Table 1
Subject information (autism group)

Subject no.	Gender	Age	CARS	IQ			Medication (mg/day)			EEG abnormality
				Verbal	Performance	Total	Neuroleptics	Antiepileptic drugs	Anticholinergic drugs	
1	M	33	33.5	NA[a]	NA[a]	38	Haloperidol 0.75	None	Biperiden 1	No
2	F	31	36.5	NA[a]	NA[a]	48	Haloperidol 2.25	Valproate 600	Biperiden 2	No
3	F	27	34.5	62	52	54	Haloperidol 0.75	None	Biperiden 1	No
4	M	20	33.0	73	73	68	Haloperidol 0.75	Valproate 600	Trihexyphenidyl 2	No[b]
5	F	20	34.0	NA[a]	NA[a]	37	Haloperidol 1.5	None	Biperiden 2	No
6	M	15	33.0	52	99	71	None	None	None	No
7	M	38	33.0	70	91	77	None	None	None	No
8	M	26	32.5	56	52	51	None	None	None	No
9	M	35	33.5	80	64	71	Bromperidol 3	None	Trihexyphenidyl 2	No

CARS, Childhood Autism Rating Scale; EEG, electroencephalogram; M, male; F, female.
[a] Verbal and non-verbal IQs were not available because the data were based on the Tanaka–Binet Test. Otherwise, the IQs were evaluated using the Wechsler Adult Intelligence Scale-Revised (WAIS-R).
[b] This subject had a history of generalized tonic–clonic seizures in his childhood, although no epileptiform EEG activities had been previously detected.

(Wechsler, 1981; Japanese standardized version, Shinagawa et al., 1990) or the Tanaka–Binet Intelligence Scale (Japanese standardized version of the Stanford–Binet test, Tanaka Institute for Education, 1987) (Table 1). No individuals with autism showed current electroencephalogram (EEG) abnormalities. One subject had a history of generalized tonic–clonic seizures in childhood, although no epileptiform EEG activities had been previously detected. Six of the individuals were treated with neuroleptics to reduce the occurrence of disabling self-injurious behavior. Mean haloperidol equivalent dose (Inagaki et al., 1999a) was 1.0 mg/day (SD 1.1). These 6 individuals also received anticholinergic drugs; mean biperiden equivalent dose (Inagaki et al., 1999b) of 0.89 mg/day (SD 0.78), to prevent the occurrence of Parkinsonism secondary to the neuroleptics. No symptoms of Parkinsonism were clinically observed. No individuals with autism received anxiolitics or hypnotics, but two were treated with sodium valoproate (600 mg/day) as a mood stabilizer.

The first language of all participants in both groups was Japanese. The exclusion criteria for both groups were a history of electroconvulsive therapy, neurological illness, traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 min, substance use or addiction, and presence of hearing or vision impairment. No individuals with autism showed evidence of tuberous sclerosis. An additional exclusion criterion for the control group was a history of psychiatric disease in themselves, or a family history of axis I disorder in their first-degree relatives.

This study was approved by the Ethical Committee of the Faculty of Medicine, University of Tokyo. After a complete explanation of the study, written informed consent was obtained from all the control subjects. Written informed consent was also obtained from all the autism participants as well as from their parents.

2.2. Task procedures

The subjects were presented with sequences of auditory stimuli consisting of standard (probability 90%) and deviant ($P = 10\%$) stimuli delivered randomly, except that each deviant stimulus was preceded by at least one standard stimulus. The interstimulus interval (ISI) was 510 ± 20 ms. The stimuli were delivered binaurally through plastic tubes. The subjects were instructed to watch a silent film to help distract them from the stimuli.

The experiment consisted of 3 conditions. The first condition was to elicit MMF in response to a duration decrement of pure-tone stimuli (tone-duration condition; standard, 100 ms duration; deviant, 50 ms duration). The second condition was to elicit MMF in response to a duration decrement of vowel stimuli (phoneme-duration condition; standard, Japanese vowel /a/ with a 150 ms duration; deviant, /a/ with a 100 ms duration). The last condition was to elicit MMF in response to a vowel across-category change (across-phoneme condition; standard, Japanese vowel /a/ with a 150 ms duration; deviant, /o/ with a 150 ms duration). These vowel stimuli were spoken by a native-Japanese-speaking actor, digitized using the NeuroStim system (NeuroScan Inc., USA), and edited to have a duration of 100 or 150 ms, loudness of 70 dBSPL and rise/fall time of 10 ms. The frequency spectra for the vowels were as follows: /a/, formant (F) $0 = 140$ Hz, $F1 = 760$, $F2 = 1250$, $F3 = 2750$, and $F4 = 3600$; /o/, $F0 = 140$ Hz, $F1 = 480$, $F2 = 770$, $F3 = 2820$, and $F4 = 3600$. The order of the 3 conditions was counterbalanced across the subjects.

2.3. Data collection and processing

The recording and analysis procedures were the same as those described elsewhere (Kasai et al., 2001, 2002, 2003). Magnetic fields were recorded in a magnetically shielded room (NKK Plant Engineering Co., Japan) with a 122 channel magnetometer (Neuromag Ltd., Finland; Knuutila et al., 1993). This whole-head magnetometer consists of 61 dual-sensor units, each with two orthogonal planar gradiometers for recording maximal signals directly above the source (Hämäläinen et al., 1993). The subjects sat on a chair with their head inside the helmet-shaped magnetometer. The position of the magnetometer with respect to the head was determined at the beginning of the task under each condition by recording the magnetic fields produced by currents fed into 3 indicator coils at predetermined locations on the scalp. The locations of these coils in relation to the preauricular points and nasion were determined with an Isotrak 3D-digitizer (Polhemus TM, USA) before the start of the experiment. One electrode was placed at the outer canthus and another one below the left eye to monitor eye movements.

MEG epochs were averaged separately for standard and deviant stimuli online. The duration of the averaging period was 512 ms, including a 64 ms prestimulus baseline. The recording bandpass was 0.03–100 Hz, with a sampling rate of 500 Hz. The first 10 stimuli were automatically excluded from averaging. Epochs coinciding with electrooculogram movement or MEG exceeding 150 μV or 3000 fT/cm were also excluded from averaging. Each condition lasted until 100 deviant stimuli without contamination of artifacts were acquired. Averaged responses were digitally filtered with a bandpass of 1–30 Hz.

2.4. MMF measurement

For each subject in each condition, equivalent current dipoles (ECDs) for MMF were calculated primarily according to the method used by Alho et al. (1998b). Briefly, the MMF was determined from the difference curves obtained by subtracting the response to standard stimuli from that to deviant stimuli. ECDs were then determined using a least-squares fit at 2 ms intervals from 100 to 250 ms. The calculation was performed separately for each hemisphere (a subset of 44 channels over the temporal brain areas), utilizing a spherical head model in which the center of the model sphere was placed 45 mm above the origin of the coordinate system (Alho et al., 1998b). ECDs with a maximal goodness of fit (GOF) $\geq 60\%$ were included in the analysis. In this procedure, we reduced the number of channels to 28–43 when the dipole was not calculated or a certain channel had a considerable number of artifacts. The mean GOFs under the 3 conditions and in the two hemispheres ranged from 78.3 to 85.7% for the autism group and 72.5 to 85.7% for the control group, and did not differ between groups for any condition or hemisphere (Mann–Whitney's U test, $Ps > 0.23$).

The subjects for whom ECDs were not reliably calculated for at least one hemisphere were 6/19 for the control group and 9/9 for the autism group. In the control group, visual inspection of the signals for these cases indicated that MMF was strongly lateralized to one hemisphere, possibly resulting in the failure to calculate ECDs in the other hemisphere. In the autism individuals, there were no gross artifacts or noises superimposed on averaged response curves that would account for the failure to calculate ECDs. In theory, the ECD is stronger when neuronal activities are synchronized and regionalized (Kasai et al., 2002, 2003). Thus, an alternative explanation for the failure to calculate ECDs may be that some individuals with autism had deficits in the synchronization and regionalization of the neuronal population involved in MMF generation.

To utilize the data of all the subjects in the statistical analyses, the magnitude of MMF responses was reassessed by applying global field power (GFP; Lehmann and Skrandies, 1980) to the analysis of MEG data (magnetic counterpart of GFP [mGFP]; Kasai et al., 2001, 2002, 2003; Kreitschmann-Andermahr et al., 1999; Rosberg et al., 2000). First, for each subject, the mGFP was calculated separately for each condition and hemisphere using the same 44 channels as those used in the dipole analysis. In this procedure, the number of channels was reduced to 28–43 when a certain channel had a considerable number of artifacts. The peak latency of MMF for each subject was determined based on the individual mGFP curve as a function of time. Second, the grand mean mGFP curves were plotted. The MMF power for each subject was then determined as the mean mGFP within a 100 ms window around the peak latency of the grand mean mGFP. This 100 ms window was chosen because it was within the length of clear evocation of MMF (Fig. 1). Our previous studies have shown the mGFP power/latency to be a good substitute for ECD strength/latency (Kasai et al., 2001, 2002, 2003).

2.5. Statistical analysis

Group differences in dipole strength and location were tested using Mann–Whitney U test. The t tests were performed for the group comparison of MMF power or latency for each condition and hemisphere. Since there were 12 multiple comparisons, the level for significance was set at $P = 0.0042$ (Bonferroni correction). Moreover, since there was unequal sample size and, in two conditions unequal variance (Levene's test for equality of variance; tone-duration, left hemisphere: $P = 0.02$; phoneme-duration, left hemisphere: $P = 0.008$; other conditions: $P > 0.28$) between the groups, we also performed non-parametric Mann–Whitney U test for confirmation purpose. Spearman's rho was calculated for correlations between MMF indices and total CARS scores in the autism group. Additionally,

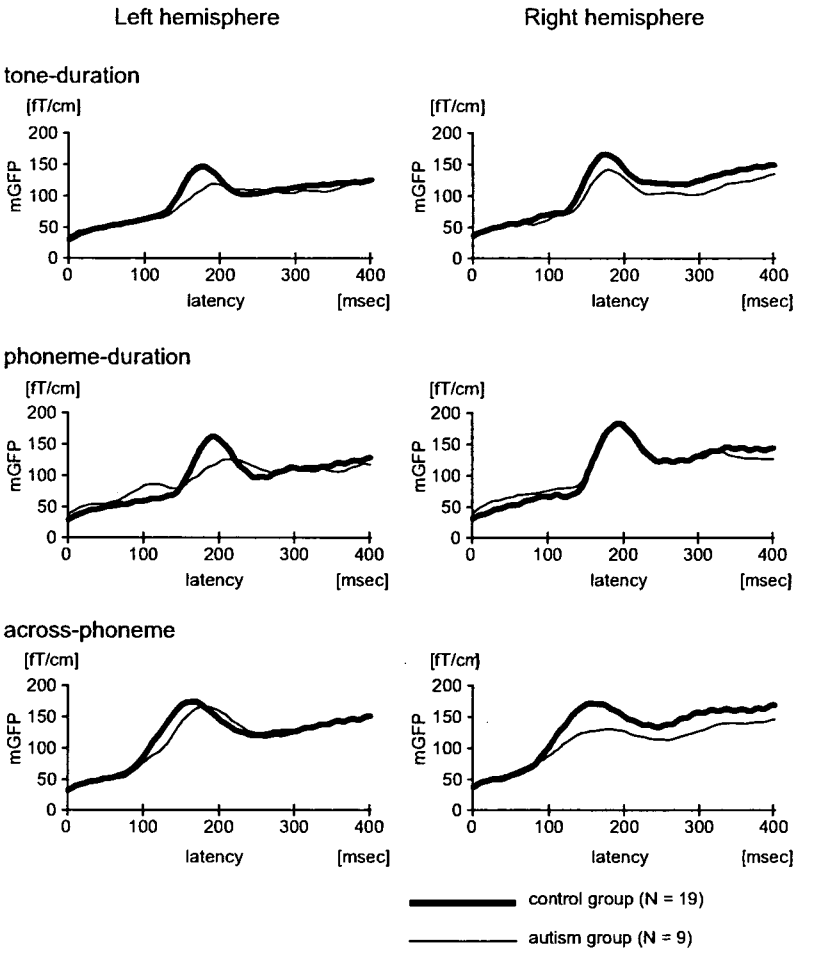

Fig. 1. Grand mean magnetic counterpart of global field power (mGFP) waveforms under the tone-duration (top), the phoneme-duration (middle), and the across-phoneme (bottom) change condition for each hemisphere. Thick lines are for the control group and thin lines for the autism group.

Spearman's correlations between MMF indices and age (for each of both groups), IQ scores (autism group only) or neuroleptics and anticholinergic dose (autism group only) were calculated to test for potential confounding status of these indices.

3. Results

3.1. Dipole analysis

An example of dipole locations superimposed on a subject's magnetic resonance imaging is shown in Fig. 2. For this subject, MMF for each condition was located in the vicinity of the posterior superior temporal gyrus in each hemisphere, coinciding with previous reports on source localization of MMF in response to pure tones (Alho et al., 1998b) or speech sounds (Alho et al., 1998a).

Group differences in dipole strengths or locations were not statistically significant for any condition or hemisphere after Bonferroni correction (the level of significance was $P=0.002$; 24 comparisons) (Table 2).

3.2. MMF power and latency

The t tests showed that the autism group was associated with significantly delayed latency of MMF under the across-phoneme condition in the left hemisphere ($t[26]=3.11$, $P=0.004$) (Table 3). Additionally, these results were confirmed by Mann–Whitney U test which showed a significance in the left hemisphere of across-phoneme condition ($Z=-2.46$, $P=0.014$), while other conditions/hemisphere did not reach significance ($P>0.07$).

3.3. Correlational analyses

Autism individuals' MMF latency in the left hemisphere under the across-phoneme condition showed a significant positive correlation with scores for CARS (rho=0.672, $N=9$, $P=0.047$). Importantly, MMF latency in the left hemisphere under the across-phoneme condition in

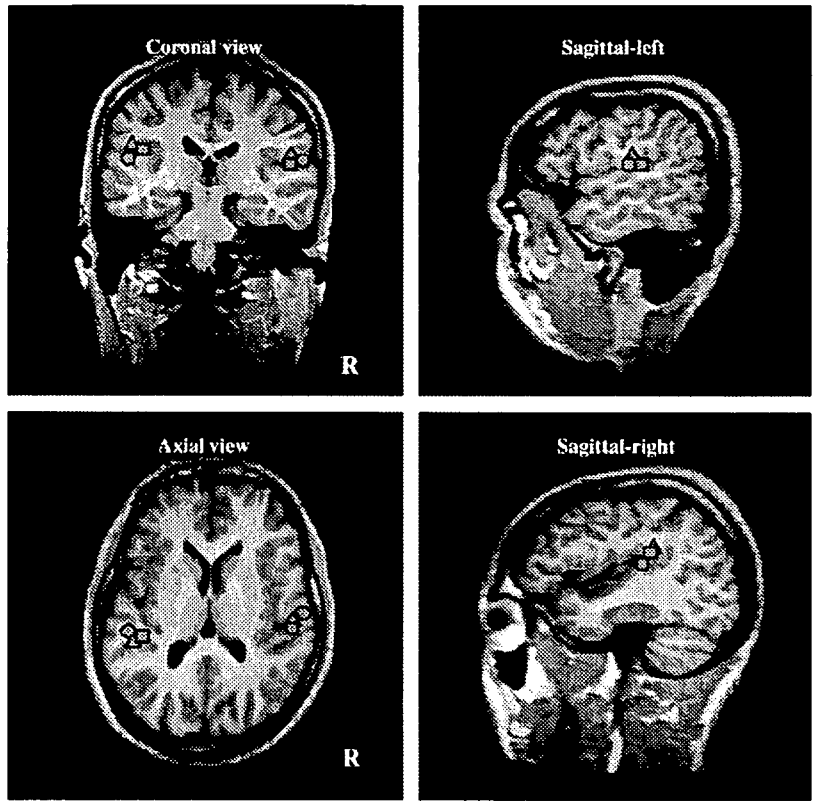

Fig. 2. Locations of equivalent current dipoles (ECDs) under each condition in each hemisphere of a control subject superimposed on a magnetic resonance imaging slice of this subject. Circle: ECD location of MMF under tone-duration condition; triangle: that under phoneme-duration condition; and square: that under across-phoneme condition.

the autism group was not significantly correlated with age (rho = −0.126, $N=9$, $P=0.75$), IQ (rho = −0.285, $N=9$, $P=0.46$), or dose of medication (neuroleptics: rho = 0.173, $N=9$, $P=0.66$; anticholinergic drugs: rho = 0.419, $N=9$, $P=0.26$).

Additional significant results were as follows: control subjects' MMF power under the across-phoneme condition showed a significant negative correlation with age (left hemisphere: rho = −0.552, $N=19$, $P=0.014$; right hemisphere: rho = −0.693, $N=19$, $P=0.001$); autism individuals' MMF latency in the right hemisphere under the phoneme-duration condition showed a significant negative correlation with IQ (rho = −0.689, $N=9$, $P=0.04$); patient's MMF latency in the left hemisphere under the phoneme-duration condition showed a significant negative correlation with neuroleptic dose (rho = −0.676, $P=0.046$) and anticholinergic drugs (rho = −0.698, $P=0.037$).

4. Discussion

The present findings represent the first physiological evidence, derived from whole-head MEG, of delayed automatic processing of change in speech sounds predominantly in the left temporal area in adults with autism. Moreover, to our knowledge, this is the first study that linked MMN/MMF abnormalities to clinical severity in autism. This study suggests that language-related dysfunction in autism may be present at the early stage of auditory processing of relatively simple stimuli such as phonemes, and not just at stages involving higher-order semantic processes. In this study, the autism group showed neither abnormal power nor lateralization for any type of MMF, while MMF latency was prolonged in the left hemisphere during across-category change detection of vowels. Although conclusions must be speculative, it may be that adults with autism have difficulties in rapid evaluation of change in speech sounds, particularly that mediated in the left auditory cortex. These results are also consonant with PET (Boddaert et al., 2003; Zilbovicius et al., 2000) and single photon emission computed tomography (Hashimoto et al., 2000; Ohnishi et al., 2000) studies that report temporal lobe hypoperfusion in autism.

Additionally, the results of the present study indicate that adults with autism did not show marked deficits in duration MMF in response to tones and vowels, although this discussion should be regarded as tentative since there appeared to be a statistically non-significant difference in the left hemisphere. Since no previous study has measured duration MMN/MMF in autism, future studies should assess

Table 2
Equivalent current dipole strength and location in control subjects and autism patients

	Control group			Autism group			Group comparison[a]	
	N	Mean	SD	*N*	Mean	SD	*Z*	*P*
ECD strength (nAm)								
Pure (L)	17	22.2	11.3	5	20.5	9.1	−0.39	0.70
Pure (R)	16	27.3	11.9	8	30.9	6.9	−0.92	0.36
a/a (L)	17	28.3	16.4	3	27.7	26.5	−0.58	0.56
a/a (R)	19	32.8	19.4	7	34.4	9.7	−0.84	0.40
a/o (L)	19	29.0	9.9	7	28.4	15.9	−0.35	0.73
a/o (R)	18	27.9	11.8	5	33.2	16.2	−0.82	0.41
ECD location[b] (mm)								
x-axis								
Pure (L)	17	−53.5	7.7	5	−49.1	6.8	−1.06	0.29
Pure (R)	16	52.7	10.8	8	49.7	8.5	−0.73	0.46
a/a (L)	17	−55.0	8.5	3	−54.5	11.2	−0.21	0.83
a/a (R)	19	51.3	10.6	7	53.4	9.2	−0.26	0.79
a/o (L)	19	−54.0	8.8	7	−49.4	10.9	−0.90	0.37
a/o (R)	18	53.5	6.4	5	56.3	5.7	−0.89	0.37
y-axis								
Pure (L)	17	6.2	8.5	5	6.6	6.5	−0.04	0.97
Pure (R)	16	11.7	8.1	8	4.4	7.5	−2.08	0.04
a/a (L)	17	8.7	8.0	3	−0.9	6.8	−1.85	0.06
a/a (R)	19	13.1	11.6	7	11.3	8.2	−0.20	0.84
a/o (L)	19	7.7	7.6	7	4.1	8.5	−0.84	0.40
a/o (R)	18	13.5	12.2	5	6.4	5.8	−1.53	0.13
z-axis								
Pure (L)	17	65.5	10.5	5	66.7	11.2	−0.04	0.97
Pure (R)	16	66.3	9.4	8	61.8	11.4	−1.13	0.26
a/a (L)	17	60.5	9.8	3	61.4	8.0	−0.16	0.87
a/a (R)	19	65.4	8.7	7	69.3	7.2	−1.19	0.24
a/o (L)	19	64.1	10.9	7	66.1	10.9	−0.14	0.89
a/o (R)	18	64.7	11.7	5	57.8	16.8	−1.04	0.30

ECD, equivalent current dipole; pure, tone-duration condition; a/a, phoneme-duration condition; a/o, across-phoneme condition; L, left hemisphere; R, right hemisphere.

[a] Mann–Whitney *U* test. The level of significance was $P=0.002$ (Bonferroni correction; 24 comparisons).

[b] The coordinate system was defined so that the *x*-axis passes through the preauricular points, with the positive *x*-axis pointing to the right. The *y*-axis passes the nasion, pointing anteriorly, and the *z*-axis points upwards.

Table 3
Global field power and latency of the magnetic mismatch field

	Autism group ($N=9$)		Control group ($N=19$)		Effect size	*T* test (df=26)	
	Mean	SD	Mean	SD		*T* value	*P* value
Global field power (fT/cm)							
Tone-duration (left)	106	26	119	33	0.39	−0.98	0.34
Tone-duration (right)	117	34	134	40	0.43	−1.11	0.28
Phoneme-duration (left)	115	36	126	40	0.28	−0.67	0.51
Phoneme-duration (right)	147	32	148	34	0.03	−0.06	0.95
Across-phoneme (left)	149	65	153	47	0.09	−0.2	0.84
Across-phoneme (right)	124	47	154	45	0.67	−1.63	0.11
Peak latency (ms)							
Tone-duration (left)	194	30	177	16	1.06	2.02	0.054
Tone-duration (right)	181	11	175	12	0.50	1.24	0.23
Phoneme-duration (left)	194	36	189	9	0.56	0.59	0.56
Phoneme-duration (right)	193	12	193	14	0.00	0.11	0.91
Across-phoneme (left)	186	23	161	19	1.32	3.11	0.004[a]
Across-phoneme (right)	172	35	162	29	0.34	0.74	0.46

df, degree of freedom.

[a] Significantly delayed in the autism group. Statistically significance level was $P=0.0042$ (Bonferroni correction for 12 comparisons).

not only frequency MMN/MMF but also duration MMN/MMF to confirm our findings. Moreover, some electrophysiological studies have suggested abnormalities in temporal lobe auditory processing as reflected by N1 component in response to tones (e.g. Bruneau et al., 1999; Lincoln et al., 1995), although a direct comparison between N1 and MMN components should be cautioned as they reflect different aspects of sound processing. Some hemodynamic studies have also reported temporal lobe hypoperfusion in the resting state, which indicate overall dysfunction in the auditory cortex (Hashimoto et al., 2000; Ohnishi et al., 2000; Zilbovicius et al., 2000). Our design did not test the hypothesis that the auditory cortex dysfunction in autism is speech-sound specific, which should be tested in future studies.

The present study found a group difference in MMF latency, but not in MMF power. Previous findings from MMN/MMF studies in autism have been mixed. Three studies reported preserved MMN amplitude (Čeponienė et al., 2003; Gomot et al., 2002; Kemner et al., 1995), two studies reported reduced MMN/MMF amplitude/power (Seri et al., 1999; Tecchio et al., 2003), and one study reported enhanced MMN in autism (Ferri et al., 2003). As for latency, only Seri et al. (1999) reported prolonged latency of MMN in response to tones, whereas Gomot et al. (2002) reported shorter latency of tonal MMN in children with autism. The interpretation of Seri et al. study should be done with caution, since they tested subjects with tuberous sclerosis, which is not a typical case of autism. Gomot et al. explained their shorter tone-MMN latency by an overlap with an early P3a component. Moreover, Gomot et al. used frequency changes in pure tones, while our study used duration changes in pure tones and vowels and phoneme changes in vowels. This difference in the type of stimuli may partly explain the difference in results between the two studies. Two other studies reported intact MMN latency (Čeponienė et al., 2003; Ferri et al., 2003), and the remaining two provided no information on latency findings (Kemner et al., 1995; Tecchio et al., 2003). Čeponienė et al. (2003) found no differences between autistic and control children in MMN elicited by speech sound changes. The subjects of our study were adults, while the subjects of theirs were children. Delayed latency of MMN in response to speech sounds may be more evident in adults with autism, possibly due to a lack of normal development of specialization (or functional plasticity) for processing of speech sounds.

To our knowledge, the present study is the first to demonstrate a significant association between mismatch abnormalities and clinical symptoms in autism. However, due to a small sample size and a restricted range of CARS scores in our sample, the results should be regarded as tentative. Future studies should clarify how MMN/MMF abnormalities are related to specific cognitive profiles and social and communication problems, in individuals with autism.

Some other methodological issues in the current study need to be commented upon. First, a follow-up experiment employing children with autism should be conducted to generalize our findings. Secondly, the subject group was not restricted to high-functioning autism individuals to match IQ to healthy subjects, nor was there an IQ-matched (intellectually disabled) control group. However, since MMF indices were not significantly correlated with IQ, employing low-functioning subjects may not have produced marked confounds in the interpretation of our findings. However, since previous studies have shown lower amplitude of phonetic MMN in subjects with learning disabilities (Bradlow et al., 1999) and in those with intellectual disabilities (Kaga et al., 1999), future studies should employ individuals with learning and intellectual disabilities as a control group to clarify whether the present findings are specific to autism. Thirdly, although we found a significant negative correlation between dose of medication and some of the MMF latency indices, these correlations were not in a predicted direction. These results should not be considered to be definitive, however, since (1) the sample size is small and (2) uncorrected *P*s of 0.046 and 0.037 may not remain significant after a correction for multiple statistical comparisons.

In conclusion, this study, using a whole-head MEG, provides physiological evidence for delayed processing of change in speech sounds in the left auditory cortex in adults with autism. Our next goal will be to elucidate the relationship of this physiological abnormality of speech sounds at the basic level with higher-order communication deficits in autism.

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Rapid communication

No association of *FOXP2* and *PTPRZ1* on 7q31 with autism from the Japanese population

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Abstract

Autism is a child-onset pervasive developmental disorder, with a significant role of genetic factors in its development. Genome-wide linkage studies have suggested a 7q region as a susceptibility locus for autism. We investigated several single nucleotide polymorphisms (SNPs) of *Forkhead Box P2* (*FOXP2*) and *Protein-Tyrosine Phosphatase, Receptor-type, Zeta-1* (*PTPRZ1*) at the 7q region in Japanese patients with autism and healthy controls. No significant difference was observed, after correction for the multiple testing, in allele, genotype or haplotype frequencies of the SNPs of *FOXP2* or *PTPRZ1* between patients and controls. No evidence was thus obtained for a major role of *FOXP2* or *PTPRZ1* in the development of autism.

Keywords: Autism; Chromosome 7q; FOXP2; PTPRZ1; Genetic association

Autism is a neurodevelopmental disorder characterized by impairment in reciprocal social interaction and communication (or language), restricted and stereotyped pattern of interest and activities, occurring within the first 3 years of life. A disrupted growth of the brain, with unknown mechanism, is suggested on the background of autism. Twin and family studies have indicated a robust role of genetic factors in the development of autism (Folstein and Rosen-Sheidley, 2001). A number of genome-wide linkage studies have been conducted in Caucasian families with autism and its spectrum disorders. Although the results of the linkage studies were controversial, several studies have provided evidence for a chromosomal 7q region (7q21-31.3) as a susceptibility locus (or loci) of autism (reviewed by Folstein and Rosen-Sheidley, 2001). Candidate genes of autism on this chromosomal region may include *Forkhead Box P2* (*FOXP2*) and *Protein-Tyrosine Phosphatase, Receptor-type, Zeta-1* (*PTPRZ1*), which are both located on 7q31.

FOXP2 encodes a forkhead family protein, which plays diverse and important roles in embryogenesis (Kaufmann and Knochel, 1996). The gene contains 17 exons spanning over 274.9 kb. *FOXP2* is expressed in confined regions of the brain during embryogenesis, in contrast to its ubiquitous expression during adulthood, suggesting its role in the prenatal brain development (Lai et al., 2001). Interestingly, mutations of *FOXP2* may be responsible for speech and language disorder in a unique three-generation pedigree and an independent individual (Lai et al., 2001). Affected members of the family had disturbances in usage of grammar as well as phonation of the language. Voxel-based morphometry (VBM) of the brain showed reduction of the

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gray matter in some of the brain region in the affected members (Vargha-Khadem et al., 2005). Thus far, a Chinese group reported a weak support for association between the *FOXP2* gene and autism (Gong et al., 2004), while other studies observed no association (Gauthier et al., 2003; Newbury et al., 2002; Wassink et al., 2002).

PTPRZ1 is also highly expressed in the brain during embryogenesis (Levy et al., 1993). The gene contains 30 exons spanning over 188.6 kb. PTPRZ1 is a large receptor-type protein of tyrosine phosphatase. Phosphorylation of tyrosine-residues plays a key role in the signaling of cell growth and differentiation. PTPRZ1 plays a role in recovery and survival of oligodendrocytes in demyelinating disease (Harroch et al., 2002). Glial cells including oligodendrocytes may be involved in neurodevelopmental disorders including autism (Dong and Greenough, 2004). Thus far, Bonora et al. (2004) observed no association between *PTPRZ1* and autism.

Here we studied these two genes in 170 unrelated Japanese patients with autism (147 males and 23 females, mean age = 20.8 years with the range of 3–41 years) and 214 unrelated healthy volunteers (145 males and 69 females, mean age = 34.6 years with the range of 21–65 years). All patients met the DSM-IV criteria for autistic disorder. The patients were recruited from the outpatient clinics of the departments of psychiatry, Tokyo University Hospital and Tokai University Hospital, and seven daycare facilities for subjects with developmental disorders. All the hospital and facilities were located around Tokyo. Apparent physical anomalies were not observed in the subjects. Controls were mainly recruited from the hospital and facility staff. All controls resided in the same area (Kanto District or around Tokyo) as the patients. All the patients and controls were ethnically Japanese, with no parents or grandparents of ethnicity other than Japanese.

Confirmation of the diagnosis was conducted as follows. Semi-structured behavior-observation of the patients and interview of them and their parents were conducted, for most of the cases, by two experienced child psychiatrists independently. When one of the parents was not available, mothers were interviewed in most of the cases. At the interview of the parent(s), the Child Behavior Questionnaire Revised (Izutsu et al., 2001) was used to assist the evaluation of the autism-specific behaviors and symptoms. Diagnosis was made according to the DSM-IV criteria. After the initial observation and interview, the patients were followed up to examine the behaviors and symptoms for several months (for at least 6 months in most of the cases) and those who were not considered to meet the DSM-IV criteria during the follow-up were excluded from the sample. The present study was approved by the Ethical Committees of the University of Tokyo and Tokai University. Informed consent was obtained from all subjects and healthy controls. Peripheral blood was obtained and genomic DNA was extracted using the standard phenol–chloroform method.

Single nucleotide polymorphisms (SNPs) of the genes were analyzed, using ABI prism 7900HT sequence detective system (Applied Biosystems Foster City, CA, USA). Five SNPs and seven SNPs of the *FOXP2* and *PTPRZ1* genes, respectively, were selected from the list of Assays-on-Demand™ Products for ABI PRISM 7900HT for this association study. SNPs with putative high minor allele frequencies in Japanese, according to the database for the Applied Biosystems Assays-on-Demand™ SNP Genotyping Assays, were predominantly selected for the study. The db SNP IDs of the SNPs are shown in Tables 1 and 2. Primers and probes of the ABI Assays-on-Demand™ kit were used for the genotyping.

Statistical analyses including chi-square tests and others were performed using the SAS/Genetics 9.1 software (SAS Institute Inc. Cary, North Carolina, USA). D′ and r2 of the linkage disequilibrium between SNPs and frequencies of haplotypes consisting of SNPs, which were at high linkage disequilibrium, were estimated. Exact *p*-values based on the likelihood ratio test with 10,000 permutations were calculated for comparison of haplotype frequencies between patients and controls.

Allele frequencies of the SNPs of *FOXP2* and *PTPRZ1* are summarized in Tables 1 and 2. The frequency of the minor allele of the SNPs was higher than 30%, except for the SNP 4 of *FOXP2* and the SNPs 4 and 6 of *PTPRZ1*. For the most of the SNPs, the allele frequencies were almost same between the patients and controls. No significant difference was observed in the allele frequencies of any of the SNPs

Table 1
Allele frequencies of five SNPs of the FOXP2 gene in autism patients and controls

Locus	db SNP ID	Allele A/B[a]	Patients		Controls		Chi-square	*p*-Value	Odds ratio	95% Confidence intervals		Chromosome position
			Allele B		Allele B							
			N	%	*N*	%				Lower	Upper	
SNP 1	rs2106900	[C/T]	102	30	124	30	0.043	0.837	0.97	0.71	1.32	113145303
SNP 2	rs2061183	[G/C]	84	25	105	25	0.007	0.933	0.99	0.71	1.37	113280812
SNP 3	rs1456029	[A/G]	143	42	203	48	2.354	0.125	1.25	0.94	1.67	113313550
SNP 4	rs1005958	[A/G]	27	8	31	7	0.140	0.709	0.90	0.53	1.54	113325658
SNP 5	rs1058335	[C/T]	114	34	156	37	0.860	0.354	1.15	0.85	1.56	113356466

The SNPs 1–5 are located at introns 1, 27, 14 and the 3′ region of the gene, respectively. Chromosome position on the SNPs is according to TCAG consortium.
[a] Alleles "B" are minor alleles.

Table 2
Allele frequencies of seven SNPs of the *PTPRZ1* gene in autism patients and controls

Locus	db SNP ID	Allele A/B[a]	Patients		Controls		Chi-square	*p*-Value	Odds ratio	95% Confidence intervals		Chromosome position
			Allele B		Allele B							
			N	%	*N*	%				Lower	Upper	
SNP 1	rs740960	[G/T]	113	34	143	34	0.014	0.904	1.02	0.75	1.38	120551052
SNP 2	rs1206504	[A/G]	102	30	144	34	1.312	0.252	1.20	0.88	1.63	120592038
SNP 3	rs1196490	[G/A]	112	33	153	36	0.721	0.396	0.88	0.65	1.19	120612421
SNP 4	rs1196509	[C/T]	20	6	26	6	0.012	0.914	1.03	0.57	1.89	120640281
SNP 5	rs1196475	[C/T]	119	35	142	33	0.362	0.547	0.91	0.67	1.23	120673905
SNP 6	rs1147489	[A/G]	62	18	67	16	0.864	0.353	0.84	0.57	1.22	120703821
SNP 7	rs1206381	[A/C]	111	33	128	31	0.584	0.445	0.89	0.65	1.21	120723515

The SNPs 1–7 are located at introns 1 (SNPs 1 and 2), 2, 4, 11, 19 and 27, respectively. Chromosome position on the SNPs is according to TCAG consortium.
[a] Alleles "B" are minor alleles.

between the groups. No significant difference was found in genotype frequencies between the groups, either (not shown in the table). When analyzed by sex, a difference was observed in allele frequencies of the SNP 3 (rs1456029) of *FOXP2* at intron 7 between male patients and controls (chi-square = 5.06, d.f. = 1, $p = 0.024$, uncorrected). The difference was not statistically significant after correction for the multiple testing. No other significant difference was observed. Hardy-Weinberg disequilibrium ($p < 0.05$) was significant for rs740960 marker in the patients (PTPRZ1, $p = 0.00570$), not for other 11 markers. The disequilibrium for rs740960 was not statistically significant after the correction for the 24 tests (cases and controls of the 12 SNPs).

Strength of linkage disequilibrium, denoted as D′, between pairs of the SNPs of *FOXP2* and *PTPRZ1* is summarized in Table 3(a) and (b), respectively. The SNP 1 and 2 of *FOXP2* were at high LD and the SNPs 4–7 of *PTPRZ1* appear to form a LD block, both in controls and patients. Haplotype frequencies of the *FOXP2* SNPs 1–2 and the *PTPRZ1* SNPs 4–7 were estimated and compared between patients and controls using permutation test. No significant difference was observed.

Table 3
The strength of LD (denoted as D′) between pairs of SNPs of (a) *FOXP2* and (b) *PTPRZ1*in autism patients (the lower diagonal) and controls (the upper diagonal)

SNP	1	2	3	4	5
(a) *FOXP2*					
1		0.94	0.37	0.35	0.14
2	0.92		0.40	0.47	0.82
3	0.53	0.43		0.21	0.22
4	0.59	0.29	0.88		0.74
5	0.15	0.54	0.09	1.00	

SNP	1	2	3	4	5	6	7
(b) *PTPRZ1*							
1		0.40	0.91	0.24	0.53	0.50	0.56
2	0.62		0.44	1.00	0.21	0.34	0.24
3	0.90	0.67		0.26	0.59	0.55	0.61
4	0.15	0.09	0.28		1.00	1.00	1.00
5	0.67	0.52	0.85	1.00		1.00	0.99
6	0.66	0.52	0.85	1.00	1.00		1.00
7	0.67	0.56	0.85	1.00	1.00	1.00	

The 95% confidence intervals of odds ratios were within 0.53 and 1.67 in 11 of the 12 SNPs of the two genes. The only exception was rs1196509, where minor allele frequency was extremely low (6%) compared with the others. Thus, our results might have adequate statistical power to contradict the effects of the genes with odds ratios of approximately 1.8 or more. However, the present sample size is not adequate to detect smaller effect of the genes.

Genetic association between these genes and autism has been explored in a limited number of studies. *PTPRZ1* was previously investigated in a study, with other six genes on the 7q area. While the study provided suggestive evidence for the role of other genes, including *LAMB1* and *NRCAM*, no support was observed for the role of *PTPRZ1* in autism, (Bonora et al., 2004). This result on *PTPRZ1* may be consistent with the present study. Regarding *FOXP2*, a Chinese group found an association of one SNP (rs1456031 at intron 9 of *FOXP2*) out of three with autism using transmission disequilibrium test (TDT) (Gong et al., 2004). The level of the significance did not reach statistical significance after correction for the multiple testing of the three SNPs. We attempted to investigate this polymorphism. However, another SNP (rs6966051) was within 2bp of the rs145631, which made the precise genotyping unable at present. Other studies that investigated the role of *FOXP2* provided no evidence for the linkage or association between the gene and autism, which may be consistent with the present result (Gauthier et al., 2003; Newbury et al., 2002; Wassink et al., 2002). Studies thus far to our knowledge, including the present one, may therefore yield no or few support for the genetic association between *PTPRZ1* or *FOXP2* with autism.

A major limitation of the present study may be the limited sample size. Although the present result may contradict the effect of the genes with odds ratio of 1.8 or more, smaller effects might not be detected in the present sample. Another concern may be population stratification of the sample,

which could affect studies in case-control design. This might not however significantly affect the present study in homogeneous Japanese population. No subjects in this study had parents or grandparents of ethnicity other than Japanese. Also acknowledged might be that the controls in this study were not age-matched to the patients. But this may not be likely to significantly affect the result, considering the homogeneity of the population and no major effect of environmental factors in autism (Folstein and Rosen-Sheidley, 2001).

In conclusion, no evidence was provided for the association of *FOXP2* and *PTPRZ1* with autism. However, a weak tendency for the association was observed in male subjects for one SNP of *FOXP2*. The present sample size may not have adequate power to detect very small effects of the genes. Further investigations with larger samples are recommended to detect more subtle effects of these genes on autism.

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Polyhistidine tract expansions in HOXA1 result in intranuclear aggregation and increased cell death

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Abstract

HOXA1 gene is part of a cluster of homeotic selector genes that regulates the anteroposterior patterning of mammals during embryonic development. HOXA1 encodes two alternatively spliced mRNAs with two isoforms, A and B, the former contains the homeodomain and expressed in early embryonic development. HOXA1 contains a string of 10 histidine repeats. However, individuals heterozygous for 7, 9, 11, and 12 histidine repeat variants were present among the Japanese population, notably in some autism cases. To determine the biological implications of the different polyhistidine repeat lengths, we expressed these variants in COS-7 and a human neuroblastoma cell line (SK-N-SH). Expression of expanded variants of HOXA1 isoform A, containing 11 and 12 polyhistidine, resulted in early and great degree of protein aggregation in the nucleus. This aggregation resulted in accelerated cell death in cells expressing 11 and 12 expanded variants compared to those transfected with 7 and 10 polyhistidine variants. Furthermore, we showed that these aggregates were ubiquitinated and were inhibited by a histidine-modifying compound, DEPC. These data suggest that HOXA1 protein with polyhistidine tract expansions misfold, aggregate, and have a toxic effect on cell.

Keywords: HOXA1; Histidine repeat; Protein aggregation; Ubiquitin; Cell death; Diethylpyrocarbonate

Homeobox genes encode for transcription factors contributing to the regulation of embryonic patterning and organogenesis [1]. The clustered homeobox genes were highly conserved from *Drosophila* to human at the genomic level. In mammals, 39 of the Hox genes were identified and they encode to the class of proteins which share the evolutionary conserved homeodomain involved in the recognition of the target DNA sites [2]. HOXA1 is the first homeobox gene expressed in the developing hindbrain [3]. Its mRNA has two alternative splice variants; one containing the homeobox which encodes isoform A, and the shorter, isoform B, lacking the homeobox [4]. One of the noticeable features of HOXA1 isoform A is a stretch of 10 histidine repeats at amino acid positions 65–74. Although, its importance has not yet been established, it may possibly interact with Polycomb, a repressor of homeotic genes. The targeted disruption of the Hoxa1 gene in mice leads to numerous developmental defects, including hindbrain deficiencies and abnormal skull ossification and ultimately to neonatal death [5,6]. Ingram et al. [7] reported

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an association of the A218G polymorphism in HOXA1 gene and autism; however, a number of contradictory reports have also been made [8,9]. Racial differences of sample populations may be the reasons for these discrepancies.

In this study, we investigated HOXA1 gene variations in a Japanese population comprised of normal and autistic individuals, and found novel histidine repeat deletions and expansions. Expression of polyhistidine expanded forms of HOXA1 protein results in early intranuclear aggregation and increased cell death. Time-dependent protein aggregation and cell death could be an indication of apoptosis associated with the process of assigning segmental identity in the developing hindbrain. Moreover, expanded forms of HOXA1 isoform A, aggregated earlier and faster, suggest that longer histidine repeats may result in accelerated apoptosis.

Materials and methods

Antibodies and reagents. Antibodies were purchased as follows, polyclonal anti-HOXA1 antibody (N-20) was from Santa Cruz, polyclonal anti-EGFP antibody was from Invitrogen, and polyclonal anti-ubiquitin antibody, Alexa fluor 546-conjugated anti-rabbit IgG antibody, and Alexa fluor 594-conjugated anti-goat IgG antibody were from Molecular Probes. Hoechst 33258 for nuclear staining was from Molecular Probes.

DNA sequencing of HOXA1 gene. Subjects consisted of 110 Japanese autistic patients, their parents ($n = 211$); and 336 normal controls. All autistic patients were diagnosed under DSM-IV criteria. This study was approved by the Ethical Committees of the Faculty of Medicine, University of Tokyo, and the Faculty of Medicine, Tottori University. Total DNA was extracted from lymphoblasts using a standard method. PCR primers were designed to amplify all exons of HOXA1, including flanking sequences. PCR products were subcloned in T-Vector (Promega) and sequenced.

HOXA1 plasmid constructions. Each HOXA1 isoform was amplified from lymphocyte mRNA of human normal controls and autistic individuals following the standard method. Since isoform A differs from isoform B primarily by the presence of the homeodomain region and expresses only in the developing embryo, isoform A constructs were generated partly from genomic DNA by PCR amplification and joined with the appropriate region from isoform B transcript. All PCR amplification steps were performed using Pfu Ultra DNA polymerase (Stratagene) and confirmed by sequencing. Each variant was ligated into pCMV-Script (Stratagene) and pEGFP-N1 (Invitrogen) to generate expression constructs.

Transfection and cell death assay. COS-7 and human neuroblastoma cell line (SK-N-SH) were maintained in DMEM (Sigma) with 10% fetal bovine serum (Gibco) and transfected with HOXA1 constructs using Fugene 6 transfection reagent (Roche) following manufacturer's protocol. After transfection, cells were maintained in DMEM supplemented with 10% serum. A mock transfection was also performed as a control. Floating dead cells from medium of transfected cells were harvested every 24 h for a period of 3 days, stained with Trypan blue (Gibco), and counted using a hemocytometer under a phase contrast microscope (Olympus IX-70). Lactate dehydrogenase (LDH) cytotoxicity assay (Wako, Tokyo) was also performed from the collected cell medium following the manufacturer's procedure.

Immunocytochemistry and fluorescence imaging. After transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and immunostained with anti-HOXA1 antibody and anti-ubiquitin antibody for 1 h at room temperature. Bounded antibodies were detected by incubation with Alexa fluor 595-conjugated anti goat IgG antibody for 1 h. For nuclear staining, cells were incubated with Hoechst 33258 for 30 min. All samples were mounted on glass slides and fluorescence images were obtained using Leica confocal microscope (TCS SP2).

Western blotting. Twenty-four hours after transfection, cells were harvested for total protein extraction. Briefly, cell samples were lysed and sonicated with 10 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA plus protease inhibitor cocktail (Roche). Protein was quantified using Color-Producing Solution (Wako). Samples were run on 10% SDS–PAGE gels and transferred on a nylon membrane (Millipore) using a semi-dry transfer blotter (Bio-Rad). Membranes were incubated in either a polyclonal rabbit anti-EGFP antibody followed by incubation in a horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham), respectively, or in a polyclonal anti-HOXA1 antibody with a horseradish peroxidase linked sheep anti-goat antibody (Amersham). Detection was performed using ECL (Amersham–Pharmacia Biotech) and images were captured in X-ray film or a LAS-1000 plus imager (Fujifilm).

DEPC treatment. Twenty-four hours after transfection, cells were treated with diethylpyrocarbonate (DEPC) with a concentration of 20 mM for 30 min or 1 mM for 24 h. Then cells were fixed and mounted on the slides. The degree of protein accumulation within the cell nuclei of the transfected cells was counted. Cells were counted from 10 randomly selected microscope fields from each sample. The ratio of the number of aggregated cells over the total number of EGFP-positive cells was then computed. The same computation procedures were performed for cells processed after 18 and 42 h of incubation.

Results

Novel variants of histidine repeats in HOXA1 gene

All exons and flanking sequences of HOXA1 gene were subjected to direct sequencing. Using this approach, novel polyhistidine variants were detected. Families affected with autism and randomly selected normal individuals were screened for sequence of HOXA1 gene. Finally 7, 9, 11, and 12 repeat variants were detected in both normal and autism patients (Table 1). Nevertheless, we were interested in further expression study because some trinucleotide repeat expansions, such as polyglutamine and polyalanine, have already been implicated neurodegenerative and congenital diseases [10–12].

Intranuclear protein aggregations in polyhistidine variants of HOXA1

To investigate the cellular localization of the different variants of HOXA1, we generated expression constructs having the 7, 10, 11, and 12 polyhistidine repeats for the

Table 1
Allele frequencies of the polyhistidine variants in HOXA1 gene between autism patients (child), their parents, and normal individuals

Type	Child	Parent	Normal
7/10	2	4	11
9/10	0	0	1
10/10	105	200	312
11/10	3	6	12
12/10	0	1	0
Total	110	211	336

The length of histidine repeats in HOXA1 gene was assessed by sequencing of genomic DNA and was classified under five types of allelic variants.

two HOXA1 isoforms fused with and without the N-terminus of GFP (Fig. 1A). COS-7 and SK-N-SH cells that were transfected with the wild-type (10 histidine repeats) HOXA1 isoform A and immunostained with anti-HOXA1 antibody revealed that HOXA1 protein is diffusely localized in the nucleus (Fig. 1B). In contrast, 11 and 12 histidine variants of HOXA1 isoform A resulted in noticeable protein aggregations in the nucleus. On the other hand, deleted form (7 his-

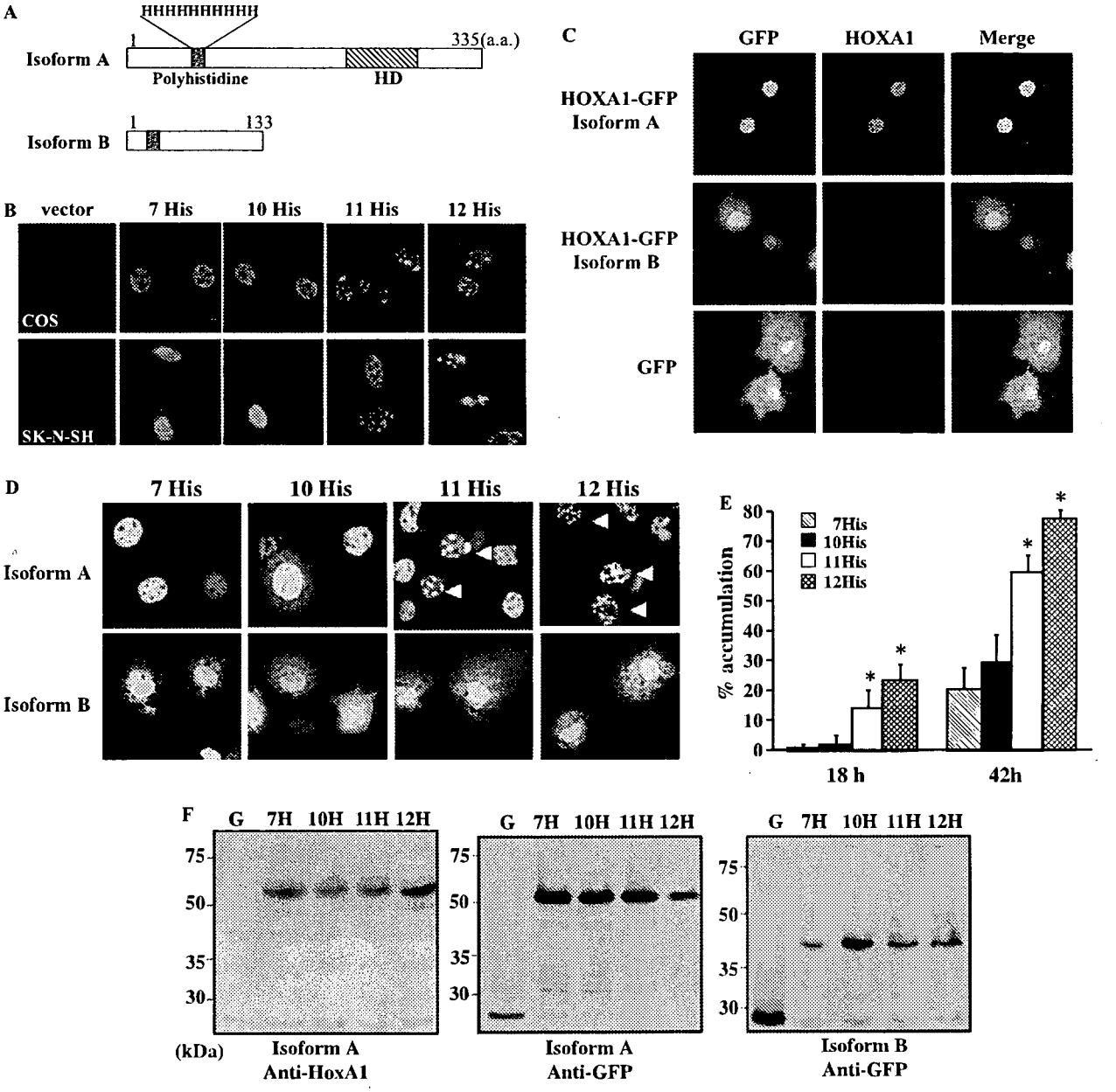

Fig. 1. Expression of 11- and 12-polyhistidine repeats of HOXA1-GFP protein results in intranuclear aggregations. (A) HOXA1 expression constructs with two isoforms A and B. Isoform A contains homeodomain (HD). Variants of the same gene were constructed containing 7, 11 or 12 polyhistidine repeats. (B) In cellular localization of HOXA1 isoform A protein. COS-7 cells and SK-N-SH cells were transiently transfected with HOXA1 isoform A with polyhistidine variants and stained with the anti-HOXA1 antibody. Nuclear aggregation of 11 and 12 histidine variants of HOXA1 protein was noticeable in both cells. (C) COS-7 cells transfected with HOXA1-GFP isoforms A and B, and stained with the anti-HOXA1 antibody. Mock GFP vector expression used as a control. (D) COS-7 cells transfected with HOXA1-EGFP fusion construct. Fluorescence images were taken 24 h after transfection. Arrows indicate HOXA1-EGFP protein aggregations in the nucleus. (E) The number of cells with protein aggregations was scored at 18 and 42 h after transfection. Values were expressed as the ratio of nuclei exhibiting aggregation over the total number of HOXA1-EGFP fluorescent cells. Each bar represents the standard error of the mean (SEM) from three independent experiments. $*P < 0.05$ significantly different from the values of 7- and 10-polyhistidine repeats. (F) Western blot analysis for HOXA1-GFP fusion protein. Protein extracts from COS-7 cells were prepared 24 h after transfection with HOXA1-GFP constructs. Western blotting was performed using anti-HOXA1 and/or anti-GFP antibodies. HOXA1 isoform A, 63 kDa; isoform B, 41 kDa; GFP, 27 kDa (G, GFP; H, histidine repeats).

tidine repeats) has the same pattern as the wild-type protein in both cells (Fig. 1B). There was little or no detectable signal from mock vector transfected cells. When cells were expressed with wild-type HOXA1 isoform A and GFP fusion protein, HOXA1-GFP was found localized in the nucleus and this was also detected by the anti-HOXA1 antibody (Fig. 1C). Since anti-HOXA1 antibody only detected isoform A, HOXA1-GFP isoform B was not stained. For the GFP fluorescence analysis, expression of 11 and 12 histidine variants of HOXA1-GFP isoform A resulted in protein aggregations in the nucleus but not in wild-type (10 histidine repeats) and deleted form (7 histidine repeats) in both COS-7 and SK-N-SH cells (Fig. 1D). This is also consistent with the untagged HOXA1 protein localization as shown in Fig. 1B. There is a significant difference in the percentage of scored cells with protein aggregations in variants with 11 and 12 histidine repeats after 18 and 42 h of transfection, although protein aggregations were also found in wild-type and 7 histidine repeat variant after 42 h (Fig. 1E). While protein aggregates were noticeable in expanded forms of isoform A, no significant aggregation was observed in expanded forms of isoform B. (Fig. 1D). The same result was also found in SK-N-SH cells (data not shown). Expression of HOXA1-GFP fusion protein was also confirmed by Western blotting with anti-GFP and anti-HOXA1 antibodies (Fig. 1F).

Polyhistidine tract expansions of HOXA1 result in increased cell death

Since there was no difference in the subcellular localization between the untagged HOXA1 and HOXA1-GFP protein, GFP-fused constructs were used for the following experiments. To test whether the expression of expanded histidine repeats of HOXA1-GFP causes an increase in cell death, we examined the rate of cell death in transfected COS cells by scoring dead cells and also using the LDH release assay. After 48–72 h of transfection, a significant increase in the percentage of dead cells was noted in cells transfected with 11 and 12 polyhistidine variants (Fig. 2A). This increase in cell death may have been a result of increased protein aggregation. These also correlated with the results from LDH cytotoxicity assay wherein the amount of LDH released into the medium was higher in cells transfected with 11 and 12 polyhistidine variants as compared to the control, 7 and 10 polyhistidine repeat variants, 48–72 h after transfection (Fig. 2B). By staining with Hoechst33258, nuclear fragmentation was observed in cells expressed with HoxA1 containing the 11 and 12 histidine repeats, which colocalized with GFP fusion fluorescence but not with wild type and 7 histidine repeat variants (Fig. 2C).

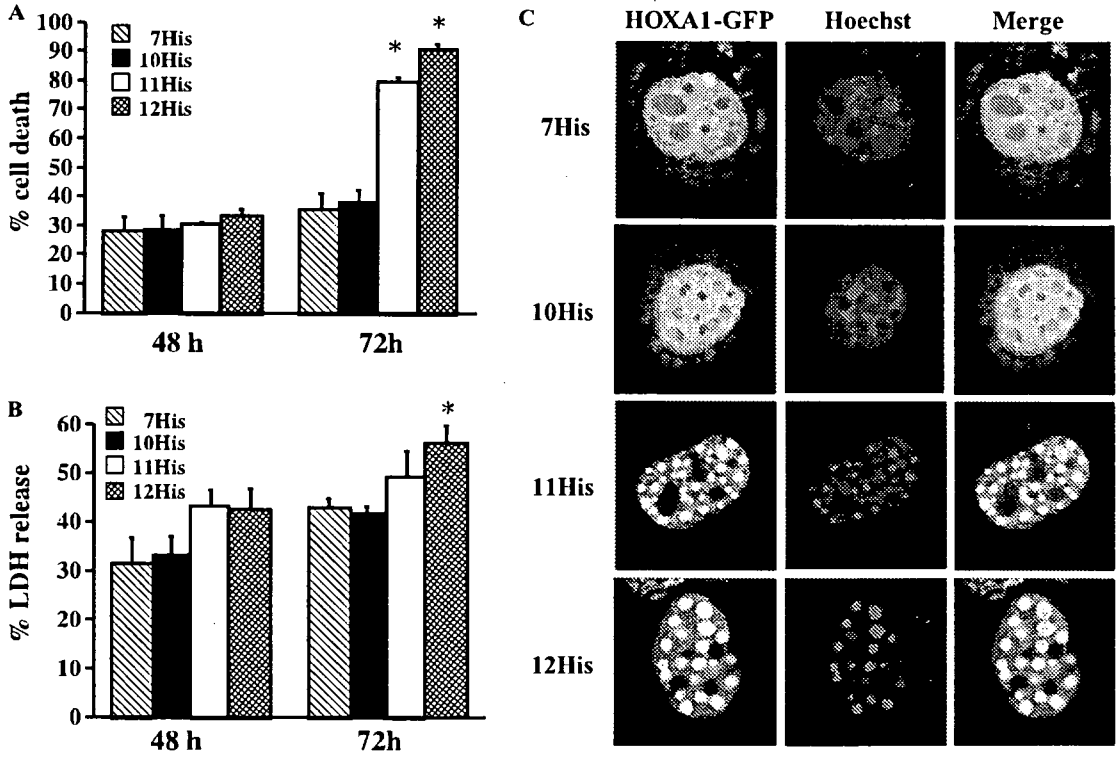

Fig. 2. Polyhistidine expansions of HOXA1-GFP results in increased cell death in COS-7 cells. COS-7 cells were transfected with HOXA1-GFP plasmid constructs. Assays were performed every 24 h for 3 consecutive days. (A) Floating dead cells from the medium were collected and stained with trypan blue and scored. Ratio of the number of dead cells over the total number of cells was computed. Values for percent cell death are expressed as means (SEM) from four independent experiments. (B) Lactate dehydrogenase (LDH) release assay. Each bar represents the mean (SEM) from three independent experiments. $^*P < 0.05$ significantly different from the value of control. (C) COS-7 cells were fixed and stained with Hoechst after transfection. The fragmented nucleus was seen in cells with 11 and 12 histidine repeats of HOXA1-GFP.

Nuclear aggregations of HOXA1 are ubiquitinated and inhibited by DEPC

Improper folding is believed to cause aggregation of cellular proteins and often cause cell death as observed in polyglutamine diseases [12]. We examined the transfected cells by immunofluorescence staining with anti-ubiquitin antibody. More than 50% of aggregated cells exhibited ubiquitin immunoreactivity in both 11- and 12-polyhistidine variants 18–24 h after transfection (Fig. 3). Although the image background was diffusely labeled for ubiquitin, immunoreactivity was concentrated at the site of aggregation. Cells transfected with 7- and 10-polyhistidine variants did not exhibit conspicuous aggregation prior to 18 h post-transfection and did not show any significant increase in ubiquitin immunoreactivity (Fig. 3).

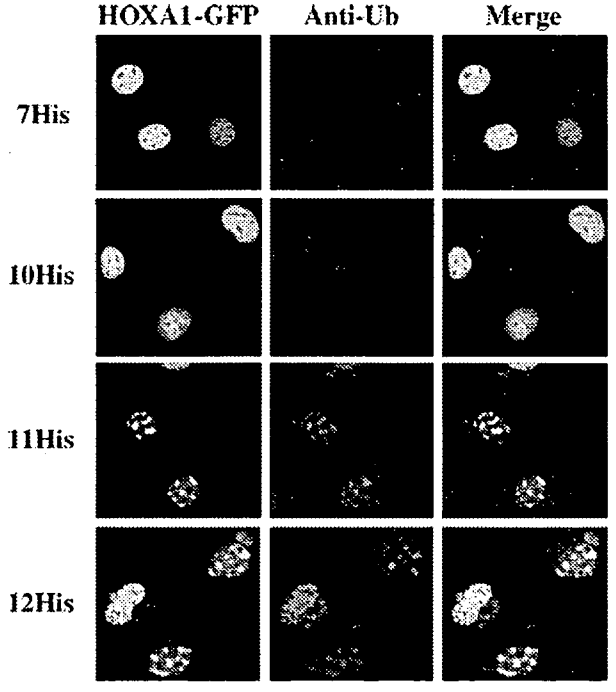

Fig. 3. Aggregations of HOXA1-GFP protein are ubiquitinated. COS-7 cells were transfected with 10, 11, and 12 polyhistidine constructs of HOXA1-GFP isoform A and fixed 24 h after transfection. Immunostaining revealed that nuclear aggregations were positive for ubiquitin in 11 and 12 histidine repeat transfectants.

To show that the observed protein aggregation was related to the presence of histidine repeats, we treated HOXA1 transfected cells with diethylpyrocarbonate (DEPC), a known histidine-modifying compound [13]. The modification of histidine repeats resulted in a significant decrease of HOXA1 protein aggregation in the nucleus for cells transfected with 11 and 12 histidine variants (Figs. 4A and B). Formation of aggregates was also observed 24 h after 1 mM treatment of DEPC in 11- and 12-poly-H variants, however, the inhibition of aggregation was temporary. Twenty-four hours later, the formation of aggregates slowed down and cells exhibited signs of apoptosis. Taken together, these results suggest a possible involvement of histidine repeat expansions in protein aggregation.

Discussion

Homopolymeric amino acid tracts exist in many human proteins, including a variety of transcription factors [14,15]. In this study, we have identified novel variants of HOXA1 gene encoding various polyhistidine repeat lengths. Since these variants can be observed in both affected and unaffected individuals, their contribution to autism pathophysiology remains controversial. At present, no report

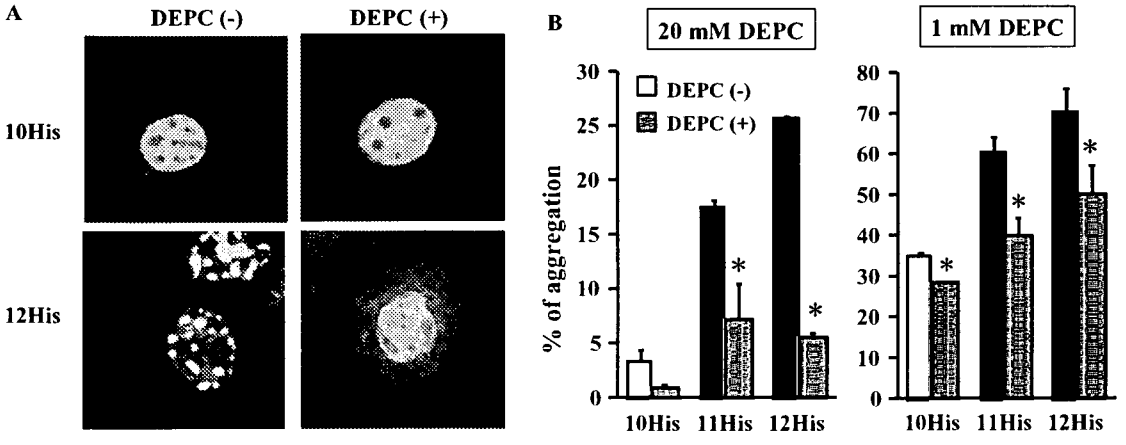

Fig. 4. DEPC delayed nuclear aggregations. (A) Fluorescent images for COS-7 transfected with 11 and 12 polyhistidine variants of HOXA1-GFP, treated with and without diethylpyrocarbonate (DEPC). Twenty-four hours after transfection, cells were treated with 20 mM DEPC for 30 min. Treatment with DEPC reduces HOXA1-GFP protein aggregates. (B) Formation of aggregates delayed after 1 mM treatment of DEPC for 24 h or 20 mM for 30 min. After treatment with DEPC, the percentage of protein accumulation over the number of transfected fluorescent cells for 11, 12, and also 10- polyhistidine variants were significantly reduced as compared to untreated cells. Each bar represents the mean (SEM) from three independent experiments. $*P < 0.05$ significantly different from untreated control.

has ever been made about polyhistidine repeat expansion and a possible link with neurodegenerative disease. An increasing number of human neurological diseases have been linked to the triplet expansion of normal tracts of single amino acid repeats such as polyglutamine repeats [10–12]. More recently, expansions of alanine tracts have been shown to cause at least nine human diseases, including mental retardation and malformations of the brain [16,17]. Repeat expansion diseases share a number of similar characteristics, including formation of ubiquitinated protein aggregations, neuronal dysfunction, and cell death [18,19]. Here, we showed that histidine repeat expansion resulted in protein aggregation in the nucleus and that this aggregation was positively correlated with time after transfection and increasing repeat length (Fig. 1E). These aggregations were highly ubiquitinated and resulted in early cell death with the fragmented nuclei (Figs. 2 and 3); however, it still remains unclear whether this cell death is accompanied by caspase activations as seen in the polyglutamine-induced cell death [20]. DEPC is known as a histidine-modifying compound. Loomans et al. [13] reported that polyhistidine activity for an antimicrobial protein, calprotectin, was reversed by addition of DEPC. DEPC decreased and delayed protein aggregations in HOXA1 transfected cells (Fig. 4), suggesting that increasing histidine repeat length is correlated with HOXA1 protein aggregation and improper protein folding caused by polyhistidine expansion. It is generally believed that an altered, aggregation prone, conformation of the mutant protein confers the loss of a normal function, the gain of a toxic new function, or both [10–12]. In HOXA1 protein, polyhistidine is thought to be the site to interact with other proteins [4] and the variation in histidine repeat lengths may influence the function of the proteins that bind to HOXA1. Alteration of protein partner binding interaction is already described to confer neuronal damage in polyglutamine diseases, such as HIP-1, HAP, and GAPDH (as well as many others) for huntingtin [21–23], LANP and GAPDH for ataxin-1 [24], and A2BP for ataxin-2 [24,25]. To analyze the interaction between HOXA1 and its cofactors, HOXB1 and EphA1, may give us more physiological relevance. Moreover, in our study, protein aggregation of HOXA1 was noticeable in cells expressing variants of isoform A, while no significant aggregation was observed for cells transfected with isoform B variants (Fig. 1D). These findings further suggest that the two isoforms of HOXA1 may have different roles in patterning during the development. Furthermore, the degree of protein aggregation correlated positively with increasing length of histidine repeats. We hypothesize that this may be a mechanism for apoptosis associated with the process of assigning segmental identity in the developing brain. In vivo studies will be necessary for the future work to identify a novel function of HOXA1 protein with histidine tract expansions in the brain.

In summary, we discovered polyhistidine repeat variants in HOXA1 protein from a Japanese population comprised of normal and autistic individuals. Certain individuals were heterozygous for 7-, 9-, 11-, and 12-histidine repeats. No homozygous case has been found for any of these variants. In vitro expression of expanded variants resulted in early nuclear protein aggregation and an increase in cell death. We speculated that polyhistidine tract expansions may have an important role for correct folding of the HOXA1 protein and function.

Acknowledgments

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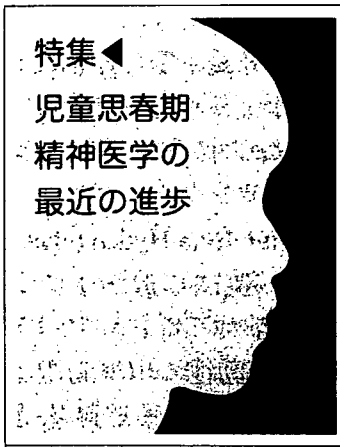

第2章 子どものこころの障害

1. チック障害とトゥーレット症候群について

金 生 由紀子

Key words: チック障害，トゥーレット症候群，注意欠陥多動性障害(ADHD)，強迫性障害(OCD)

はじめに

チック障害はチックという運動症状で定義された症候群であり，その中で複数の運動チックと音声チックを有する慢性チック障害がトゥーレット症候群(Tourette Syndrome；TS)である。TSを中心として海外では多側面から研究が積み重ねられており，2006年には『Advances in Neurology』の99号，『Journal of Child Neurology』の21巻8号という2つの雑誌がTSの特集号を組んでいた。それらおよび2006～2007年に出版された原著論文を中心に研究領域を大きく5つに分けて最近の進歩を紹介したい。

症候学研究，神経心理研究，精神生理研究

チックは不随意運動であるが部分的にまた一時的に随意的に抑制できることが特徴とされる。この特徴に関連して，TSの児童でストップーシグナル課題を行ったところ健常対照と差が認められず，運動チックには随意的側面があるのではないかとさえ指摘された[19]。また，チック障害の特徴は不随意運動のみならず，反射的に誘発される運動を抑制することや異なる運動セットの間ですばやく選択したり変更したりすることが困難なのではないかとの仮説から，眼球運動切り替え課題を用いた検討が行われた。ところが，TSの児童・青年では，運動時の認知的統制のレベルが健常対照よりも逆説的に高く，チックを常に抑制する必要があるために抑制的統制が増強する可能性が示唆された[15]。

チック障害，特にTSは他の精神神経障害をしばしば併発するが，その中でも注意欠陥多動性障害(Attention-Deficit/Hyperactivity Disorder; ADHD)や強迫性障害(Obsessive-Compulsive Disorder; OCD)は高率であり，関連性が検討されている。TSとADHDの有無の組み合わせから児童・思春期80名を4群に分けて行動および認知の表現型を検討したところ，行動でも認知機能でもADHDの影響がより大きかったが，感情面の症状や不安は3つの臨床群間で大差なくいずれも健常対照よりも強かったとの報告がある[22]。同様に慢性チック障害とADHDの有無の組み合わせから児童・思春期467名を4群に分けてCBCLの8つの下位尺度を検討した研究でも，ほとんどの尺度でADHDの影響が強く，慢性チック障害とADHDの併発では相加が強く示唆されたという[23]。

TSとADHDとの併発については精神生理学的検討も行われている。TSとADHDの有無の組み合わせから児童53名を4群に分けて事象関連電位

Tic disorders and Tourette syndrome

KANO Yukiko 東京大学医学部附属病院「こころの発達」診療部〔〒113-8655 東京都文京区本郷 7-3-1〕