Dosage-dependent over-expression of genes in the trisomic region of Ts1Cje mouse model for Down syndrome

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Down syndrome (DS) is the most common chromosomally caused form of mental retardation and is caused by trisomy of chromosome 21. The over-expression of genes located on the trisomic region has been assumed to be responsible for the phenotypic abnormalities of DS, but this hypothesis has not been confirmed fully and the very existence of gene dosage effects has been called into question. We have therefore investigated global gene expression profiles in Ts1Cje, a mouse model for DS that displays learning deficits and has a segmental trisomy of chromosome 16 orthologous to a segment of human chromosome 21 spanning from Sod1 to Znf295. DNA microarray analyses of six Ts1Cje and six normal littermate (2N) mouse brains at postnatal day 0 with probe sets representing approximately 11 300 genes revealed that the number of expressed genes and their identities in Ts1Cje mice were almost same in 2N mice. Notably, the expression levels of most genes in the trisomic region were increased ~1.5-fold, and the top 24 most consistently over-expressed genes in the Ts1Cje mice were all located in the trisomic region. In contrast, the expression levels of genes on other chromosomes or the euploid region of chromosome 16 were largely the same (1.0-fold) in Ts1Cje and 2N mice. These results indicate that the genes in the trisomic region of Ts1Cje are over-expressed in a dosage-dependent manner and are implicated in the molecular pathogenesis of DS.

INTRODUCTION

Down syndrome (DS) is the most frequent chromosomal cause of mental retardation and results from the presence of an extra copy of human chromosome 21 (HSA21). The incidence of DS is approximately one in 700 live-born infants. In addition to mental retardation, which is virtually always present, there are several other phenotypes, including congenital heart disease and gastrointestinal anomalies, that are not present in all persons with DS (1). Although the trisomy of HSA21 is thought to be directly or indirectly responsible for the mental retardation and other phenotypic abnormalities of DS, studies of global gene expression profiles in DS and its mouse models have been in conflict (2–6), and the precise molecular mechanisms underlying the development of the DS phenotype are still not clear.

The distal end of mouse chromosome 16 (MMU16) is orthologous to most of HSA21. Several mouse models of DS with full or segmental trisomy of MMU16 have been produced. The Ts16 mouse (7) has three full copies of MMU16 which contains not only the region orthologous to HSA21 but also ones orthologous to HSA3, 8, 16 and 22. Although Ts16 shows some of the characteristics of DS (8), its value as a model for DS is limited because of both the presence of triplicated genes on other than the HSA21 orthologous region and its lethality in utero. Ts65Dn (9), Ts1Cje (10) and Ms1Ts65 (11) mice that only contain regions orthologous to HSA21 are segmentally trisomic for MMU16.

(13). It contains about 136 genes having orthologs in human and harbors the region that has been reported to be critically responsible for the observable DS phenotype (12,14). The trisomic region of Ts1Cje is part of that of Ts65Dn and spans from $sodl$ (superoxide dismutase 1) to $znf295$. It contains about 97 genes having orthologs in human, with $sodl$ being functionally inactivated. Ms1Ts65 is trisomic from $app$ to $sodl$, a part of the trisomic region in Ts65Dn but not in Ts1Cje. Ts65Dn and Ts1Cje mice display learning and behavioral abnormalities that model the mental retardation associated with DS (10,11,15).

In this study, we investigated global gene expression in whole brains of the Ts1Cje mice at postnatal day 0 by using DNA microarrays and found that most of triplicated genes showed~1.5-fold over-expression. The expression of genes outside of the trisomic region was not perturbed, and the results, which demonstrate that trisomy of a segment of MMU16 results in gene dosage effects of~1.5-fold without dysregulating the expression of genes outside of the trisomic region, are compatible with the hypothesis that the increased expression of genes on the trisomic segment is responsible directly for the pathogenesis of DS.

**RESULTS**

**Identical sets of genes are expressed in Ts1Cje and 2N mouse brains**

Using brains of six Ts1Cje and six normal euploid (2N) littermate mice at postnatal day 0 (P0), we performed Affymetrix GeneChip analysis with 24,935 probe sets representing approximately 11,300 genes. To compare the number of genes expressed in Ts1Cje and 2N mice, we averaged the ‘detection $P$-value’ of six Ts1Cje and six 2N mice for each probe set. The detection $P$-value indicates the significance of the ‘detection call’ and is an index for estimating whether the transcript was expressed or not judged by the Affymetrix Microarray Suite 5.0 software. We used the default settings of this software to evaluate the detection calls of ‘present’ (expressed), ‘marginal’ or ‘absent’ (not expressed). Among all 24,935 probe sets, 9,218 (37.0%) sets received present calls and 15,717 (63.0%) received marginal or absent calls for Ts1Cje. For 2N mice, 9,475 (38.0%) probe sets received present calls and 15,460 (62.0%) sets received marginal or absent calls (Supplementary Material, Table S1). 8,878 (96.3%) probe sets with present calls in Ts1Cje were identical to those in 2N mouse. These results suggest that the number of genes and the genes themselves expressed in Ts1Cje mice at P0 are virtually same as in 2N mice, regardless of the presence of an extra copy of a segment of MMU16.

In subsequent analyses, 10,602 probe sets with a present or marginal call in either or both Ts1Cje and 2N mice were used (Supplementary Material, Table S2), and the 14,333 probe sets that had an absent call in both Ts1Cje and 2N mice were excluded.

We have deposited the raw, unfiltered data (accession no. GSE1294) to the public repository, Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo/).

**Genes with over-expression are localized on the trisomic segment of Ts1Cje**

We next examined the expression level of genes in Ts1Cje mice and 2N mice. The ‘change $P$-value’ indicates the significance of the ‘change call’ and is an index for estimating a change in transcript level between a baseline array and an experiment array. We calculated the change $P$-value for each probe set in all possible comparisons between each of six Ts1Cje and six 2N mice (36 comparisons in total), and then evaluated the change calls of ‘increase’, ‘marginal increase’, ‘no change’, ‘marginal decrease’ or ‘decrease’ using the default settings of the Affymetrix Microarray Suite 5.0 software. Table 1 lists the probe sets that revealed increase or decrease calls in more than 18 of 36 comparisons (see also Supplementary Material, Tables S3 and S4). Four genes ($donson$, $son$, $ifngr2$ and $dscr5$) had an increase call in two different probe sets, and three ESTs ($AW124994$, $AI844478$ and $AI842074$) represented the same locus, synaptojanin 1 homolog. Altogether, there were 24 genes or ESTs with increase calls and only one gene with a decrease call. Quite notably, all of the 24 over-expressed genes and ESTs are localized on the trisomic region of MMU16.

**Triplicated genes in Ts1Cje are~1.5-fold over-expressed**

Figure 1 summarizes the fold changes of genes and ESTs on MMU16 in Ts1Cje mice when compared with 2N mice (see also Supplementary Material, Table S5). The fold changes were calculated from averaged values of ‘signal log ratio’ which is an index for estimating the change in expression level for a transcript between a baseline and an experiment array (see Materials and Methods). The figure contains 230 genes and ESTs on MMU16 except for the probe sets that were not expressed in both Ts1Cje and 2N mouse brains. When the probe sets represented the same genes or ESTs, we averaged their fold changes. Notably, the fold changes of genes in the trisomic and the euploid regions are clearly discriminated (Fig. 1 and Supplementary Material, Table S6). Most of the 192 genes and ESTs located on the euploid region (proximal part of $sodl$) showed no change (averaged 1.012-fold), except for 5,430404L10Rik and Rab13, which showed~0.8-fold (0.776 and 0.722, respectively), and 98,3004M20Rik, Bcl6 and BC027231, which showed~1.2-fold (1.201, 1.209 and 1.233, respectively). In addition, the averaged fold change value for the genes located on euploid regions, including those on other chromosomes, is 1.016. Only a few genes on euploid regions are expressed differentially in Ts1Cje mice when compared with 2N mice. Among approximately 10,000 genes and ESTs on euploid regions, only 59 genes and ESTs show~0.8-fold change and 199 genes and ESTs show~1.2-fold change (Supplementary Material, Table S7). In contrast, 37 out of 38 genes and ESTs in the trisomic region (distal to $sodl$) showed>1.2-fold increases, and the remaining gene, Prdm15, showed a 1.18-fold increase. The mean value of the fold changes for the expression levels of these triplicated genes is 1.435, which is quite close to the theoretically expected value of 1.5.
Expression profiles of genes outside of the trisomic region in Ts1Cje mice are indistinguishable from those of 2N mice

We performed principal components analysis (PCA) (16) to compare the global gene expression profiles between Ts1Cje and 2N mice. In the plot of all genes expressed in Ts1Cje and/or 2N mice, the gene expression profiles were not distinguishable between Ts1Cje and 2N mice, and the first two principal component axes accounted for 53.4% of the variance in the data set (Fig. 2A). Next, we applied PCA to two samples of expressed genes: the genes inside of the trisomic region of MMU16 and the genes outside of the trisomic region of MMU16 or on other chromosomes. In the PCA plot of the genes outside of the trisomic region, the gene expression profiles were also indistinguishable between Ts1Cje and 2N mice, and the first two principal component axes accounted for 53.4% of the variance in the data set (Fig. 2B). In contrast, the expression profiles of genes in the trisomic region were clearly separated between Ts1Cje and 2N mice, and the first two principal component axes accounted for 73.1% of the variance in the data set (Fig. 2C). These results further indicate that the over-expressed genes in Ts1Cje mice are located primarily on the trisomic region, and that very few genes are expressed differentially outside of the trisomic region.

Quantitative PCR analyses confirm the dosage-dependent over-expression of the triplicated genes in Ts1Cje

To confirm the results of the GeneChip analysis, we performed quantitative real-time PCR using the TaqMan system. Donson (downstream neighbor of SON), Mprps6 (mitochondrial ribosomal protein S6), Tic3 (tetratricopeptide repeat domain 3) and Pcp4 (Purkinje cell protein 4) genes on the trisomic region in Ts1Cje, which showed 1.72-, 1.61-, 1.42- and 1.50-fold changes in Ts1Cje, respectively, in the GeneChip analysis (Fig. 1), showed 1.58-, 1.55-, 1.56- and 1.80-fold changes, respectively, in the quantitative RT–PCR analysis (Fig. 3). The Atp5c1 (ATP synthase, Hþ–transporting, mitochondrial F1 complex, gamma polypeptide 1), Prkcb (protein kinase C, beta) and App genes, localized on MMU2, MMU7 or outside of the triplicated region of MMU16, showed 1.00-, 1.00- and 0.97-fold changes, respectively, in the GeneChip analysis of Ts1Cje, and 0.93-, 1.07- and 1.00-fold, respectively, in the RT–PCR analysis (Fig. 3). A housekeeping gene, Gapdh on MMU2, was used to normalize data.

We also investigated the actual status of a few triplicated genes that did not show values close to 1.5-fold increases in the GeneChip analysis by using the quantitative RT–PCR. Ifnar2 (interferon (alpha and beta) receptor 2), Il10rb (interleukin 10 receptor, beta), Il10r1 [interferon (alpha and beta) receptor 1] and Ifnar1 (interferon (alpha and beta) receptor 1) genes showed 0.93-, 1.00-, 1.00- and 1.00-fold changes, respectively, in the quantitative RT–PCR analysis (Fig. 3).
receptor 1], Dyrk1a [dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a], Hmgn1 (high mobility group nucleosomal binding domain 1) and Prdm15 (PR domain containing 15) comprise most of (six out of eight) the triplicated genes that showed <1.3-fold changes in Ts1Cje in the GeneChip analysis, with 1.30-, 1.29-, 1.25-, 1.26-, 1.28- and 1.18-fold changes, respectively (Fig. 1). However, in the quantitative RT–PCR analysis they showed 1.49-, 1.34-, 1.63-, 1.48-, 1.49- and 1.41-fold changes, respectively (Fig. 3). These results further substantiated the ∼1.5-fold dosage-dependent over-expression of the triplicated genes in Ts1Cje.

DISCUSSION

We examined the global gene expression profiles in the Ts1Cje DS mouse model with segmental trisomy 16 by using Affymetrix Murine GeneChips and consistently detected significant differences in the expression levels of genes on the triplicated segment. DNA microarrays are useful for estimating the changes in global gene expression levels, the accuracy of data is supported by reports comparing the results obtained by DNA microarrays with those using other approaches such as TaqMan analysis (17), northern blot analysis (18) and oligonucleotide arrays and quantitative real-time PCR analysis (19). Our results of GeneChip analysis confirmed by TaqMan analysis indicate that comparisons among six samples of each genotype can reliably detect small (∼50%) differences in gene expression.

The completion of the initial mouse genome draft sequence revealed that both the human and the mouse genomes contain approximately 30 000 genes. The proportion of mouse genes with a single identifiable ortholog in the human genome is ∼80%, and the proportion of mouse genes without any homolog detectable in the human genome is <1% (20). The Affymetrix Murine GeneChips can examine the expression levels of 11 300 genes, and we therefore assume that approximately one-third of the mouse genes are represented on the chip. Since the trisomic segment of Ts1Cje contains approximately 97 genes, we consider the coverage provided by this microarray to be reasonable for investigating and comparing the expression of genes in euploid (2N) and Ts1Cje mice.

Two opposing hypotheses have been advanced to explain the pathogenesis of DS, the gene dosage effect hypothesis and the amplified developmental instability hypothesis (21,22). The gene dosage effect hypothesis holds that the phenotype is a direct result of the cumulative effects of the imbalance of the individual gene(s) located on the triplicated chromosome or chromosomal region. In contrast, the amplified developmental instability hypothesis claims that most manifestations of DS are caused by a non-specific disturbance genomic regulation and expression, which results in a disruption of developmental homeostasis.

To test these hypotheses, several studies have been conducted to analyze the differences in mRNA or protein levels in cells or tissues from fetuses with DS, and the results of these studies have been in conflict. The down-regulation of REST-regulated genes (SCG10, L1, Synapsin 1 and β4 tubulin), which are not on HSA21, was reported in
neuronal precursor cells (2). In another report on primary cultures, 10 of the most up-regulated genes were not located on HSA21, although the largest increases in expression were observed for genes on HSA21 (3). A third group described unchanged levels of proteins encoded by many genes on HSA21 and suggested that the DS phenotype cannot be explained simply by gene dosage effects (23–26). In contrast to all of these studies, up-regulation specific to genes on HSA21 was shown in cerebral cortex extracts and astrocyte cell lines (4) (Table 2).

For mouse models of DS, the first global gene expression profile was reported for Ts65Dn P30 mouse brain using SAGE (serial analysis of gene expression) (5). Out of 45 856 unique tags in the mouse brain transcriptome 330 showed significant differences between Ts65Dn and normal mice. Fourteen of these genes encode ribosomal proteins, and it was suggested that abnormal ribosomal biogenesis was occurring. Possible increases in expression of three genes (Ifnar2, Ifngr2...
<table>
<thead>
<tr>
<th>Studies</th>
<th>Materials</th>
<th>Tissues</th>
<th>Sample type</th>
<th>Methods</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bahn et al.  (2)</td>
<td>Human, DS patients</td>
<td>Neuronal stem cells and progenitor cells</td>
<td>Non-pooled</td>
<td>Differential display</td>
<td>Down-regulation of REST-regulated genes, not on HSA21, in DS patients</td>
</tr>
<tr>
<td>FitzPatrick et al. (3)</td>
<td>Human, DS patients</td>
<td>Fetal amniocyte primary culture</td>
<td>Pooled</td>
<td>cDNA microarray (9128 cDNAs)</td>
<td>Up-regulation of genes on HSA21 in DS patients was observed, but 10 of the most up-regulated genes were not on HSA21</td>
</tr>
<tr>
<td>Cheon et al. (23–26)</td>
<td>Human, DS patients</td>
<td>Fetal cerebral cortex</td>
<td>Non-pooled</td>
<td>Western blotting</td>
<td>Unchanged levels of proteins encoded by the triplicated genes in DS patients</td>
</tr>
<tr>
<td>Mao et al. (4)</td>
<td>Human, DS patients</td>
<td>Fetal cerebral cortex, astrocyte cell lines</td>
<td>Non-pooled</td>
<td>GeneChip (1 2652 or 22 283 probe sets)</td>
<td>Specific up-regulation of genes on HSA21 in DS patients</td>
</tr>
<tr>
<td>Chrast et al. (5)</td>
<td>Mouse, Ts65Dn</td>
<td>Whole brain and spleen (P30)</td>
<td>Pooled</td>
<td>SAGE (45 856 unique tags)</td>
<td>Misregulation of 330 genes including those encoding ribosomal proteins. Expression levels of triplicated genes were too low to achieve statistical significance</td>
</tr>
<tr>
<td>Saran et al. (6)</td>
<td>Mouse, Ts65Dn</td>
<td>Cerebellum (3- to 4-month-old)</td>
<td>Pooled and non-pooled</td>
<td>GeneChip (1 2488 probe sets)</td>
<td>Up-regulation of genes on the trisomic region was observed, but the expressions of 7000 genes throughout the genome was also significantly perturbed</td>
</tr>
<tr>
<td>Present study</td>
<td>Mouse, Ts1Cje</td>
<td>Whole brain (P0)</td>
<td>Non-pooled</td>
<td>GeneChip (2 4935 probe sets)</td>
<td>Dosage-dependent up-regulation of genes on the trisomic region. No significant perturbation of expression levels for genes on euploid regions</td>
</tr>
</tbody>
</table>

In this report we have described the specific ~1.5-fold over-expression of gene expression in cerebellum of 3- to 4-month-old Ts65Dn mice. Therefore, the elevated expression of the triplicated genes in cerebellum was reported using the microarray technique, with a mean increase in expression of 1.45-fold. However, the expression of almost all of the expressed genes and ESTs on other chromosomes used and/or species. Whereas our study focused on samples, triplicated segments studied, tissues analyzed, methodologies used, and species.

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**Genotyping**

Because the trisomic segment of MMU16 contains a mutated Sod1 gene disrupted by a neomycin resistance sequence, screening of mice was performed by using multiplex PCR with a primer pair of Neo3: 5’ CTACACCTTGCTCTGGCCGAG 3’ and Neo4: 5’ CTCATGCTCTGTCCTCAGATCATC 3’, with a pair of primers Grik1F2: 5’ CCCCTTAGCAATACGACCGAG 3’ and Grik1R2: 5’ GGACCGAGACAGACT-GAG 3’ as internal controls. PCR was performed using the following reaction conditions; ‘hot start’ followed by 30 cycles of 94°C for 45 s, 55°C for 60 s in a 25 μl reaction mixture containing DNA (50–100 ng), 0.2 mM dNTPs, 0.5 μM of each primer, 1× Expand™ High-Fidelity buffer (with 1.5 mM MgCl2) and 0.875 U Expand™ High-Fidelity System DNA polymerase mixture (Roche Diagnostics Corp., Indianapolis, IN, USA). PCR reactions were performed in a DNA Engine Peltier Thermal Cycler (PTC-200; MJ Research, Inc., Waltham, MA, USA).

**Sample preparation and GeneChip hybridization**

Total RNA was extracted from each mouse brain separately using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). mRNA was isolated with a μMACS mRNA Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and converted to double-strand cDNA (Superscript Choice System; Invitrogen Corp.) and then to biotinylated cRNA (BioArray High Yield RNA Transcription Labeling Kit; Enzo Biochem, Inc., Farmingdale, NY, USA). After fragmentation and quality confirmation with the Affymetrix Test-2 Array, 20 μg of the biotinylated cRNA were hybridized to Affymetrix Murine Genome U74A and U74B GeneChips (24 935 probe sets) (Affymetrix, Inc., Santa Clara, CA, USA). The chips were washed, stained with streptavidin–phycoerythrin and scanned with a probe array scanner (HP GeneArray Scanner, Hewlett-Packard Company, Palo Alto, CA, USA).

**GeneChip data analysis**

Data were analyzed with Affymetrix Microarray Suite 5.0 software (Affymetrix, Inc.) and GeneSpring 5.0 software (Silicon Genetics, Redwood City, CA, USA). The Suite 5.0 software uses the one-sided Wilcoxon’s signed-rank test to generate a detection P-value and a detection call to decide statistically whether a transcript is expressed on a chip. The software generates the detection call based on the detection P-value for each transcript: present (P < 0.04), marginal (0.04 < P < 0.06) or absent (P > 0.06). On each chip, the mouse housekeeping genes β-actin, Gapdh, transferrin receptor, pyruvate carboxylase and 18S rRNA served as controls. When comparing the data, the Suite 5.0 software normalized the values of expression levels using all of these controls. Statistical comparisons of expression levels between Ts1Cje and 2N mice were performed by using the Mann–Whitney U-test. After normalization, the software scaled the data for each chip and then generated a change P-value, a change call and a signal log ratio using Wilcoxon’s signed-rank test. During the comparison analysis, each probe set on the experiment array was compared with its counterpart on the baseline array to calculate the change P-value that was used to generate the change call of increase (P < 0.0025), marginal increase (0.0025 < P < 0.003), decrease (P > 0.9975), marginal decrease (0.997 < P < 0.9975) or no change (0.003 < P < 0.997). Fold changes were calculated using the formula: \( \frac{\text{signal log ratio}}{P} \).

PCA was performed with the normalized raw data using GeneSpring 5.0 software. The raw data generated by the Affymetrix Microarray Suite 5.0 software were exported to GeneSpring 5.0 software and were then normalized using the default settings for the parameters according to the manufacture’s recommendations.

**Quantitative PCR analysis**

Total RNA was extracted from each mouse brain separately using Trizol reagent. cDNA synthesis was performed in triplicate. For each 20 μl reverse transcription reaction, 1 μg of total RNA was mixed with 0.5 μg of oligo(dT)12–18 primer (Invitrogen Corp.) and incubated for 5 min at 65°C. After cooling on ice, the solution was mixed with 1× first strand buffer, 0.1 mM dithiothreitol, 0.25 mM dNTPs, 40 U of RNaseOUT (Invitrogen Corp.) and 200 U of SuperScript II reverse transcriptase (Invitrogen Corp.). Reactions were performed for 60 min at 42°C and terminated by incubating for 15 min at 70°C. TaqMan MGB probes and PCR primers for 13 target genes (Atp3cl, Prkcb, App, Donson, Mrps6, Tc3, Pcp4, Ifnar2, Il10rb, Ifnar1, Dyrk1a, Hmgn1 and Prdm15) (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA, USA) were used. Values of Gapdh were used for normalization of data. Gapdh showed 1.02-fold change in Ts1Cje compared with 2N mice when normalized to total amount of RNA. TaqMan PCR assays for each gene target on four Ts1Cje and four 2N mice were performed in triplicate on each cDNA sample in 96-well optical plates on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). For each 50 μl TaqMan reaction, 2 μl of cDNA was mixed with 20.5 μl of PCR-grade water, 25 μl of 2× TaqMan Universal PCR Master Mix, No AmpErase UNG and 2.5 μl of 20× Assays-on-Demand Gene Expression Assay Mix. PCR was performed using the following reaction conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

All TaqMan PCR data were captured using Sequence Detector Software (SDS version 1.7; Applied Biosystems). A standard curve was plotted for each gene by using one 2N sample, and the mean threshold cycle (Ct) for each of the four Ts1Cje and the remaining three 2N mouse samples was expressed as an arbitrary value relative to the standard. The arbitrary values were divided by the corresponding value for Gapdh and expressed as a ratio.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
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