genes showed reduced transcription and loss of cohesin binding to TSSs in CdLS cells. There is a big CpG island embedded at the *CAPN2* promoter region; two probes, cg01566170 and cg14972271 on the HumanMethylation27 array, were located 455 bp and 465 bp upstream of the TSS of *CAPN2*, respectively. The 252-bp fragment (chr1:221,966,278-221,966,529) amplified by primers CAPN2 329/330 covers these two probe CpG sites in addition to seven other CpG dinucleotides included in this fragment. DNA isolated from four controls and four severely affected CdLS probands with *NIPBL* mutations tested on methylation arrays above were tested again by BS. Ten to twelve clones were sequenced for each sample. Figure 7 shows the increased number of 5-methylcytosine at CpG sites in CdLS probands, indicating elevated DNA methylation levels in this chromatin region at the *CAPN2* promoter which is consistent with the methylation microarray findings. In addition, the BS result from control samples also illustrated varied levels of DNA methylation in the healthy population. Sample 11 obviously has much higher methylation level than sample 46 and 48, although, on average, 48.8% of CpGs in control and 80.3% in CdLS are methylated. A second promoter region, the promoter of the *LMO2* gene, was also tested by BS in six control and six CdLS individuals. A 358-bp fragment that is located at the *LMO2* promoter region was amplified by primers LMO2 337/338. This examined fragment is located on chr11:33,870,140-33,870,497 surrounding probe cg11822932 on the HumanMethylation27 array, which is 120 bp upstream of the TSS of *LMO2*. The *LMO2* promoter is very CpG poor and harbors no region that meets the criteria of the 'CpG island'. There are only three CpG dinucleotides in this tested region including the probe CpG. We could demonstrate similar methylation changes at the *LMO2* locus as demonstrated by the array study (Supplementary Figure S4). In conclusion, a consistent methylation pattern was obtained from both BS and HumanMethylation27 analysis.

Specific histone modifications and repetitive sequences are involved in the differential methylation in CdLS by EpiGRAPH analysis

EpiGRAPH (http://epigraph.mpi-inf.mpg.de/WebGRAPH/) is an online software to analyze genomic and epigenomic features that are enriched in given DNA fragments (35–37). As described above, we have identified 924 CpG sites correlated to 902 genes that are differentially methylated in CdLS ($P < 0.01$), out of which the methylation level of 361 CpGs (356 genes) are decreased and the methylation level of 563 CpGs (546 genes) are increased. The EpiGRAPH web service was used to analyze DNA features enriched in regions with differential methylation in CdLS. One kilobase DNA sequence (± 500 bp) surrounding each of the 924 CpG dinucleotides was identified and downloaded from the UCSC genome browser (http://genome.ucsc.edu/), processed in Galaxy (http://galaxy.psu.edu/) and uploaded to EpiGRAPH to identify overrepresented genomic or epigenomic features by comparing the 361 sequences harboring hyper-methylated CpGs and the 563 sequences harboring the hypo-methylated CpGs in CdLS. We chose a size of 1000 bp for the analyses because in normal tissues the extended distance of DNA methylation is generally shorter than 1000 bp (13). Significantly distinct histone modifications were found in chromatin regions with both differential DNA hypo-methylation and hyper-methylation, indicating a tight correlation between the alteration of DNA methylation and the diverse chromatin structure in CdLS (Table 1, Supplementary Table S5). The finding that repetitive sequences are enriched in chromatin regions harboring differentially methylated CpG sites is very interesting because cohesin binding is dysregulated in CdLS and the association between cohesin and repetitive sequences has been suggested to be involved in multiple biological roles (15). ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was further used to look for consensus sequences covering the differentially

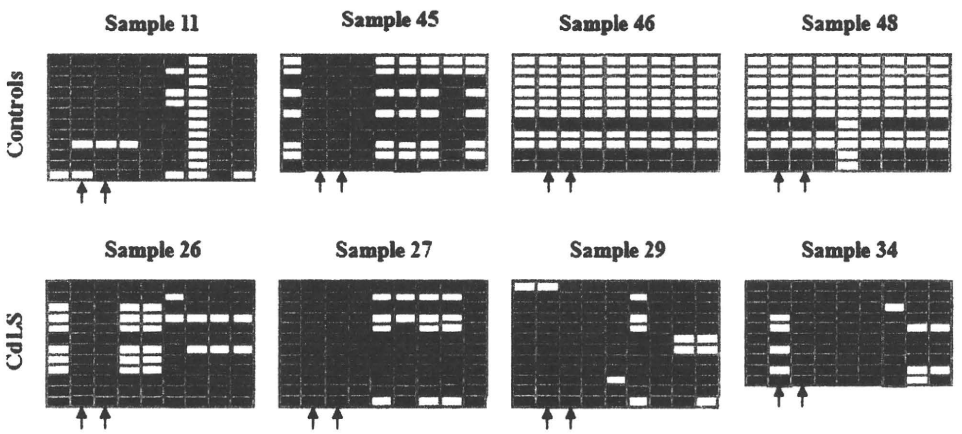

Figure 7. Bisulfite sequencing of a 252-bp fragment that is located at the *CAPN2* promoter region in four control and four CdLS samples. The examined fragment is located on chr1:221,966,278-221,966,529 surrounding probes cg01566170 and cg14972271 on the HumanMethylation27 array, which are 455 bp and 465 bp upstream of the TSS of *CAPN2*, respectively. There are nine CpG dinucleotides in this region. A solid block represents the methylated 'C' allele that remains as 'C' after bisulfite conversion and an empty block represents the unmethylated 'C' allele that was converted to 'T' after bisulfite treatment. Ten to twelve clones were selected for sequencing for each sample. Each row represents one sequenced clone and each column represents one examined 'CpG' site. The arrows point to probes cg01566170 and cg14972271.

Table 1. Specific histone modification markers and repetitive genomic sequences are enriched in chromatin regions harboring differentially methylated CpGs in CdLS

	Hyper_M	Hypo_M
Histone modification markers	H2A_H4R3me2 H3K27me1, H3K27me2 H3K36me1	H2A_Z H3K27me3 H3K4me2, H3K4me3 H3K9me1, H3K9me3 H3R2me1, H3R2me2 H4K20me1, H4K20me3 H3K79me3
Repetitive sequences	LINE SINE L2	repClass_Low_complexity repFamily_Low_complexity

EpiGRAPH analysis was performed on 361 1-kb DNA fragments surrounding differentially hyper-methylated CpGs and 563 1-kb fragments surrounding differentially hypo-methylated CpGs in CdLS.
Hyper_M: chromatin region with differential hyper-methylation in CdLS; Hypo_M: chromatin region with differential hypo-methylation in CdLS.

methylated CpG sites; however, no consensus sequences could be identified (data not shown).

DISCUSSION

CdLS is a dominant genetic disorder with multiple-organ system abnormalities, including characteristic facial features, limb defects, mental retardation, developmental delay and gastrointestinal problems. Mutations in the cohesin regulatory protein NIPBL and cohesin subunits SMC1A and SMC3 account for ~65% of confidently diagnosed probands, while the remaining ~35% of probands have no identifiable gene mutations (19). Prior studies (19) have shown that CdLS probands with *SMC3* and *SMC1A* mutations present with a milder clinical picture than the classic form of CdLS typically associated with protein truncating mutations in *NIPBL*. Since NIPBL is a regulator of the cohesin complex and both SMC3 and SMC1A are actual structural components of the cohesin complex, the exact mechanisms by which mutations in these proteins manifest their effects on development and gene regulation are likely to be quite different. The cohesin complex consists of four major subunits, SMC1A, SMC3, RAD21 and STAG1/STAG2, forming a ring structure holding sister chromatid together during mitosis and meiosis (39). Additionally, cohesin has been suggested to play pivotal roles in fundamental biological events in humans such as gene expression (22,26), double-strand DNA repair (40,41), genome instability (42), carcinogenesis (43) and chromatin loop formation (44). The co-localization of cohesin and CTCF in human cells further suggests functional cooperation and overlap may exist between these two proteins (25). Of note, mutations in cohesin accessory factors such as NIPBL, ESCO2 and ATRX have also been identified in human developmental disorders with similar but quite distinct clinical presentations which are currently collectively named 'cohesinopathies' (15). The cohesinopathies provide valuable experimental models with naturally occurring mutations to study the biological functions of the cohesin pathway in general and the specific proteins in particular in human cells. Recently, the term 'genomic disorder' was proposed to describe groups of developmental disorders or human maligancies in which epigenetic mechanisms including DNA methylation play important roles in the pathogenesis (33). It is not known whether similar mechanisms could also be involved in CdLS.

We chose to use a seemingly phenotypically unrelated tissue type, LCL, in this proof-of-principle study on a human developmental disorder, CdLS, based on two considerations: availability of the sample and goals of the project. As opposed to primary fibroblasts which represent a limited resource, has uncontrollable environmental exposure (such as diet or medications), and has significant variation of growth and survival rate across different samples in culture, patient-derived LCLs for genome-wide DNA methylation studies on human subjects has obvious advantages including (i) easier growth under controlled conditions to minimize the environmental influence on DNA methylation, (ii) ease of identification of sufficient numbers of matched samples (gender, age, clinical manifestations, etc.) for valid statistical analysis that would be much more difficult using fibroblasts (especially for groups engaged in the study of rare human disorders) and (iii) provides continually renewable and stable biomaterials that ensure for sequential integrated genomic analyses. Of note, GM12878, a well-circulated model LCL, has been universally used in the HapMap, ENCODE, and other major projects. Publicized DNA methylation data generated for GM12878 on the same Illumina platform demonstrated good correlation with our LCL samples with $r = 0.947$ when the low-quality measurements were removed (Zhang *et al.*, manuscript in preparation). In addition, LCLs have been widely used as surrogates or as cellular models to study epigenetic changes in neuropsychiatric illnesses such as autism, bipolar disorder, schizophrenia and in central nervous system disorders such as Parkinson disease (45). Genetic disorders, such as ataxia telangiectasia (46) and Nijmegen breakage syndrome, have also been investigated on patient derived LCLs yielding valuable insights into the pathobiology of these disorders (47).

Methylation and demethylation of regulatory sequences in the genome are known to have profound effects on cellular behavior and fate. In this study, we have performed genome-wide DNA methylation analysis in CdLS using Illumina Methylation27 bead chip carrying 27 578 CpG dinucleotides that represents 14 495 cognate genes in the human genome. The power of this array is that a very large number of sites can be determined simultaneously, allowing highly reproducible global patterns to be discovered. Both unsupervised PCA based on results of all data from available CpG sites and the supervised clustering signatures based on differentially methylated autosomal CpG sites shown in Figure 1 indicate that CdLS has a distinct methylation profile as compared to controls. Of note, *NIPBL* transcription has only dropped 30% in those CdLS cells presenting the unique disease-specific DNA methylation pattern in the current

study (26). The hierarchial clustering also demonstrated that the methylation profile correlates to disease severity and to the specific mutated genes: the mildly affected probands stay midway between severe probands and controls; mildly affected probands with *NIPBL* mutations were clustered as a transient group between the mildly affected individuals with mutations in other genes (*SMC1A* or *SMC3*); and the severe individuals with *NIPBL* mutations suggesting a genotype–phenotype correlation (Figure 1B). However, CdLS DNA methylation profile alone is not sensitive enough to serve as a diagnostic tool (Supplementary Figure S5). Leave-One-Out cross validation using $P < 0.001$ on 22 healthy controls and 22 severely affected CdLS probands with *NIPBL* mutations was conducted. Controls and probands could be roughly separated; however, 11 samples were mis-classified after 44 rounds with a classification accuracy of only 75%.

We next attempted to correlate the data from the three sets of genome-wide analyses on DNA methylation, gene expression and cohesin binding in CdLS. Venn diagram analysis did not reveal a single gene that had significant alterations in all of the three biological events (Supplementary Figure S6); therefore, no direct correlation between the three dysregulated biological events could be revealed in CdLS, indicating that additional unknown mechanisms might be involved in the pathogenesis of CdLS. That there is no remarkable correlation between gene expression and DNA methylation identified in CdLS probably can be explained by (i) A relatively big variation of DNA methylation level exists in healthy controls as seen on both array and BS (Supplementary Figure S1B and Figure 7) which adds to the difficulty of identifying an unambiguously changed DNA methylation pattern in CdLS. (ii) Global DNA methylation is a more stable type of epigenetic modification modulating the transcriptional plasticity in the human genome than we have realized (48). One study reporting the high-resolution methylation states on 1.9 million CpGs on human chromosomes 6, 20 and 22 from 12 different tissues failed to correlate DNA methylation with mRNA expression levels for 63% of the genes. Therefore, the author suggested that differential promoter methylation might have only a permissive role, such as establishing an open chromatin conformation, for the transcription regulation. In combination with other factors or mechanisms that drive transcription but not alone, DNA methylation could regulate the transcription of the cognate genes (49). To support this argument, in our study we have found keratin genes that are not expressed in the studied LCLs, most promoters of the two keratin gene clusters located on chromosome 12 and 17 are constantly under hyper-methylation in control cells. In CdLS, uniform methylation was found again at the two clusters but with even higher methylation levels (Supplementary Figure S7A and B). Keratin genes remain silent in both control and CdLS cells with differential promoter hyper-methylation levels, suggesting gene expression is not solely controlled by DNA methylation. (iii) The average global DNA methylation level in LCLs is low, and overall methylation changes between control and CdLS are quite subtle, making it more difficult to identify minor but significant changes. (iv) As seen in *Drosophila*, NIPBL or cohesin may regulate transcription mainly by the association with enhancers or other remote regulatory elements first and only subsequently with promoters. The CpG dinucleotides examined on the Methylation27 platform in this study have minimal coverage of the remote cis elements, which could lead to the lack of detection of any DNA methylation alteration on enhancers, silencers, insulators, locus control regions, etc. We therefore propose whole-genome BS to be the next experimental approach.

In humans, most genes are expressed from both alleles in diploid cells; however, more and more genes are being identified that are only expressed from a single allele. Mammalian X inactivation, imprinting (e.g. *IGF2* and *H19*), and allelic exclusion (e.g. olfactory receptor genes, immunoglobulin genes, T cell receptors, interleukins and natural killer cell receptors) are classic examples of monoallelic gene expression. A recent report suggests at least 1000 autosomal human genes are subject to random monoallelic expression (50). Regulation of this group of genes is clearly epigenetic and the role of DNA methylation in imprinting has been well recognized, for example, allele-specific DNA methylation has been observed for the immunoglobulin gene and few other monoallelically expressed genes (50–52). Hence, we combined the data set from this study with our previous genome-wide gene expression study and ChIP–chip assay to test whether differential DNA methylation affects monoallelic expression in CdLS and whether it is also involved in the altered cohesin–chromatin association in CdLS. Information on documented human imprinted genes was obtained from the online database 'Catalogue of Parent of Origin Effects' (http://igc.otago.ac.nz/Summary-table.pdf), a list of X-linked genes with their X inactivation status and a list of monoallelically expressed autosomal genes were obtained from the literature (50,53). Twenty-nine imprinted genes, 465 X-linked genes and 191 randomly (paternal or maternal) monoallelically expressed autosomal genes have data available from all the three assays (DNA methylation, expression and ChIP–chip). Fisher's exact tests did not reveal any correlation between differential DNA methylation and the differential expression for monoallelically expressed genes in severely affected CdLS probands with *NIPBL* mutations (Supplementary Table 6). In addition, correlation between the differential DNA methylation and altered cohesin binding (loss or addition) at transcription start sites (TSSs), and correlation between the altered cohesin binding at TSSs and the differential gene expression for the monoallelically expressed genes were not identified either (Supplementary Table S7). In conclusion, the current data are not able to support an impact of CdLS-related disruption of cohesin on monoallelic gene expression. This may be due to the tissue or developmental stage specificity of expression of these genes.

DNA methylation state is influenced by a number of endogenous and exogenous parameters such as gender, age, tissue type or passage of cell cultures (54). To be consistent, all samples for expression and methylation studies were from Caucasians, all three genome-wide

assays used the same tissue type (LCLs), and the same set of samples were used for all the studies whenever possible. Gender was strictly matched for the differential expression and DNA methylation analyses. Only two factors, the age and the culture passage, could not be controlled for stringently due to the limitation of resources. Most of our CdLS probands are children and some of the healthy controls are adults. The LCLs we have used are stock cell lines that have been continuously collected in our laboratory for decades. However, in one report, no detectable global changes in average DNA methylation levels can be recognized between a group of 26-year-olds and the second group of 68-year-olds (49), and, in another report, the methylation change during prolonged passage in culture is suggested to be insignificant (55).

At present, genome-wide information of gene expression, DNA methylation and cohesin binding in CdLS is available from our integrative studies. The tip of the iceberg revealed in these studies will help us to design new assays to further understand how cohesin regulates critical biological pathways in humans. For example, '6C' could be conducted to identify epigenetic chromatin looping structure mediated by cohesin at specific loci that associate with a particular gene transcription pattern. Allele-specific DNA methylation cannot be studied on the current array platform but could be possible if combined with the SNP information. The combined data will help us to illustrate NIPBL and cohesin's role in monoallelic expression in the human genome, knowledge of which is currently lacking. ChIP–chip or ChIP–Seq studies to identify specific histone modifications in CdLS are also a needed next step to understand how chromatin structure is involved in human developmental disorders. Finally, animal models with complete or partial NIPBL or cohesin knockdown may help us to interpret cohesin-dependent cellular functions *in vivo*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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DONEPEZIL SIGNIFICANTLY IMPROVES ABILITIES IN DAILY LIVES OF FEMALE DOWN SYNDROME PATIENTS WITH SEVERE COGNITIVE IMPAIRMENT: A 24-WEEK RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL

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ABSTRACT

Objective: Down syndrome (DS) patients share certain neuropathological features with Alzheimer disease patients. A randomized, double-blind, placebo-controlled study was performed to investigate the efficacy and safety of donepezil, an Alzheimer disease drug, for DS patients. *Method*: Twenty-one DS patients with severe cognitive impairment were assigned to take donepezil (3 mg daily) or a placebo for 24 weeks, and evaluated for activities in daily lives by concisely modified International Classification of Functioning, Disability and Health (ICF) scaling system. *Results*: ICF scores significantly increased without any adverse effects in the donepezil group in comparison to those in the placebo control. Among the individual functions tested, there was a dramatic improvement in the global mental functions and in specific mental functions. *Conclusions*: Donepezil may effectively and safely improve overall functioning of DS patients with severe cognitive impairment.

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Key Words: Down syndrome, severe cognitive impairment, randomized, double-blind, placebo-controlled study, donepezil, overall functioning

INTRODUCTION

Down syndrome (DS) is the most common chromosomal disorder resulting in an intellectual disability [1]. Although 75% of DS patients aged 60 years or older are clinically diagnosed with Alzheimer disease (AD) [1], most DS

patients develop AD-like neuropathological changes to their brain even before the disease becomes clinically apparent [2]. In addition, cholinergic dysfunction has been associated with both DS and AD [3-6]. Aside from AD-like dementia, young adult DS patients occasionally develop neurobehavioral disorders of unknown causes, thus leading to rapid progressive deterioration of the activities in daily life [3].

A choline esterase inhibitor, donepezil, was approved for AD patients in the United States in 1996 and in Japan in 1999. Based on the similarities in the neuropathogenesis between AD and DS, donepezil has been tested therapeutically in DS patients; however, its efficacy has not been shown consistently and substantial adverse reactions have often been observed [7-11]. An open-labeled trial of donepezil therapy recently conducted in a small number of DS patients demonstrated that plasma concentrations of donepezil were generally higher in DS patients than in other individuals and that low-dose (3 mg daily) donepezil could be given to DS patients without any adverse effects [12, 13]. Under such low dosage, donepezil appeared to effectively improve activities in the daily lives of DS patients including young adults with the above-mentioned rapid and progressive deterioration of activities in daily life [13, 14].

Such promising preliminary results prompted a double-blind randomized trial of donepezil to determine whether it improves activities in the daily lives of DS patients and to identify the functions that specifically improved.

METHOD

Participants

The participants in the present trial were recruited from DS patients at the Megumi and Nozomi Homes of Misakaenosono (Isahaya, Nagasaki, Japan), which are dedicated to females with intellectual disabilities having little or no medical problems except for cognitive impairment. Among 206 females who were cared for at the two homes, there were 21 DS patients; they were aged from 32 to 58 years with the mean of 45.6 years. The exclusion criteria included the presence of clinically significant diseases or a history of major surgical procedures. None had a history of major surgical procedures; two had hypothyroidism and congenital arterio-ventricular septal defect, which were not considered significant. The measurements of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, blood urea nitrogen, serum creatinine, amylase, creatine kinase, free triiodothronin, free thyroxin, and thyroid stimulating hormone were all within normal limits in all DS patients; electrocardiogram was also normal in all patients. We therefore enrolled all of the 21 DS patients in the present study (see Figure 1). Any participant was to be withdrawn from the study when she developed adverse reactions of grade 3 or 4 or when she showed other clinically significant conditions.

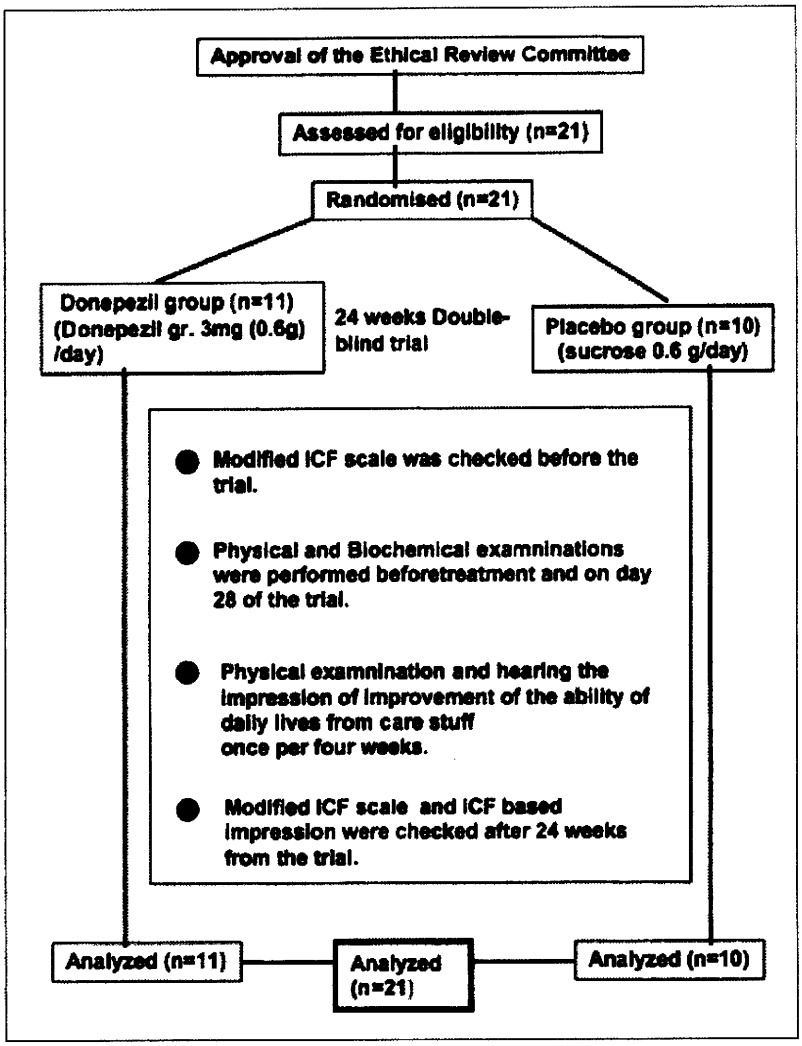

Figure 1. CONSORT flow diagram.

Informed Consent and IRB Approval

The present study was approved by the Ethical Review Committee of Nagasaki University and by the faculty of the social welfare corporation "The Holy Family Association, Misakaenosono." The written informed consent was obtained from the guardian of each participant.

Procedures

The trial started in November 2006 and ended in May 2007. We allocated 11 patients out of the 21 participants to the group receiving donepezil (Aricept®, Eisai Co., Ltd., Japan) for 24 weeks using simple random sampling, and allocated the remaining 10 patients to the group receiving placebo. Our experiences indicated that the improvement of overall functioning was felt 1 to 4 months after administration of donepezil, and we therefore set the length of this trial as 24 weeks. The random allocation was carried out by one of the authors (M. Nakashima, MNa), and the list of allocation was kept securely in the pharmacy of Nagasaki University Hospital and was not available to anyone except for MNa, who never had contact with the patients, their guardians, their caretakers, or others involved in the present study during the 24-week study period. The dose of donepezil was fixed at 3 mg once daily throughout this trial, based on the results of previous studies [12-14], and supplied as a lyophilized power in packages containing 3 mg donepezil and 600 mg lactose. The placebo was identically supplied and formulated except that it contained no donepezil. These packages were delivered directly from MNa to the Megumi and Nozomi Homes. The code was not disclosed to other researchers until the data including laboratory analyses collected through the trial were completed and fixed.

The patients' health conditions were checked every 4 weeks during the trial. Furthermore, peripheral blood was drawn on the 24th day of the trial to measure the aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, serum creatinine, amylase, and creatine kinase levels, since abnormalities in those measurements had previously been reported as adverse effects in AD patients on donepezil therapy (data collected by Eisai Co., Ltd.). The plasma trough levels of donepezil were measured 24 weeks after the commencement of the treatment.

Assessments

We evaluated the efficacy of donepezil on the basis of an abridged edition of *International Classification of Functioning, Disability and Health (ICF)* scales, which was developed by two of the authors (AK and HI) in 2007 for efficient measurement of chronological changes in psychological and motor functions in handicapped or aged persons; the details are given in the Appendix. In brief, the following 63 items were chosen for easy handling that appeared sensitive enough to assess psychological and motor functions in the above-mentioned

individuals: global mental functions of 18 items (b126, b1260-1267, b1300-1304, and b1340-1343); specific mental functions partial of 20 items (b1400-1403, b1470-1471, b1520-1522, b1600-1602, b1640-1646, and b176); voice and speech functions of 6 items (b3300-3303 and b3400-3401); functions of the digestive system of 7 items (b5100-5106); urinary functions of 3 items (b6200-6202); and movement-related functions of 9 items (b755, b7600-7603, and b7650-7653). Each item is assessed from the two viewpoints (i.e., the degree of need for support at a point of time and the impression on the change in the degree of the quality of life (QOL) called as ICF based impression (IBI)). The assessment from the first viewpoint is classified into five grades: not need support (grade 5), occasionally need support (grade 4), sometimes need support (grade 3), almost extensive supports need (grade 2), and extensive supports need (grade 1); and the assessment from the second viewpoint is also classified into five grades: improved (grade 5), slightly improved (grade 4), no change (grade 3), slightly deteriorated (grade 2), and deteriorated (grade 1).

The details will be reported elsewhere, but the validity of this abridged edition of the ICF scales has been confirmed in many settings including DS patients [15]. In the previous study [15], DS patients with and without rapid progressive deterioration of the activities in daily life were assessed by the aforementioned checklist. Total points on the ICF-based checklist was significantly lower in the former group than in the latter.

In the present study, two respective persons assessed the above-mentioned 63 items for each participant at the two points of time, at enrollment and 24 weeks after the commencement of the treatment, while they assessed the same items from the second viewpoint 24 weeks after the commencement of the treatment. One of the two persons was participant's primary care taker and the other was one of the two key scorers supervising these caretakers in the institute. Final assessment was made after mutual discussion.

Analysis

We summarized the quantitative data by the triplet of the 1st, 2nd, and 3rd quartiles; the 2nd quartile is the median. We scored the abridged edition of ICF scales by summing up the grade of 63 items assessed from the first viewpoint at enrollment and 24 weeks after the treatment; we also scored the six subscales such as global mental functions in a similar way. Primary evaluation of the efficacy of donepezil on DS patients was performed on the basis of the difference in the abridged edition of ICF scales assessed at the two points of time, namely, at enrollment and 24 weeks after the commencement of the treatment. Similar difference calculated within respective six subscales was used for auxiliary evaluation of the efficacy. We compared the aforementioned difference between the two groups receiving donepezil and placebo on the bases of Wilcoxon rank-sum test. We used the IBI for secondary evaluation of the efficacy in the following

way: if the number of items graded 5 or 4 exceeded the number of items graded 2 or 1, we defined the QOL of the participant was improved, and compared the frequency of participants with QOL improved between the two groups using Fisher's exact test for 2 × 2 contingency table; similar comparison was made within 6 subscales for auxiliary evaluation. The *p*-values shown are all two-sided. FREQ, NPAR1WAY, and UNIVARIATE in the SAS® system were used for the calculation.

RESULTS

All of the 21 participants completed the study showing normal conditions in both of physical examinations and laboratory analyses throughout the trial, although one was hospitalized for about 2 weeks during the trial because of severe pneumonia; this patient was found to be in placebo group after the code was disclosed. Two patients with hypothyroidism and congenital arterio-ventricular septal defect at enrollment were found to have been in donepezil group, and one patient with hypothyroidism at enrollment was found to have been in placebo group; their health status except for dementia was stable during the trial.

Table 1 compares the baseline characteristics between the donepezil and placebo groups, and no significant imbalance was observed between the two groups with respect to the baseline measurements of age, height, IQ, and abridged edition of ICF scales including subscales; although the weight was larger in the placebo group than in the donepezil group and the difference was statistically significant, the *p*-value was just below 0.05 ($p = 0.0486$). Their IQs were very low and did not reach 20, except for three patients whose IQs were 30, 31, and 35, respectively. Their motor performances were relatively well: they could walk by themselves or with some support, although one patient could not sit on bed or walk even with full support.

Efficacy of Donepezil on DS Patients

The change in the scores of the abridged edition of ICF scales and subscales assessed at enrollment and 24 weeks after the commencement of the treatment from the first viewpoint for participants is shown in Figures 2 and 3 for the two groups, respectively. On the whole, the scores in the donepezil group demonstrated an increasing tendency, while showed a decreasing or unchanged tendency in the placebo group. The difference in the scores of abridged edition of ICF scales assessed at two time points was significantly larger in the donepezil group than in the placebo group (median = 14 and 0, respectively; $p = 0.0001$). Similar results held for the subscales of global mental functions (median = 4 and 0, respectively; $p = 0.0001$), specific mental functions partial (median = 6 and 0, respectively; $p = 0.0002$), and voice and speech functions (median = 1 and 0, respectively; $p = 0.0005$), while not for the functions of the digestive system

Table 1. Distribution of Baseline Characteristics in Donepezil Group and Placebo Group

Characteristics	Group[a] Donepezil (11)	Placebo (10)	p-Value[b]
Age (years)	(42, 46, 53)[c] 36-58[d]	(38, 43.5, 51) 32-55	0.5256
Height (cm)	(130.0, 137.0, 142.5) 124.0-144.8	(138.8, 140.4, 141.8) 137.8-143.8	0.1586
Weight (kg)	(38.9, 43.1, 45.5) 33.1-51.0	(45.0, 47.0, 53.1) 30.6-58.9	0.0486
IQ[e]	(17, 19, 23) 15-31	(16, 18.5, 27) 6-35	0.8699
Abridged edition of ICF scales	(184, 213, 237) 76-259	(199, 221, 255) 157-264	0.4384
Global mental functions	(51, 60, 71) 20-86	(61, 70.5, 76) 46-81	0.2039
Specific mental functions partial	(36, 53, 66) 20-75	(41, 54, 70) 30-75	0.7780
Voice and speech functions	(6, 11, 12) 6-19)	(6, 10.5, 18) 6-18	0.9713
Functions of the digestive system	(26, 29, 33) 13-35	(29, 35, 35) 18-35	0.1177
Urinary functions	(10, 13, 15) 3-15	(13, 14.5, 15) 9-15	0.2910
Movement-related functions	(39, 44, 45) 13-45	(41, 44.5, 45) 34-45	0.7372

[a]Number of participants in parentheses.
[b]Based on Wilcoxon rank-sum test; two sided.
[c]The triplet denotes the 1st, 2nd, and 3rd quartiles from the left, respectively.
[d]Minimum-maximum.
[e]The IQ could not be assessed in two participants of donepezil group.

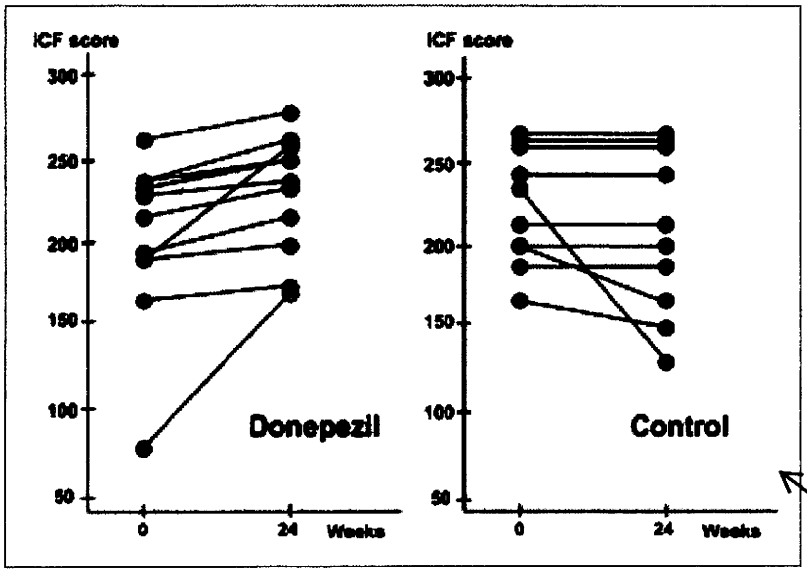

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Figure 2. Changes in the score of the abridged edition of ICF scales in the donepezil and placebo groups assessed from the first viewpoint at the two points of time, at enrollment and 24 weeks after the commencement of the treatment. Each closed circle depicts each patient. The changes were significantly different between the two groups ($p = 0.0001$).

(median = 0 and 0, respectively; $p = 0.0149$), urinary functions (median = 0 and 0, respectively; $p = 0.1376$), and movement-related functions (median = 0 and 0, respectively; $p = 0.0340$); since there were six subscales, we considered the significance of the results adjusting for the multiple comparison on the basis of Bonferroni inequality.

The frequency of participants with improved IBI defined that the number of items graded 5 or 4 exceeded the number of items graded 2 or 1 was significantly higher in the donepezil group than in the placebo group for abridged edition of ICF scales (11/11 *vs* 4/10, $p = 0.0039$), and subscales of global mental functions (11/11 *vs* 3/10, $p = 0.0010$), specific mental functions partial (10/11 *vs* 0/10, $p < 0.0001$), and voice and speech functions (9/11 *vs* 2/10, $p = 0.0089$), while no significant difference was observed between the two groups for functions of the digestive system (2/11 *vs* 0/10, $p = 0.4762$), urinary functions (5/11 *vs* 1/10, $p = 0.1486$), and movement-related functions (2/11 *vs* 0/10, $p = 0.4762$). Most caregivers of DS patients in the donepezil group got the impression that overall functioning was clearly improved between 4 and 16 weeks after administration. Once any effect was obtained, it lasted during the study period.

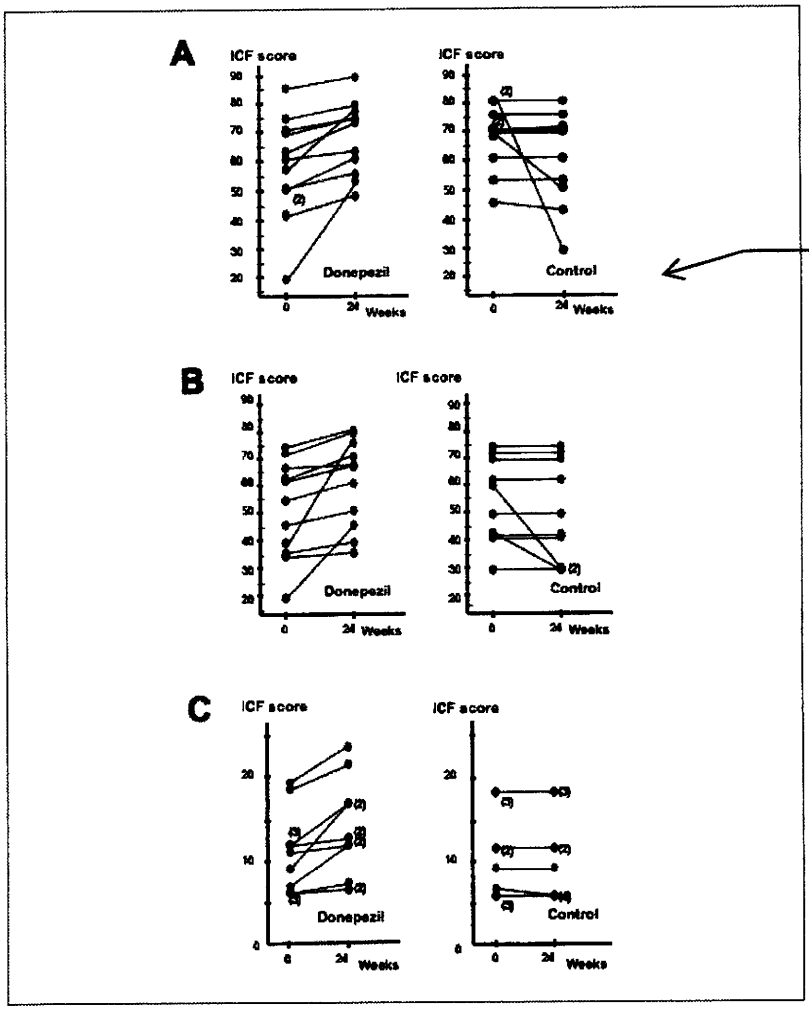

Figure 3. Changes in the score of the subscales for abridged edition of ICF scales in the donepezil and placebo groups assessed from the first viewpoint at the two points of time, namely, at enrollment and 24 weeks after the commencement of the treatment. Each closed circle depicts each patient except for one with parenthetic entries indicating the number of patients. These changes were significantly different between the two groups for: **A.** Global mental functions (p = 0.0001); **B.** Specific mental functions (p = 0.0002); and **C.** Voice and speech functions (p = 0.0005). The changes in other functions, namely, functions of the digestive system, urinary functions and movement-related functions did not differ significantly between the two groups.

Safety of Donepezil on DS Patients

Minor adverse reactions such as soft stool and skin rash were seen both in donepezil group (2 patients) and placebo group (1 patient). Mild skin rash was found in two patients in placebo group only. Those symptoms were improved spontaneously without any treatments. No other harmful events were observed in the donepezil group throughout the trial, and the mean plasma concentration (trough level) of donepezil was 19.2 ng/ml, ranging from 9.9 to 30.5 ng/ml, as expected from the previous studies [12-14].

DISCUSSION

Our previous and present studies have demonstrated that donepezil treatment could efficiently and safely provide an improved QOLs to DS patients. Previous studies showed that plasma concentrations of donepezil are significantly higher in DS patients than in controls and that donepezil can be used safely for DS patients under careful monitoring of the plasma concentration. DS patients treated with donepezil in the previous study showed such dramatic improvement that their parents living with them got very impressed; however, it was not easy to precisely score such improvement. Many objective scaling systems, including the Tanaka-Bennett IQ test, Alzheimer's Disease Assessment Scale and Social Maturity Scale, as well as subjective scaling systems such as the Adaptive Behavior Scale, are difficult to perform with DS patients. The reasons why scaling systems that are effective with AD patients do not sufficiently work remain obscure, but may include the facts that while the baseline intelligence of AD patients is normal, the cognitive function of DS patients is congenitally impaired. In addition, psychological instability is observed more frequently in DS patients than in AD patients [16].

The present study adopted the abridged edition of ICF scales to obtain the data for evaluating the effects of donepezil on DS patients as much objectively and precisely as possible. The ICF has been recognized as an extremely useful system for classification of functioning and disability, and scaling systems based on the ICF are useful for precise and comprehensive assessment of degrees of and changes in disability and handicaps. Its major drawback, however, is that it is too extensive to perform with DS patients. The abridged edition of ICF scaling system adopted in the present study has been used for the chronological assessment of DS patients at Tokyo Gakugei University for the last 2 years, and proved to be convenient and useful to assess their ability in their daily lives [15]. To further examine its aptitude for the assessment of activities in daily lives of DS patients, we used the same system for a questionnaire survey of 14 other DS patients who had been treated with donepezil since 2002. Their IQs and activities in daily lives were various. The abridged edition of ICF scales was very sensitive to reflect the conditions of daily activities of severely impaired

DS patients. In contrast, since DS patients who had relatively high abilities in daily lives got nearly full scores in this system, chronological changes that their families remarked upon donepezil therapy were not reflected on the scores (data not shown).

Before this trial, none of the participants could communicate with the staff members in the homes or follow their advice; however, a gradual but dramatic improvement in communication skills was observed in a substantial number of the study participants, all of whom later turned out to be assigned to donepezil treatment. The efficacy became apparent approximately 3 months after the initiation of treatment.

One patient in the placebo group developed severe pneumonia during the trial. Since she showed a very large decrease in the score of the abridged edition of ICF scales and subscales, we re-analyzed the data after excluding this patient. No change in the significance was observed except the voice and speech function assessed on the basis of IBI: the p-value was 0.0089 (9/11 vs 2/10) in the analysis of all participants, while it was 0.0216 (9/11 vs 2/9) in the analysis after excluding the patient.

The present double-blind study had several implications regarding the efficacy of donepezil on DS patients. First, donepezil treatment was beneficial for DS patients at ages of 30 to 50 years when many of these individuals develop AD-like progressive cognitive dysfunction. Second, most DS patients in the present study had IQs below 20, suggesting that donepezil treatment would be beneficial even for severely impaired patients. Third, the ability in daily activity in donepezil group was apparently improved several months after the commencement of the treatment, and was never reduced since then throughout the trial. Prolonged administration may be needed to get fully satisfactory results. We got an impression that the efficacy of donepezil therapy against DS patients differs from that against AD patients in two points: one is that it will take longer time to improve QOLs in DS patients than in AD patients, and the other is that clinical efficacy of donepezil treatment will continue longer in DS patients than in AD patients. The DS patients upon donepezil therapy since 2002 have been keeping in good condition. Pathological deterioration of the brain may develop slower in DS patients than in AD patients, despite the similarity of neuropathological characteristics between the two clinical entities. Finally, donepezil has turned out not to be equally effective in all items tested: it improved global mental functions and specific mental functions more efficiently than others.

Safety issues are critical when considering broader and more constant administration of donepezil to DS patients. Besides this report, we have tested this drug in a total of 60 DS patients over a period of seven and one-half years. Daily administration of as low as 3 mg of donepezil can achieve therapeutic plasma levels in many of these individuals. Only a small proportion of patients require a higher dose (5 mg daily). Therefore, low dosage and monitoring of plasma concentrations of donepezil will guarantee its safety. No serious adverse reactions

have been observed. Minor adverse reactions such as diarrhea were acceptable for most patients and their families. Therefore, donepezil could safely be administered to DS patients for extended periods of time.

In conclusion, the current study demonstrated that donepezil could efficiently and safely improve overall functioning of DS patients. Although further investigation including a larger scale study is clearly needed to demonstrate whether donepezil can provide better QOL to any DS patient, the results of the present study have favorable implications for the "pharmacotherapy for DS, a chromosomal disorder."

APPENDIX
The Abridged Edition of ICF Scales

A. Demographic Information

A.1 NAME A.2 SEX A.3 DATE OF BIRTH A.4 WRITTEN DATE
A.5 PREVIOUS WRITTEN DATE A.6 WRITTEN NAME
A.7 DEGREE OF MENTAL DEFICIENCY: PROFOUND, SEVERE, MODERATE, MILD
A.8 MEDICAL DIAGNOSIS: MENTAL RETARDATION, DOWN SYNDROME, AUTISM, OTHER
A.9 OTHER HANDICAPPED: VISUAL, HEARING, SPEECH, BODY
A.10 HEIGHT A.11 WEIGHT A.12 MENTALLY STATE: MA, IQ, SCALE

B. Check List

Global Mental Functions

SA01. General mental functions of constitutional disposition of the individual to react in a particular way to situations, including the set of mental characteristics that makes the individual distinct from others. [b126 Temperament and personality functions]

SA02. Mental functions that produce a personal disposition that is outgoing, sociable and demonstrative, as contrasted to being shy, restricted and inhibited. [b1260 Extraversion]

SA03. Mental functions that produce a personal disposition that is cooperative, amicable, and accommodating, as contrasted to being unfriendly, oppositional and defiant.[b1261 Agreeableness]

SA04. Mental functions that produce personal dispositions such as in being hard-working, methodical and scrupulous, as contrasted to mental functions producing dispositions such as in being lazy, unreliable and irresponsible.[b1263 Psychic stability]

SA05. Mental functions that produce a personal disposition that is even-tempered, calm and composed, as contrasted to being irritable, worried, erratic and moody. [b1262 Conscientiousness]

SA06. Mental functions that produce a personal disposition that is curious, imaginative, inquisitive and experience-seeking, as contrasted to being stagnant, inattentive and emotionally inexpressive.[b1264 Openness to experience]

SA07. Mental functions that produce a personal disposition that is cheerful, buoyant and hopeful, as contrasted to being downhearted, gloomy and despairing. [b1265 Optimism]

SA08. Mental functions that produce a personal disposition that is self-assured, bold and assertive, as contrasted to being timid, insecure and self-effacing.[b1266 Confidence]

SA09. Mental functions that produce a personal disposition that is dependable and principled, as contrasted to being deceitful and antisocial.[b1267 Trustworthiness]

SA10. Mental functions that produce vigour and stamina.[b1300 Energy level]

SA11. Mental functions that produce the incentive to act; the conscious or unconscious driving force for action.[b1301 Motivation]

SA12. Mental functions that produce a natural longing or desire, especially the natural and recurring desire for food and drink.[b1302 Appetite]

SA13. Mental functions that produce the urge to consume substances, including substances that can be abused.[b1303 Craving]

SA14. Mental functions that regulate and resist sudden intense urges to do something. [b1304 Impulse control]

SA15. Mental functions involved in the time spent in the state of sleep in the diurnal cycle or circadian rhythm.[b1340 Amount of sleep]

SA16. Mental functions that produce the transition between wakefulness and sleep.[b1341 Onset of sleep]

SA17. Mental functions that sustain the state of being asleep.[b1342 Maintenance of sleep]

SA18. Mental functions that produce the natural sleep leading to optimal physical and mental rest and relaxation.[b1343 Quality of sleep]

Specific Mental Functions

SB01. Mental functions that produce concentration for the period of time required. [b1400 Sustaining attention]

SB02. Mental functions that permit refocusing concentration from one stimulus to another.[b1401 Shifting attention]

SB03. Mental functions that permit focusing on two or more stimuli at the same time.[b1402 Dividing attention]

SB04. Mental functions that permit focusing on the same stimulus by two or more people, such as a child and a caregiver both focusing on a toy.[b1403 Sharing attention]

SB05. Mental functions that regulate the speed of behaviour or response time that involves both motor and psychological components, such as in disruption of control producing psychomotor retardation (moving and speaking slowly; decrease in gesturing and spontaneity) or psychomotor excitement (excessive behavioural and cognitive activity, usually nonproductive and often in response to inner tension as in toe-tapping, hand-wringing, agitation, or restlessness.)[b1470 Psychomotor control]

SB06. Mental functions that produce nonverbal behaviour in the proper sequence and character of its subcomponents, such as hand and eye coordination, or gait.[b1471 Quality of psychomotor functions]

SB07. Mental functions that produce congruence of feeling or affect with the situation, such as happiness at receiving good news.[b1520 Appropriateness of emotion]

SB08. Mental functions that control the experience and display of affect.[b1521 Regulation of emotion]

SB09. Mental functions that produce the spectrum of experience of arousal of affect or feelings such as love, hate, anxiousness, sorrow, joy, fear and anger. [b1522 Range of emotion]

SB10. Mental functions that govern speed of the thinking process.[b1600 Pace of thought]

SB11. Mental functions that organize the thinking process as to its coherence and logic. [b1601 Form of thought]

SB12. Mental functions consisting of the ideas that are present in the thinking process and what is being conceptualized.[b1602 Content of thought]

SB13. Mental functions of creating general ideas, qualities or characteristics out of, and distinct from, concrete realities, specific objects or actual instances. [b1640 Abstraction]

SB14. Mental functions of coordinating parts into a whole, of systematizing; the mental function involved in developing a method of proceeding or acting. [b1641 Organization and planning]