable of oral ingestion decreased in MyD patients. Not surprisingly, the evaluations of gastrostomy placement by patients and their families were better for DMD than MyD.

Most complications in our study were those commonly observed in other populations who receive gastrostomies, and included cutaneous symptoms, problems with the gastrostomy tube, and gastrointestinal symptoms. The overall rate of complications associated with gastrostomy placement generally ranged from 5% to 50% in previous studies [9–13]. On the other hand, Martigne et al. reported that complications occurred in 21 of 25 patients with DMD (84%), but caused no mortalities [3]. They described some complications as apparently being more specific to DMD, e.g., aspiration pneumonia at the beginning of enteral feeding or shortly after general anesthesia. In our study, the complication rate was 13.7% during the perioperative period and 25.9% after the perioperative period; the overall rate of complications was 42.1%, i.e., similar to that observed in other groups of patients, but respiratory failure in the perioperative period and peritonitis appear to be highly problematic for patients with muscular dystrophy. Respiratory failure is the most important complication that must be considered in muscular dystrophy patients. Patients with unstable respiratory status are at risk for developing acute respiratory failure in the perioperative period. The clinician should take care when using sedative drugs. For patients who need NPPV support for 24 h, safe insertion of the endoscope may be difficult. The use of a small-caliber endoscope nasally, a laryngeal mask airway, or manual ventilation with a standard flow-inflated anesthesia bag attached to a nasal mask is recommended [14,15]. We should consider tracheostomy before gastrostomy for patients who hope to switch from NPPV to TPPV in order to avoid emergency tracheal intubation. In addition, peritonitis is among the most severe complications. Benign pneumoperitoneum is common after gastrostomy [13]. Pneumoperitoneum is usually self-limiting and of clinical concern only when intra-abdominal air worsens or when it is found in the presence of signs of peritonitis. This may occur due to the failure of tract formation possibly because of a very loose external bolster or disruption of the formed tract [10]. In our study, dissection of the abdominal and gastric walls was the cause of peritonitis in 2 patients, even though gastropexy had been performed in both. These two patients were extremely thin and presented with severe intestinal gaseous distention. Therefore, we must be cautious regarding the occurrence of peritonitis in such patients. In our study, the timing of gastrostomy placement was relatively late in the courses of muscular dystrophy patients. In other regions of the world, gastrostomy placement is mostly performed in the teens or third decade of life [3,4]. High mortality in Japan might be associated with the procedures being performed on an older patient population with more advanced disease as compared to other regions. Given these facts, we should consider gastrostomy placement in the early stage of swallowing difficulty before respiratory disturbance or body weight loss progresses. In addition, gastrostomy placement should be a nutritional treatment option for patients and their families in the early stages of muscular dystrophy.

Our study has several limitations. First, the mean follow-up period after gastrostomy placement was only 1.8 (±1.4) years in DMD patients and 2.3 (±2.0) years in MyD patients. Second, few patients were examined for body weight decrease during the follow-up period. The number of MyD patients in whom body weight was analyzed was especially small. Third, the evaluations of malnutrition, swallowing difficulty and respiratory disturbance were predominantly subjective. Therefore, further investigation of a larger population with a longer follow-up period applying objective methods for evaluations of nutritional status, swallowing and respiratory function are needed in order to confirm whether gastrostomy feeding actually contributes to improvements in quality of life and longevity of patients with muscular dystrophy.

5. Conclusions

Many benefits were observed after the introduction of gastrostomy feeding in patients with muscular dystrophy. Especially in DMD patients, mean body weight significantly increased after gastrostomy placement. Although most complications were also commonly observed in other populations, respiratory failure and peritonitis are apparently more problematic in patients with muscular dystrophy. Given these facts, we recommend the following: (I) Gastrostomy placement should be considered in the early stages of swallowing difficulty, before respiratory disturbance or body weight loss progresses. (II) Careful respiratory management is necessary in the perioperative period for patients with unstable respiratory status, especially those requiring NPPV support for 24 h.

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Case report

A novel mutation in the *LMNA* gene causes congenital muscular dystrophy with dropped head and brain involvement

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Abstract

We describe a 22-month-old girl with axial muscle and diaphragmatic weakness as well as motor developmental delay without mental retardation. The striking clinical feature was a dropped head, although she could walk unaided. T2/FLAIR brain MRI revealed a focal abnormality with high signal intensity in the white matter including U-fibers. A muscle biopsy showed active necrotic and regenerative processes. These distinct clinical findings prompted a mutational analysis of the *lamin A* (*LMNA*) gene, and we identified a novel heterozygous mutation in *LMNA* (c.1330_1338dup9). This is the first report of an Asian patient with *LMNA*-related congenital muscular dystrophy (L-CMD) and a dropped head.

Keywords: A-type lamin; *LMNA*; L-CMD; Congenital muscular dystrophy; Dropped head; Brain abnormality

1. Introduction

The *LMNA* gene encoding A-type lamins that are nuclear envelope proteins is located on chromosome 1q11–q23. Mutations in *LMNA* are associated with various diseases, including autosomal dominant and recessive Emery–Dreifuss muscular dystrophy, limb-girdle muscular dystrophy type 1B, cardiomyopathy with conduction defects, familial partial lipodystrophy, Charcot-Marie-Tooth disease type 2B and premature aging syndromes [1]. *LMNA*-related congenital muscular dystrophy (L-CMD) characterized by severe axial muscle weakness with a dropped head has recently been described [2]. We describe the first Asian patient with L-CMD and a dropped head associated with a novel duplication mutation in *LMNA*.

2. Patient

A 22-month-old Japanese girl was the third child of healthy and non-consanguineous parents. Neuromuscular diseases were not evident in the family history. The patient was born at term with no abnormalities during the pregnancy and neonatal period. Psychomotor development was normal except for poor head control. She sat upright at 9 months of age and walked unaided at 13 months.

A neurological examination at the age of 22 months revealed severe axial muscle weakness predominantly in the neck. She had a proximal dominant muscle weakness, used the Gowers' maneuver to stand up, and had difficulty in raising her arms. She could walk independently with a

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dropped-head posture, but often fell, and was unable to run. Diaphragmatic weakness was observed by fluoroscopy, and transcutaneous blood gas monitoring showed hypercapnia during sleep (Fig. 1B). Deep tendon reflexes were absent. No joint contractures and spinal rigidity were evident.

Her serum CK level was 1317 IU/L (normal <200 IU/L), and electromyography showed myopathic patterns with normal motor and sensory nerve conduction. T2-weighted and FLAIR MRI of the brain showed areas of focal high signal intensity in the white matter including U-fibers (Fig. 2). Findings of electrocardiography and echocardiography were normal. A muscle biopsy taken from the biceps brachii at age 22 months showed myopathic changes with necrotic and regenerating fibers (Fig. 1). Immunohistochemical assessment identified positive staining for dystrophin, sarcoglycans, alpha-dystroglycan, emerin, merosin and collagen VI. Mutational analysis of *LMNA* revealed a heterozygous c.1330_1338dup9 (p.E444_D446dup) in exon 7. This duplication was not found in 100 control Japanese DNA samples.

3. Discussion

Reports of L-CMD with a dropped head are rare, and all of them have been from Europe [2–6]. Here we describe the first Asian patient with L-CMD accompanied by a dropped head caused by a novel mutation in the *LMNA*. The muscle weakness in this patient was distinctive in that she was unable to steadily hold up her head and crawl, although she could walk independently. Due to the characteristic muscle involvement with dystrophic muscle pathol-

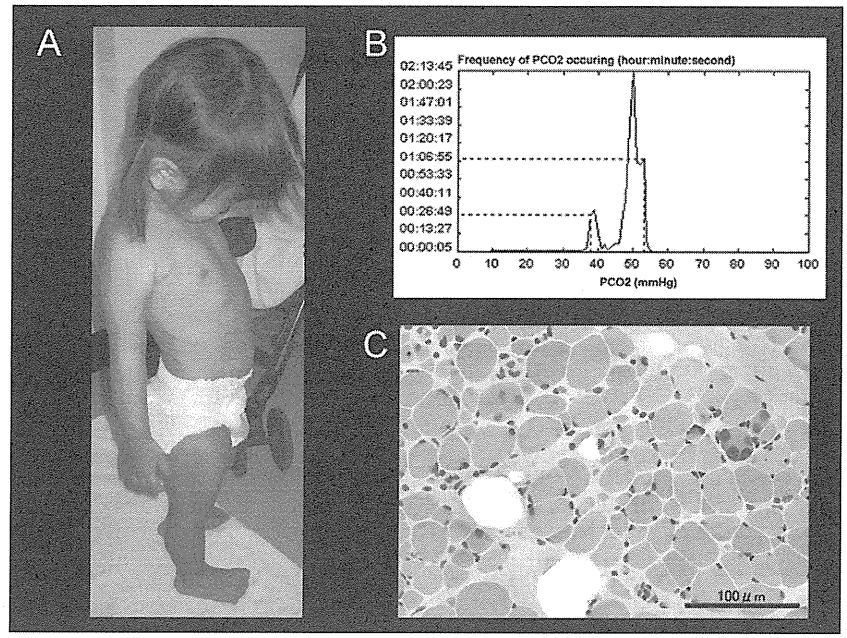

Fig. 1. Patient at age 22 months shows dropped head (A). She can stand and walk independently. Transcutaneous blood gas monitoring of PCO_2 during sleep (B). Muscle biopsy stained with hematoxylin and eosin (C). Necrotic, regenerating and other fibers remarkably vary in size.

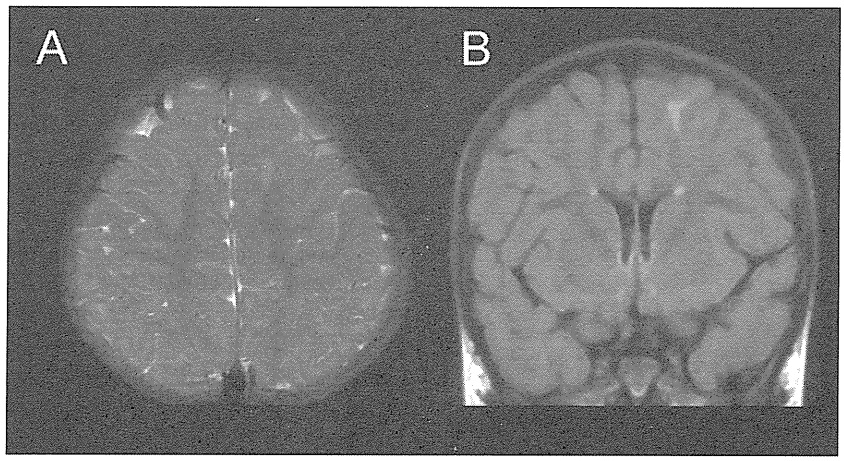

Fig. 2. MRI image of brain. Axial T2 weighted (A) and coronal FLAIR (B) images show focal high signals in white matter including U-fibers.

ogy, we performed a mutational analysis of the *LNMA* and discovered a novel 9-bp duplication that produced a duplication of three amino acids (p.E444_D446dup). A heterozygous missense mutation in *LMNA* is the usual cause of L-CMD and this is the first report of L-CMD caused by an in-frame duplication mutation.

Quijano-Roy et al. clinically subdivided L-CMD in 15 patients into severe and dropped-head types [2]. Nine patients with the dropped-head type acquired independent ambulation between the ages of 13 months and 2.5 years. However, most of them lost the ability to walk within a few years. Respiratory involvement was also evident in the eight of the patients with dropped-head type, and seven of them required non-invasive positive pressure ventilation. Cardiac arrhythmia was another important clinical finding in four of the 15 patients, and one suddenly died despite the outcomes of cardiac studies being normal [2]. Our patient was classified as having the dropped-head type of L-CMD with a diaphragmatic weakness and mild hypercapnia that indicated respiratory insufficiency. Considering published clinical information about L-CMD, respiratory and cardiac management is crucial, because respiratory failure and/or arrhythmia may develop later in life. Polysomnography and 24-h Holter electrocardiography will be performed as soon as our patient is old enough to cooperate, and ventilatory support will be provided if the respiratory insufficiency progress.

Brain MRI revealed focal white matter changes in our patient. Changes in the white matter of the brain without molecular data have been associated with Emery–Dreifuss muscular dystrophy [7], but brain involvement has not been associated with *LMNA* mutations until now. Such MRI findings might have occurred independently. Further studies and careful follow up of central nervous system involvement are required for patients with *LMNA* mutations.

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RESEARCH ARTICLE

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Functional Genetic Variation at the *NRGN* Gene and Schizophrenia: Evidence From a Gene-Based Case–Control Study and Gene Expression Analysis

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Genome-wide association and follow-up studies have reported an association between schizophrenia and rs12807809 of the *NRGN* gene on chromosome 11q24.2. We investigated the association of five linkage disequilibrium-tagging SNPs and haplotypes that cover the *NRGN* gene with schizophrenia in a Japanese sample of 2,019 schizophrenia patients and 2,574 controls to determine whether rs12807809 is the most strongly associated variant for schizophrenia in the vicinity of the *NRGN* gene. We found that the rs12807809–rs12278912 haplotype of the *NRGN* gene was associated with schizophrenia (global $P = 0.0042$). The frequencies of the TG and TA haplotypes of rs12807809–rs12278912 in patients were higher (OR = 1.14, $P = 0.0019$) and lower (OR = 0.85, $P = 0.0053$), respectively, than in the controls. We did not detect any evidence of association of schizophrenia with any SNPs; however, two nominal associations of rs12278912 (OR = 1.10, $P = 0.057$) and rs2075713 (OR = 1.10, $P = 0.057$) were observed. Furthermore, we detected an association between the rs12807809–rs12278912 haplotype and *NRGN* expression in immortalized lymphoblasts derived from 45 HapMap JPT subjects ($z = 2.69$, $P = 0.007$) and confirmed

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the association in immortalized lymphoblasts derived from 42 patients with schizophrenia and 44 healthy controls ($z = 3.09$, $P = 0.002$). The expression of the high-risk TG haplotype was significantly lower than the protective TA haplotype. The expression was lower in patients with schizophrenia than in controls; however, this difference was not statistically significant. This study provides further evidence of the association of the *NRGN* gene with schizophrenia, and our results suggest that there is a link between the TG haplotype of rs12807809–rs12278912, decreased expression of *NRGN* and risk of developing schizophrenia.

Key words: schizophrenia; *neurogranin* (*NRGN*); single nucleotide polymorphism (SNP); genome-wide association study (GWAS); gene expression

INTRODUCTION

Schizophrenia is a common and complex psychiatric disease with strong genetic components. Schizophrenia has an estimated heritability of approximately 80% [Cardno and Gottesman, 2000; Tsuang, 2000], and many genes have been implicated in the pathogenesis of schizophrenia [Sun et al., 2008].

Genome-wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) investigate thousands of DNA samples from patients and controls, and these studies are a powerful tool for identifying common risk factors in complex diseases. Stefansson et al. [2009] combined the samples (from 12,945 patients with schizophrenia and 34,591 controls) from three large GWAS (the SGENE-plus, the International Schizophrenia Consortium and the Molecular Genetics of Schizophrenia GWAS) and conducted follow-up studies in 4,999 patients and 15,555 controls from four sets of samples from Europe, including from the Netherlands, Denmark, Germany, Hungary, Norway, Russia, Sweden, Finland, and Spain. The authors detected several significant association signals. Seven markers gave P values smaller than the genome-wide significance threshold of approximately 1.6×10^{-7} in the combined samples: five markers, rs6913660, rs13219354, rs6932590, rs13211507, and rs3131296, which spanned the major histocompatibility complex (MHC) region on chromosome 6p21.3-22.1; a marker, rs12807809, located 3,457 bases upstream of the *neurogranin* (*NRGN*) gene on chromosome 11q24.2; and a marker, rs9960767, in intron 4 of the transcription factor 4 (*TCF4*) gene on chromosome 18q21.2. Of the seven SNPs, four SNPs, rs6913660, rs13219354, rs13211507, and rs9960767, were not polymorphic in the HapMap Japanese in Tokyo (JPT) samples. The minor allele frequencies (MAFs) for two SNPs, rs6932590 and rs3131296, were less than 5%. Because only one marker, rs12807809, in the *NRGN* gene was a common SNP in the HapMap JPT samples (MAF greater than 5%), we focused on this SNP and the *NRGN* gene in the present study.

The *NRGN* gene is the human homolog of the neuron-specific rat RC3/*neurogranin* gene. *NRGN* encodes a postsynaptic protein kinase substrate that binds calmodulin (CaM) in the absence of calcium and has been implicated in dendritic spine formation and synaptic plasticity [Baudier et al., 1991]. NRGN plays an important role in the Ca^{2+}–CaM signaling pathway [Hayashi, 2009]. Ca^{2+} influx-induced oxidation of NRGN leads to the postsynaptic activation of CaM-dependent protein kinase II (CaMKII) by CaM, which is associated with strengthened *N*-methyl-D-aspartate (NMDA) receptor signaling [Li et al., 1999]. Reduced NRGN activity may mediate the effects of NMDA hypofunction implicated in the pathophysiology of schizophrenia.

The *NRGN* gene spans 7.3 kb of genomic DNA and contains four exons [Martinez de Arrieta et al., 1997]. Part of exon 1 and exon 2 encode a 78-amino-acid protein, and exons 3 and 4 contain untranslated sequences. A thyroid hormone response element (TRE) has been identified in intron 1 [Martinez de Arrieta et al., 1999]. An association between the *NRGN* gene and schizophrenia has previously been reported in a small population of male Portuguese and Brazilians [Ruano et al., 2008], although the associated SNP in the study, rs7113041, was not tightly correlated with the genome-wide supported SNP, rs12807809 (HapMap CEU $r^2 = 0.07$, JPT $r^2 = 0.01$). In addition, two separate studies reported no association between the genetic variants of *NRGN* and schizophrenia in Bulgarian [Betcheva et al., 2009] and Chinese populations [Li et al., 2010]. The genome-wide supported SNP and other SNPs in the *NRGN* gene were not genotyped in the GWAS of schizophrenia in Japanese populations because of a difference in the genotyping chips used among the separate GWAS, which the Illumina HumanHap 300 or 550 BeadChips, Affymetrix Genome-Wide Human SNP Array 5.0 and Affymetrix GeneChip Mapping 100 K microarrays [Stefansson et al., 2009; Ikeda et al., 2011; Yamada et al., 2011] were used. Here, we first investigated the association between the *NRGN* gene and schizophrenia in a Japanese population using a gene-based approach to determine whether rs12807809 is the most strongly associated variant for schizophrenia near the *NRGN* gene. Second, we examined whether the associated haplotype of *NRGN* influenced *NRGN* expression in immortalized lymphoblasts derived from the HapMap JPT samples and our Japanese case–control samples.

MATERIALS AND METHODS

Subjects

Subjects for the genetic association analysis included 2,019 unrelated patients with schizophrenia (54.5% males, with a mean age ± SD of 44.7 ± 15.1 years) and 2,579 unrelated healthy controls (49.4% males, 45.4 ± 19.4 years). The mean age did not differ significantly between cases and controls ($P = 0.24$); however, the male to female ratio of the patients was significantly higher than in the controls ($P < 0.05$). Age and sex-matched subjects for *NRGN* expression analysis consisted of 42 patients with schizophrenia (58.1%, 38.4 ± 11.2 years) and 44 healthy subjects (56.8% males, 38.0 ± 11.4 years). These subjects were included in the genetic association analysis. All subjects used in both analyses were biologically unrelated, of Japanese ethnicity and were recruited from four geographical regions in Japan: Osaka, Aichi, Tokushima, and Tokyo [Yamaguchi-Kabata et al., 2008; Ohi et al., 2009]. Cases were recruited from outpatient and inpatient facilities at university hospitals and psychiatric hospitals. Each subject with schizophrenia had been diagnosed by at least two trained psychiatrists based on an unstructured clinical interview; diagnoses were made based on the

criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

SNP Selection and SNP Genotyping

This study was designed to examine the association between the *NRGN* gene and schizophrenia by selectively tagging SNPs in the *NRGN* gene and flanking regions (±5 kb). We selected five tagging SNPs using the TAGGER algorithm (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger) with the criteria of r^2 greater than 0.80 in "pair-wise tagging only" mode and an MAF greater than 5%, which was implemented in Haploview 4.2 using HapMap data release 27 Phase II + III, Feb 2009, on NCBI B36 assembly, dbSNP b126 [Japanese in Tokyo (JPT), Chr 11: 124,109,952.124,127,307]. The five tagging SNPs were rs1939214, rs12807809, rs12278912, rs2075713, and rs11219769. Markers are shown in Table I; orientation and alleles are reported on the genomic plus strand (rs12807809 is reported as T/C, as has been reported in previous GWAS [Stefansson et al., 2009]). Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA) as previously described [Hashimoto et al., 2006, 2007]. Detailed information on the PCR conditions is available upon request. Genotyping call rates were 98.9% (rs1939214), 98.5% (rs12807809), 99.3% (rs12278912), 99.3% (rs2075713), and 99.5% (rs11219769). No deviation from Hardy–Weinberg equilibrium (HWE) in the examined SNPs was detected in the patients with schizophrenia or healthy controls ($P > 0.05$). The positions of the five SNPs analyzed in the present study are shown in Figure 1.

Quantitative Measurement of *NRGN* Gene Expression

Isolation and immortalization procedures of lymphocytes from blood using the Epstein–Barr virus (EBV) were performed by SRL of Tokyo, Japan. Immortalized, patient-derived lymphocytes were grown in culture media supplemented with 20% fetal bovine serum. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). The total yield of RNA was determined by absorbance at 260 nm, and the quality of the RNA was determined using agarose gel electrophoresis.

According to the manufacturer's protocol, total RNA was treated with DNase to remove contaminating genomic DNA using DNase Treatment and Removal Reagents (Ambion, Austin, TX). Total RNA (10 μg) treated with DNase was used in a 50-μl reverse transcriptase reaction to synthesize cDNA with the SuperScript

TABLE I. Genotype and Allele Distributions for SNPs in the *NRGN* Gene Between Patients With Schizophrenia and Controls in a Japanese Population

Marker				SCZ (n = 2019)			CON (n = 2579)			Genotypic	MAF		Allelic	
SNP IDs (M)	Position[a]	M/m[b]	Gene	M/M	M/m	m/m	M/M	M/m	m/m	*P*-value (χ^2)	SCZ	CON	*P*-value (χ^2)	OR (95% CI)
rs1939214 (A)	124110500	A/G	5′	0.67	0.30	0.04	0.66	0.30	0.04	0.51 (1.3)	0.19	0.19	0.29 (1.1)	1.06 (0.95–1.18)
rs12807809 (T)[c]	124111495	T/C	5′	0.58	0.35	0.07	0.56	0.37	0.07	0.44 (1.6)	0.25	0.26	0.25 (1.3)	1.06 (0.96–1.16)
rs12278912 (G)[d]	124117369	G/A	Intron1	0.61	0.34	0.05	0.59	0.35	0.06	0.13 (4.1)	0.22	0.23	0.057 (3.6)	1.10 (1.00–1.22)
rs2075713 (A)	124123149	A/C	3′	0.65	0.31	0.04	0.62	0.33	0.05	0.17 (3.5)	0.20	0.21	0.057 (3.6)	1.10 (1.00–1.22)
rs11219769 (G)	124125357	G/T	3′	0.57	0.37	0.06	0.55	0.38	0.07	0.24 (2.8)	0.25	0.26	0.09 (2.8)	1.09 (0.99–1.19)

SCZ, patients with schizophrenia; CON, healthy controls; M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio.
[a]db SNP build 129.
[b]The first alleles shown are major alleles. All the alleles are represented according to the plus strand DNA sequence.
[c]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[d]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

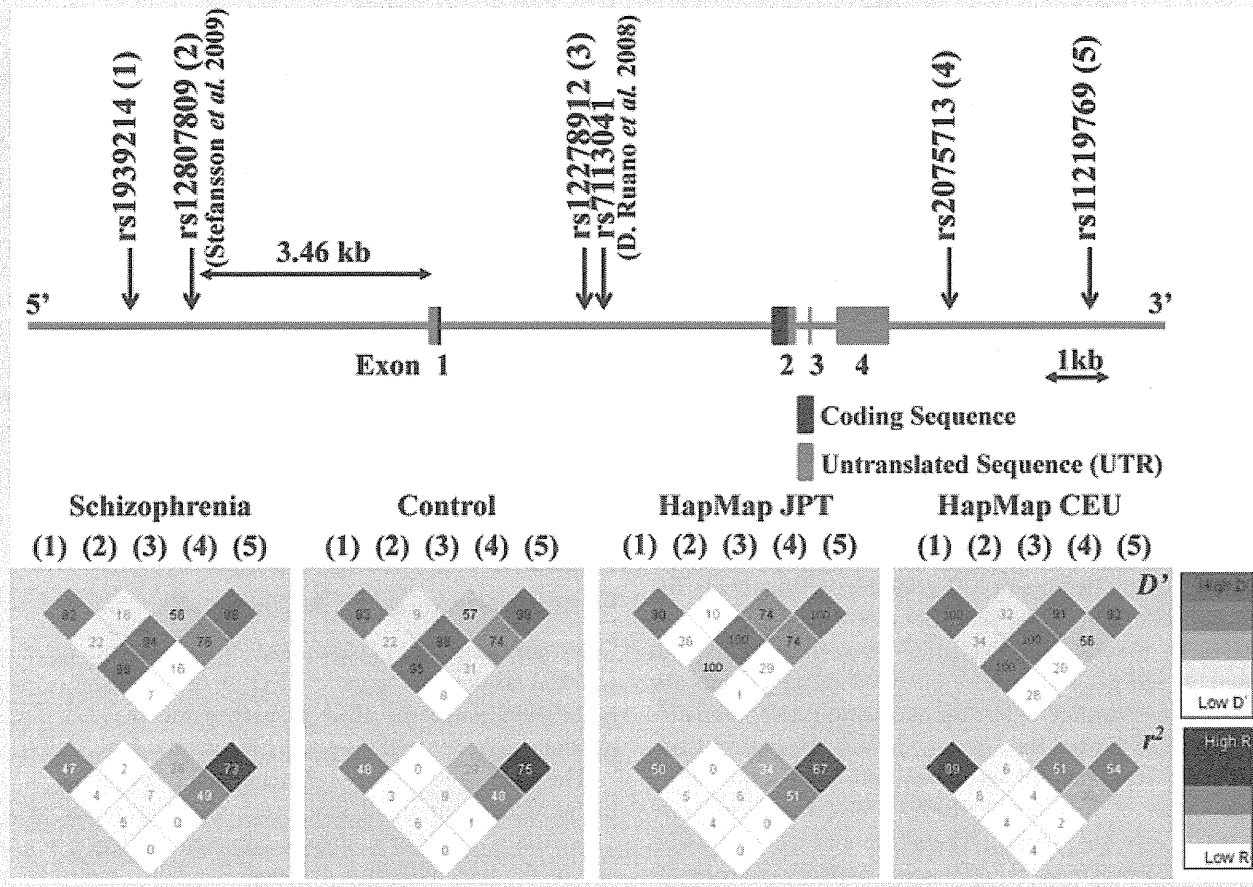

FIG. 1. The genomic structure of *NRGN*, including the locations of the five tagging SNPs studied and linkage disequilibrium of these SNPs in the patient, control, HapMap JPT, and CEU groups. Based on an entry in the Entrez Gene database (National Center for Biotechnology Information), the genomic structure of *NRGN* is shown above. The locations of the SNPs analyzed in this study are indicated by arrows, with numbers indicated in parentheses. The numbers indicated in parentheses refer to the numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of exons–introns and intermarkers are drawn to scale. The LDs between pairwise SNPs are shown using the D′ (upper) and r^2 (lower) values at the bottom of the map of the gene structure separately for cases, controls, the HapMap JPT samples and the HapMap CEU samples. High levels of LD are represented by black (D′ and r^2) coloring, with increasing color intensity from 0 to 100, as shown by color bars.

First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Detailed information on the PCR conditions is available upon request.

To measure mRNA expression levels of housekeeping (β-*actin*) and *NRGN* genes, we used the Pre-Developed TaqMan Assay Reagent kit (Applied Biosystems). Primer information (gene name: assay ID, transcript ID, target region) is as follows; *NRGN*: Hs00382922_m1, NM_001126181.1 and NM_006176.2, Exon1-2; β-actin: 4326315E, NM_001101, no region indicated (Applied Biosystems). Expression levels of these genes were measured by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a 384-well format as previously described [Yamamori et al., 2011; Yasuda et al., 2011]. PCR data were obtained using Sequence Detector software (SDS version 2.1; Applied Biosystems) and quantified using a standard curve. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold C_t, which is where the fluorescence generated within a reaction crossed the threshold, versus the quantity of RNA. All samples were measured using a single plate per target gene, and their C_t values were in the linear range of the standard curve. The quantity of each sample was predicted by C_t values. The qRT-PCR reaction was performed in triplicate, and the expression level of the gene was taken as the average of three independent measurements. Standard curves were obtained using serial dilutions (1:4) of pooled complementary DNA prepared from 300 ng total RNA derived from immortalized lymphocytes. The standard curves of β-*actin* and *NRGN* showed that these genes were expressed in immortalized lymphocytes. In each experiment for β-*actin* and *NRGN*, the R^2 value of the standard curve was >0.99, and no-template control assays resulted in no detectable signal. The individual expression levels of the *NRGN* gene were normalized to the housekeeping gene (raw target gene expression level divided by raw housekeeping gene expression level) and were used for statistical analysis.

Haplotype Associated With *NRGN* Expression (eQTL)

To identify whether the haplotypes in *NRGN* associated with schizophrenia may be expression quantitative trait loci (eQTL), we analyzed *NRGN* expression in two datasets of lymphoblast-derived HapMap JPT samples and in the Japanese case–control samples. For the HapMap JPT samples, we extracted genotypes and *NRGN* lymphoblastoid expression data from the HapMap JPT samples (n = 45) deposited in GeneVar (http://www.sanger.ac.uk/humgen/genevar/ [Stranger et al., 2007]). For the Japanese case–control samples, we used our genotypes and *NRGN* lymphoblastoid expression data obtained using the method described above.

Statistical Analyses

We performed power calculations using the Power Calculator for Two-Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [Skol et al., 2006]). The power estimate was based on an allele frequency of 0.83 at rs12807809, an odds ratio of 1.19, which was indicated by Stefansson et al. [2009], a prevalence of 0.01, and an alpha level of 0.05 using a multiplicative model.

Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for sex and the Mann–Whitney *U*-test for age using PASW Statistics 18.0 software (SPSS Japan, Inc., Tokyo, Japan). Deviation from HWE was tested separately in test cases and controls using χ^2 tests for goodness of fit using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The allelic and genotypic distributions of *NRGN* polymorphisms between patients and controls were analyzed using χ^2 tests with SNPAlyze V5.1.1 Pro software. The number of effective independent SNPs assayed was estimated to correct for multiple testing by the spectral decomposition method of Nyholt using the SNPSpD software [Nyholt, 2004]. The effective number of independent marker loci was 4.13 and corrected *P*-value for allelic and genotypic associations was set at $P < 0.012$. Pairwise linkage disequilibrium (LD) analyses expressed by D′ and r^2 were applied to detect the intermarker relationships in each group using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated using the method of maximum likelihood with genotyping data using the expectation–maximization (EM) algorithm from SNPAlyze V5.1.1 Pro software. Rare haplotypes detected in less than 3% of patients and controls were excluded from the haplotypic association analysis, as previously described [Ohi et al., 2009, 2010]. We performed 10,000 permutations for significance tests to determine empirical significance using a 2 × 2 contingency table approach. We used a 2- to 5-window fashion analysis. Since Bonferroni correction for multiple testing is considered to be too conservative to apply to genetic association analyses [Nyholt, 2001], method of Nyholt [Nyholt, 2004] for allelic and genotypic associations and permutation tests [Dudbridge, 2003] for haplotypic associations are considered to be appropriate for these analyses.

The difference in expression levels between Japanese patients with schizophrenia and controls was analyzed using linear regression in PASW Statistics 18.0 software. Age and sex, which may influence gene expression, were corrected for in the expression analysis. HPlus (http://qge.fhcrc.org/hplus) is a software application for estimating haplotype frequencies and inferring individual haplotypes based on EM and progressive ligation (PL) algorithms [Li et al., 2003], and most significantly assessing haplotypic associations with various types of phenotypes using linear regression. Differences of expression levels among haplotypes were analyzed using linear regression in HPlus software. Each genotype was treated as the number of major alleles (0, 1, and 2) in the expression analysis. For the joint haplotype analysis in HPlus software, each haplotype was tested against the reference haplotype (equal to most frequent haplotype) using linear regression. As age and sex were not available for the HapMap samples, these confounding factors were not corrected for in the expression analysis. Expression levels in Japanese cases, control samples and in the combined samples were corrected for age and sex in the analyses. We applied a Bonferroni correction in expression analysis (three tests). The significance level for statistical tests was set at two-tailed $P < 0.05$.

RESULTS

Genetic Association Analysis

Our study size of 2,019 cases and 2,579 controls had sufficient power (>80%) to detect a genetic effect at ORs of 1.19 or greater for rs12807809 when the allele frequency was 0.83, as described in previous GWAS (SGENE-plus) [Stefansson et al., 2009].

The genotype and allele frequencies of five tagging SNPs located in the *NRGN* gene and flanking regions are summarized in Table I. There was no allelic or genotypic association with schizophrenia for any of the five SNPs (uncorrected $P > 0.05$). However, nominal differences in allele frequencies between patients and controls were observed in rs12278912 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$) and rs2075713 ($\chi^2 = 3.6$, $P = 0.057$, corrected $P = 0.24$). The major allele frequencies of both SNPs were higher in patients than in controls. Consistent with previous GWAS [Stefansson et al., 2009], the frequency of the major T allele of rs12807809 was higher in patients (75.4%) than in controls (74.4%) in our Japanese population, although the results did not reach statistical significance [$\chi^2 = 1.3$, $P = 0.25$, OR (95% confidence interval (CI)) = 1.06 (0.96–1.16)].

We focused on haplotypic association between patients with schizophrenia and healthy subjects using a 2- to 5-window fashion analysis. Haplotype analysis showed a significant association with schizophrenia (rs12807809–rs12278912, $\chi^2 = 13.1$, global $P = 0.0042$) (Supplementary Table I). The frequency of the major TG haplotype of rs12807809–rs12278912 was higher in patients (62%) than in controls (58%) [$\chi^2 = 9.4$, $P = 0.0019$, OR (95% CI) = 1.14 (1.05–1.24)] (Table II). On the other hand, the frequency of the TA haplotype of rs12807809–rs12278912 was lower in patients (14%) than in controls (16%) [$\chi^2 = 7.3$, $P = 0.0053$, OR (95% CI) = 0.85 (0.76–0.96)] (Table II). There was no haplotypic association with schizophrenia for any other haplotypes. These findings suggest that the major TG haplotype of rs12807809–rs12278912 may be related to an increased risk of schizophrenia, and the TA haplotype may have a protective role against the susceptibility to schizophrenia. These results of allelic, genotypic, or haplotypic associations were not affected by excluding 86 samples used for expression analyses (data not shown).

TABLE II. Differences in the rs12807809–rs12278912 Haplotype Between Patients With Schizophrenia and Healthy Subjects

Haplotype rs12807809[a]–rs12278912[b]	Frequency: Patients	Frequency: Controls	Individual P (χ^2)	OR (95%CI)	Global P (χ^2)
					0.0042 (13.1)
TG	0.62	0.58	**0.0019 (9.4)**	1.14 (1.05–1.24)	
CG	0.17	0.18	0.07 (3.4)	0.90 (0.81–1.01)	
TA	0.14	0.16	**0.0053 (7.3)**	0.85 (0.76–0.96)	
CA	0.08	0.08	0.57 (0.3)	1.05 (0.90–1.22)	

Significant P values are shown as bold-faced and underlined type.
[a]The genome-wide supported SNP for schizophrenia [Stefansson et al., 2009].
[b]Because a high linkage disequilibrium between rs12278912 and rs7113041 [Ruano et al., 2008] was found in the HapMap JPT samples ($r^2 = 0.93$), rs12278912 was selected as the tagging SNP by the TAGGER program.

The LD relationships between the markers are provided in Figure 1. The LD pattern observed in our controls was similar to our patients and the JPT HapMap samples; however, it was different from that of the CEU HapMap samples. The strengths of the LD patterns of rs1939214–rs12807809 and rs12278912–rs2075713–rs11219769 were different between Japanese populations and the CEU HapMap samples. The low LD pattern of rs12807809–rs12278912 was similar among the groups ($D' < 0.50$, $r^2 < 0.10$).

NRGN Gene Expression Analysis

The *NRGN* expression level was lower in patients with schizophrenia ($n = 42$, mean $\pm$ SD, 0.86 ± 0.58) than in controls ($n = 44$, 1.00 ± 0.75). However, the results did not reach statistical significance ($r = -0.14$, beta $= -0.11$, SE $= 0.14$, $t = -0.97$, $P = 0.34$).

Based on the results from the genetic association analysis, we investigated whether the rs12807809–rs12278912 haplotype of the *NRGN* gene was an eQTL in two datasets. The rs12807809–rs12278912 haplotype related to schizophrenia was significantly associated with *NRGN* expression in healthy HapMap JPT samples. The *NRGN* gene expression of the high-risk TG haplotype of rs12807809–rs12278912 was significantly lower than that of the protective TA haplotype ($z = 2.69$, $P = 0.007$). We confirmed that the rs12807809–rs12278912 haplotype was significantly associated with *NRGN* expression normalized to the β-*actin* expression in the controls and combined samples (Fig. 2 and Table III, control samples: $z = 2.30$, $P = 0.021$, combined samples: $z = 3.09$, $P = 0.002$). The association occurred in the same direction among the HapMap JPT, control, and combined samples. In case samples, the expression level of rs12807809–rs12278912 was lower in samples with the high-risk TG haplotype than in those with the protective TA haplotype, although the result did not reach statistical significance ($z = 1.49$, $P = 0.14$). The association in the HapMap JPT and combined samples remained significant after correction for multiple tests (HapMap JPT samples: corrected $P = 0.021$, combined samples: corrected $P = 0.006$). However, there was no significant association after applying the correction in control samples (corrected $P = 0.063$).

DISCUSSION

In this study, we provided evidence that haplotypes, including the genome-wide-screen-supported SNP of the *NRGN* gene, were associated with an increased risk of schizophrenia. Our in silico analysis showed that the high-risk rs12807809–rs12278912 haplotype of the *NRGN* gene may be associated with a low expression level of the *NRGN* gene in lymphoblasts derived from the HapMap JPT samples. We confirmed the association between the haplotype and *NRGN* expression in the combined case–control samples. Our results suggest that the schizophrenia-associated haplotype at the

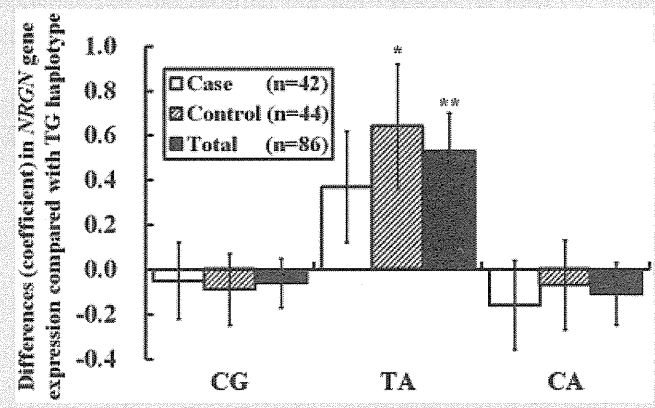

FIG. 2. The association between the rs12807809–rs12278912 haplotype of the *NRGN* gene and *NRGN* expression in lymphoblasts. Expression of the protective TA haplotype of rs12807809–rs12278912 was significantly higher than that of the high-risk TG haplotype in controls and combined case–control samples. The error bars represent standard errors of the coefficient. Estimated frequencies of each haplotype were as follows—TG haplotype: Case, 69%; Control, 61%; Total, 65%; CG haplotype: Case, 16%; Control, 20%; Total, 18%; TA haplotype: Case, 7%; Control, 11%; Total, 9%; CA haplotype: Case, 8%; Control, 9%; Total, 8%. *$P < 0.05$, **$P < 0.01$.

NRGN gene may be a functional variant, and the results support an association between the *NRGN* gene and schizophrenia.

This report is the first investigation of the association of tagging SNPs and haplotypes covering the *NRGN* gene with schizophrenia. To our knowledge, five genetic studies have investigated whether the *NRGN* gene is implicated in schizophrenia. A genome-wide linkage study has shown that the chromosomal region 11q23.3-24 including the *NRGN* gene is linked to schizophrenia in British and Icelandic populations [Gurling et al., 2001]. Subsequently, an association study determined that rs7113041, which displays high LD with rs12278912 and is located on intron 1 in the *NRGN* gene, is related to the risk of developing schizophrenia in male subjects of Portuguese origin [Ruano et al., 2008]. In addition, three GWAS and follow-up studies have shown that rs12807809 is associated with schizophrenia in large European samples [Stefansson et al., 2009]. However, two studies reported no association between *NRGN* and schizophrenia in Bulgarian or Chinese populations [Betcheva et al., 2009; Li et al., 2010]. In the present study, we determined that the rs12807809–rs12278912 haplotype is associated with an increased risk of schizophrenia in a Japanese population. However, there were no significant associations between any SNP, including rs12807809 and rs12278912, and schizophrenia in the population. The inconsistency of association among the previous studies and the present study might result from ethnic differences or type I or II errors for the different sample sizes: Portuguese, 315 cases, 295 controls and 73 trios [Ruano et al., 2008]; European Caucasian, 12,945 cases and 34,591 controls [Stefansson et al., 2009]; Japanese, 2,019 cases and 2,579 controls (present study); Bulgarian, 185 cases and 184 controls [Betcheva et al., 2009]; and Chinese, 2,496 cases and 5,184 controls [Li et al., 2010]. In addition, the SNPs investigated in each study were different. Ruano et al. [2008] and Betcheva et al. [2009] examined rs7113041, which has high LD with rs12278912 but not with rs12807809, whereas Stefansson et al. [2009] and Li et al. [2010] examined rs12807809 but not rs12278912. However, none of these studies examined haplotypes for the *NRGN* gene. Because the rs12807809–rs12278912 haplotype may be the most significant genetic variant in this region, further study is required to confirm the association between the rs12807809–rs12278912 haplotype and schizophrenia in other populations.

Differences in the relative *NRGN* expression levels between patients with schizophrenia and healthy subjects were not demonstrated. This result may be due to the small sample sizes in this study, which may have resulted in the failure to identify a modest difference in *NRGN* expression in this complex disease. We determined that the major TG haplotypic and the TA haplotypic frequencies of rs12807809–rs12278912 were higher and lower, respectively, in patients with schizophrenia than in healthy controls. In addition to these findings, we found that *NRGN* gene expression of the high-risk TG haplotype was significantly lower than that of the protective TA haplotype in lymphoblasts derived from our Japanese case–control subjects as well as the JPT HapMap sample. The low LD patterns of rs12807809–rs12278912 were similar across populations. This region may be vulnerable to recombination. Combinations of the TG and TA of rs12807809–rs12278912 could play an important role in the pathogenesis of schizophrenia. In this study, gene expression data derived from lymphoblasts raised the possibility that the rs12807809–rs12278912 haplotype may be a functional variant of *NRGN*. Further biological studies of the function of rs12807809–rs12278912 are required to verify the expression results.

Smith et al. [2011] analyzed *NRGN* expression in several brain tissues derived from a dataset of at least 130 individuals of European ancestry. However, they showed that neither the genome-wide supported SNP nor any individually correlated SNPs were associated with *NRGN* expression. They did not examine any association between haplotype and *NRGN* expression. There are several challenges in investigating expression findings in the postmortem

TABLE III. The Association Between the rs12807809–rs12278912 Haplotype and mRNA Expression

Haplotypes	Frequency	Coefficient	SE	CI	*P*-value (Z-score)
Schizophrenia ($n=42$)					
TG	0.69	0 (ref)	—	—	—
CG	0.16	−0.05	0.17	(−0.39–0.29)	0.76 (−0.30)
TA	0.07	0.37	0.25	(−0.12–0.86)	0.14 (1.49)
CA	0.08	−0.16	0.20	(−0.55–0.24)	0.43 (−0.78)
Healthy control (n = 44)					
TG	0.61	0 (ref)	—	—	—
CG	0.20	−0.09	0.16	(−0.39–0.22)	0.58 (−0.55)
TA	0.11	0.64	0.28	(0.09–1.18)	**0.021 (2.30)**
CA	0.09	−0.07	0.20	(−0.46–0.32)	0.73 (−0.34)
Total subjects (n = 86)					
TG	0.65	0 (ref)	—	—	—
CG	0.18	−0.06	0.11	(−0.28–0.15)	0.57 (−0.57)
TA	0.09	0.53	0.17	(0.19–0.87)	**0.002 (3.09)**
CA	0.08	−0.11	0.14	(−0.39–0.17)	0.45 (−0.75)

Joint Association Analysis (the reference haplotype is the most frequent haplotype).
For the joint haplotype test, several haplotypes were tested against the reference haplotype.
Significant *P* values are shown as bold-faced and underlined type.