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Transcriptomic Analysis of Cell-Free Fetal RNA Suggests a Specific Molecular Phenotype in Trisomy 18

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Abstract

Background—Trisomy 18 is a common human aneuploidy that is associated with significant perinatal mortality. Unlike the well-characterized “critical region” in trisomy 21 (21q22), there is no corresponding region on chromosome 18 associated with its pathogenesis. The high morbidity and mortality of affected individuals has limited extensive investigations.

Objective—To better understand the molecular mechanisms underlying the congenital anomalies observed in this condition, we investigated the *in utero* gene expression profile of second trimester fetuses affected with trisomy 18.

Methods—Total RNA was extracted from cell-free amniotic fluid supernatant from aneuploid fetuses and euploid controls matched for gestational age and hybridized to Affymetrix U133 Plus 2.0 arrays. Individual differentially-expressed transcripts were obtained by two-tailed *t*-tests. Over-represented functional pathways among these genes were identified with DAVID and Ingenuity® Pathways Analysis.

Results—Three hundred fifty-two probe sets representing 251 annotated genes were statistically significantly differentially expressed between trisomy 18 and controls. Only 7 genes (2.8% of the annotated total) were located on chromosome 18, including *ROCK1*, an up-regulated gene involved in valvuloseptal and endocardial cushion formation. Pathway analysis indicated disrupted function in ion transport, MHCII/T-cell mediated immunity, DNA repair, G-protein mediated signaling, kinases, and glycosylation. Significant down-regulation of genes involved in adrenal development was identified, which may explain both the abnormal maternal serum estriols and the pre and postnatal growth restriction in trisomy 18. Comparison of this gene set to one previously generated for trisomy 21 fetuses revealed only six overlapping differentially-regulated genes.

Conclusion—This study contributes novel information regarding functional developmental gene expression differences in fetuses with trisomy 18.

Keywords

amniotic fluid; trisomy 18; transcriptome

INTRODUCTION

Trisomy 18 (Edwards syndrome) is a common human aneuploidy, with a fetal prevalence of 1 in 4272 at 18 weeks and a live born prevalence of 1 in 8333 (Embleton et al. 1996). Well-described clinical characteristics of trisomy 18 include severe psychomotor and growth restriction, microcephaly, microphthalmia, micrognathia, cardiac malformations, pulmonary hypoplasia, omphalocele, ileal atresia, and adrenal hypoplasia (Edwards et al. 1960; Smith et al. 1960; Turner and O’Herlihy 1984).

Trisomy 18 is associated with significant perinatal mortality. Approximately three-quarters of fetuses diagnosed with trisomy 18 are miscarried or stillborn between 12 weeks of gestation and term (Morris and Savva 2008). Postnatally, the median survival time is 3–6 days. Less than 50% of infants will survive for a week, and only about 5–10% will survive for a year (Rasmussen et al. 2003; Baty et al. 1994). The high mortality rate is typically due to the presence of cardiac and renal malformations, feeding difficulties that result in aspiration pneumonia, sepsis, and apnea caused by central nervous system abnormalities. Long-term survival in trisomy 18 has been reported, but this occurs primarily in the context of mosaicism (Kohn et al. 1987; Bettio et al. 2003). Interestingly, in mosaic individuals there is no correlation between the physical and intellectual findings and the percentage of trisomic cells found in either peripheral leukocytes or skin fibroblasts (Mewar et al. 1993).

Unlike the well-characterized “critical region” in trisomy 21 (21q22) (Shapiro 1997; Korenberg et al. 1990), there is no corresponding region on chromosome 18 that is associated with Edwards syndrome. The high morbidity and mortality of affected individuals limits extensive investigation into its pathogenesis. Furthermore, studies of partially trisomic individuals performed to determine if a critical region is present in this syndrome have produced inconsistent results. For example, a case report of a patient described as having a typical full trisomy 18 phenotype showed that this individual had a duplication of 18q21.1-qter (Matsuoka et al. 1981). Another study described patients with duplications of flanking regions (i.e., 18q12.3-qter and 18q21.2-qter) who did not manifest the typical trisomy 18 phenotype (Turleau and de Grouchy 1977). A clinical, cytogenetic, and molecular study of four individuals with partial duplications of the long arm of chromosome 18 concluded that either there is more than one critical region for trisomy 18, that there is no single contiguous critical region, or that the characteristic trisomy 18 phenotype could result from the additive effect of trisomy for multiple, potentially interacting segments of 18q (Mewar et al. 1993).

In a prior study utilizing global transcriptional profiling of RNA isolated from residual amniotic fluid (AF) supernatant samples, we demonstrated that widespread transcriptional disruption is present in second trimester fetuses with Down syndrome, as compared to gestational age and gender-matched controls (Slonim et al. 2009). In the current study, using a similar approach, we investigated the *in utero* gene expression profile associated with trisomy 18 to better understand the molecular mechanisms underlying the congenital anomalies observed in this condition.

PATIENTS AND METHODS

Patients

The Institutional Review Boards at Tufts Medical Center and Women and Infants’ Hospital approved the study. Residual second trimester AF supernatant samples were obtained from women carrying singleton fetuses undergoing antenatal genetic testing for routine clinical indications. All samples were anonymized before receipt in the research laboratory, although the karyotype results and gestational ages were known. AF was stored at –80°C until RNA

extraction. The study set consisted of AF samples with the following confirmed metaphase karyotypes: 47, XX,+18 (n=5) and 46, XX (n=6). The gestational ages of the samples ranged from 17 5/7 to 20 6/7 weeks.

RNA extraction

Total RNA was extracted from 10 mL AF supernatant with 30 mL TRIzol LS Reagent (Invitrogen, Carlsbad, CA) and 8 mL chloroform. After RNA extraction, RNA was purified and DNA was removed using the RNeasy® Maxi Kit, including the DNase step, according to the manufacturer's protocol (QIAGEN, Valencia, CA). RNA was precipitated using 3M NaOAc and 100% ethanol, and 80% ethanol was added after 4 hours incubation at -20°C. cDNA, synthesized from extracted RNA, was amplified and purified using the WT-Ovation™ Pico RNA Amplification System (NuGEN, San Carlos, CA) and the DNA Clean & Concentrator™-25 (Zymo Research, Orange, CA). The quality and quantity of amplified cDNA was measured on the Agilent Bioanalyzer 2100 Expert software (Agilent, Foster City, CA) with the RNA 6000 Nano kit (Agilent).

Fragmentation, labeling, and hybridization

The FL-Ovation™ cDNA Biotin Module V2 (NuGEN) was used to obtain fragmented and labeled cDNA (minimum 5 µg) that was suitable for hybridization to Affymetrix U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Arrays were washed, stained with streptavidin-phycoerythrin, scanned with the GeneArray Scanner, and analyzed using the GeneChip Microarray Suite 5.0 (Affymetrix). Array quality was assessed in R (version 2.7.2) using the simpleaffy package in BioConductor (version 1.7; www.bioconductor.org).

Data analysis and statistics

Normalization was performed with the three step command from the AffyPLM package in BioConductor, using ideal-mismatch background/signal adjustment, quantile normalization, and the Tukey biweight summary method (Gentleman et al. 2005). This summary method included a logarithmic transformation, which improved the normality of the data. Identification of individual differentially-expressed probe sets (comparing trisomy 18 to euploid samples) was performed via two-sided *t*-tests using the multtest package in BioConductor, with the Benjamini-Hochberg false-discovery rate (FDR) adjustment for multiple testing (Benjamini and Hochberg 1995).

Hierarchical clustering was performed in R, using complete-linkage hierarchical clustering (the hclust function in the stats package), and heatmaps created via the heatmap.2 function (with row z-score normalization) in the gplots package.

Functional analysis of the statistically-significantly differentially-regulated probe sets was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) toolkit (Dennis et al. 2003; Huang et al. 2009), with the Panther functional annotation classes (Thomas et al. 2003) in addition to the default pathway selections, which include Gene Ontology (GO) terms (Ashburner et al. 2000), pathways defined from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000) and BioCarta (www.biocarta.com) databases, InterPro protein families (Apweiler et al. 2000), and Protein Information Resource keywords (Barker et al. 2000).

The differentially-expressed Affymetrix probe set identifiers and corresponding Benjamini-Hochberg-adjusted probability values were uploaded into Ingenuity Pathways Analysis (IPA) software, version 7.5 (Ingenuity® Systems, www.ingenuity.com) to identify the networks, diseases and disorders associated with the differentially expressed genes. The probe sets were mapped to the corresponding IPA gene objects. These “focus” genes were

combined with Ingenuity's proprietary data and knowledge base to construct a molecular network. We also used an IPA tool called "Canonical Pathways Analysis" to identify the most statistically significantly over-represented pathways from the IPA pathway library in the data set (by Fischer's exact test). Graphical network models of some of the implicated pathways were generated by the IPA software, using literature-based pathway links to connect the focus genes and other genes in the knowledge base.

Finally, differential expression and functional enrichment were simultaneously assessed using Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005; Mootha et al. 2003), software that can identify consistent differential expression of pre-defined sets of genes even if the differential expression of each individual gene is not statistically significant by itself. We ran GSEA with 500 permutations per run and with the gene-set permutation method recommended for data sets with fewer than 7 samples per class (Subramanian et al. 2005). Gene collections assessed in our GSEA experiments included groups of genes from the same chromosomal band and those with the same GO molecular function keywords (collections c1 and c5 from the Molecular Signature Database [MSigDB]) (Subramanian et al. 2005), as well as a collection of 10 gene sets related to developmental processes that we mined from published gene expression data using techniques described elsewhere (Turcan et al. 2010).

RESULTS

Differential expression in trisomy 18 vs. controls

We initially identified 356 probe sets, which corresponded to 253 annotated genes that were statistically-significantly differentially expressed between the trisomy 18 and euploid samples (adjusted p-value < 0.05). However, in two cases two probe sets representing one gene (*NUMA1* and *KIAA1219*) showed conflicting results (i.e. both up- and down-regulation); these data were excluded from further analysis. Therefore, a total of 352 probe sets, representing 251 annotated genes, were included for bioinformatic analysis. We called this the "Identified Gene Set" (Supplemental Table 1). Only 7 genes (2.8% of the annotated total) were located on chromosome 18. These included *LOC339290*, *ROCK1*, *ATP8B1*, *MGC11082*, *ZNF516*, *EMILIN2* and *ONECUT2*. Twenty three genes (9.2% of the annotated total) were located on chromosome 19, which was the chromosome with the largest number of individual differentially regulated genes. Of the 251 annotated genes, 171 (68.1%) were up-regulated and 80 (31.9%) were down-regulated in trisomy 18 compared to controls. Cluster analysis of the Identified Gene Set, even excluding the few genes on chromosome 18, was sufficient to separate the euploid and trisomic samples (Figure 1). In the interest of including all statistically significant gene expression differences, we did not perform any filtering of genes with low expression and/or low variance. We have identified those genes (39 of 251 annotated genes) with expression levels in the lowest decile among all of the microarray data (Supplemental Table 1, shown in bold).

We compared the Identified gene set in trisomy 18 to the group of 414 probe sets representing 311 annotated genes in our prior study of trisomy 21 (Slonim et al. 2009). We found that only six of the annotated genes were differentially-regulated in both aneuploidies compared to normal controls (Figure 2). Of these, four (*NKX1-1*, *CCDC85B*, *TLL12*, and *ROCK1*) were up-regulated, and two (*CYP2C9* and *SLC25A33*) were down-regulated in both trisomies 21 and 18.

Functional analysis of differentially expressed genes

Pathway analysis of the Identified Gene Set was performed using DAVID. Using a strict significance cutoff adjusted for multiple testing (FDR < 0.05), we found 28 functions over-

represented in the Identified Gene Set (Table 1). The functions disrupted in trisomy 18 include ion and cation transport, MHCII/T-cell mediated immunity, DNA repair, G-protein mediated signaling, kinases, and glycosylation. In addition, DAVID's clustering function highlighted groups of related gene sets that were differentially regulated; these included cytoskeletal and cell adhesion proteins, zinc finger proteins, notch signaling, immunoglobulin-like proteins, and neurological development.

To further characterize the relationships between differentially-regulated genes, we used IPA to examine the 80 down-regulated genes and the 171 up-regulated genes. For down-regulated genes, all associated network functions that included more than one focus gene are shown in Table 2. The top associated network among the down-regulated genes involved "organismal function," which included pathways associated with adrenal gland development. Other network functions included cardiovascular and respiratory system development, function, and disease. Regarding adrenal development, the down-regulated gene network included 35 genes, 17 of them focus genes (Figure 3). Further characterization of these 17 focus genes identified 8 genes related to canonical pathways with functions that include signaling for corticotropin releasing hormone (CRH), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin like growth factor-one (IGF-1); all are required for normal morphological and functional development of the fetal adrenal cortical zone after 15 gestational weeks (Kempná and Flück 2008).

For up-regulated genes, all associated network functions that included more than one focus gene are shown in Table 3. The most significantly associated network identified among the up-regulated genes was comprised of 35 genes, of which 22 were focus genes. Further functional analysis of these 35 genes identified pathways related to cardiovascular development, function, and disease, although IPA did not categorize this as one of the "top" functions associated with these genes (Table 3). Rather, these network functions were identified by IPA as Endocrine Function, Lipid Metabolism and Molecular Transport. Nevertheless, *ROCK1*, a gene that is known to be involved in cardiac development and was statistically significantly up-regulated in both trisomies 18 and 21 was included in this network. Other networks identified by IPA included immunological and neurological system development, function, and disease (Table 3).

Differentially expressed gene sets

Further consideration of the expression variation of pre-defined sets of genes can help to detect consistent expression changes that might be missed by a gene-by-gene analytic approach. Accordingly, we used the GSEA package to look for differential expression of gene sets corresponding to each chromosomal band (Subramanian et al. 2005). The only significantly up-regulated set of genes (with an FDR q-value < 0.05) was the set of genes from chromosomal band 18q22 (q < 0.033). The next two most-significantly up-regulated gene sets were from two other bands on chromosome 18, 18p11 (q < 0.085), and 18q11, (q < 0.216).

In addition, we looked for dysregulation of gene sets from the molecular function gene set collection (c5 from MSigDB), which contains sets of genes with the same GO molecular function key words. Although none of these gene sets was over-represented with FDR q-value below 0.05, several were up-regulated in the trisomy 18 samples with q-values between 0.10 and 0.25. Such q-values are still of potential interest (Subramanian et al. 2005). Among these were several groups related to neurotransmitters (including G protein coupled receptors and sodium channel activity) and cytokine/chemokine activity.

Finally, we looked for differential expression in a collection of ten gene sets that we had previously mined from public data with the specific goal of better characterizing fetal

developmental processes. We found that one of these ten gene sets was significantly up-regulated in the trisomy 18 samples, with an FDR q-value below 0.002. Since little is known about the functional roles of the genes in this set, we selected the 79 genes (Supplemental Table 2) in its “leading edge” gene set (the genes that contributed the most to the enrichment statistic calculated by the GSEA algorithm) (Subramanian et al. 2005), nine of which had expression levels in the lowest decile among all data, and performed a functional analysis of these genes in DAVID using the methods described earlier. Significantly enriched functions in this differentially-expressed developmental gene set include lipid, fatty acid and steroid metabolism and signal transduction.

DISCUSSION

Differential gene expression analysis

In this study we used cell-free fetal RNA in AF supernatant to perform developmental gene expression analysis in second trimester fetuses with trisomy 18, and compared the results to euploid fetuses matched for gestational age and gender. The results showed a consistent phenotypic pattern common to all affected fetuses, without much individual variation, and they clearly differed from the normal second trimester fetuses. The data demonstrated that only 2.8% of the differentially-regulated annotated genes were physically located on chromosome 18. Using a different tissue (first trimester chorionic villi), Altug-Teber et al. (2007) also showed that in fetuses with trisomy 18 only 36 of 628 differentially-regulated genes (5.7%) mapped to chromosome 18. Thus, there are many downstream molecular effects of trisomy 18, as evidenced by differential expression of genes from chromosomes other than 18. Overall, only five of the 251 differentially regulated annotated genes in our study were shared with that of Altug-Teber et al. (2007); histone H3b (*H3F3B*), nuclear factor I/B (*NFIB*), protein phosphatase 1 (*PPP1R7*), and two hypothetical proteins. This lack of overlap between the gene lists from the two studies is most likely a reflection of the different biological material used, as their analysis was of RNA isolated from amnion and chorionic villus cells in culture, while ours was of RNA from AF supernatant, which is hypothesized to represent a variety of fetal tissue types (Hui and Bianchi 2010). Indeed, we suggest that transcriptomic analysis of the supernatant fraction of AF is more appropriate and valuable for the assessment of fetal development than the cellular fraction due to the representation of various organ systems (e.g. respiratory, gastrointestinal, urinary, skin) in the cell-free component of AF (Hui and Bianchi 2010). It also has an advantage over other maternal body fluids, specifically plasma or serum, for monitoring fetal development in that the RNA in AF supernatant is exclusively fetal in origin.

These results have both similarities to and differences from those of a prior study in which we analyzed gene expression in cell-free AF from fetuses with trisomy 21 (Slonim et al. 2009). In that study, only five of over three hundred individually differentially-expressed annotated genes (1.6%) were located on chromosome 21, but GSEA also implicated coordinated up-regulation of gene sets from the aneuploid chromosome. The specific differentially-regulated genes identified for trisomies 18 and 21, however, were largely different from each other. The fact that each aneuploidy had hundreds of non-overlapping differentially regulated genes (as compared to euploid fetuses) suggests that each aneuploidy has a specific unique molecular phenotype. Interestingly, while only six annotated genes were differentially regulated in both trisomy 18 and 21 compared to controls, the expression differences for these genes were in the same direction for both aneuploidies. It is unclear at present whether this represents random chance or common mechanisms. However, at least one of these genes (*ROCK1*, discussed below) is involved the development of an organ system (i.e. cardiovascular) that is abnormal in both aneuploidies. The possibility of gene expression changes representing common mechanisms between different genetic anomalies warrants further investigation.

Pathway and functional analyses

Endocrine system—IPA specifically implicated adrenal gland development among the down-regulated pathways in the trisomy 18 samples. Gross and microscopic hypoplasia of the adrenal cortical zone has previously been reported in autopsy studies of fetuses with trisomy 18 (Turner and O’Herlihy 1984). This study suggested that adrenal cortical hypofunction is a frequent, but generally underappreciated, feature of trisomy 18. In the first trimester, fetal adrenal growth is independent of adrenocorticotrophic hormone (ACTH). After 15 weeks, while ACTH is required for normal morphological and functional development, other factors of fetal and/or placental origin are also needed (Langlois et al. 2002). These include fibroblast growth factor (FGF), epidermal growth factor (EGF), and insulin like growth factor-one (IGF-1), which were all implicated by the IPA network analysis as down-regulated in the trisomy 18 samples. We suggest, therefore, that abnormal gene expression related to the adrenal network may contribute to the growth restriction that is known to be an antenatal and postnatal feature of trisomy 18. Furthermore, this may explain the abnormal maternal serum estriol levels that are found in this condition. Unfortunately, due to patient confidentiality issues, we only had karyotype information but no clinical follow-up on study subjects. Thus, we were unable to examine the adrenal glands from fetuses with trisomy 18 in the present study, but we propose such analysis as future work.

It was intriguing to discover that in addition to the genes described above that were down-regulated and known to be associated with adrenal gland development, other genes involved in endocrine system development and function were found to be up-regulated in trisomy 18. These results may suggest an overall dysregulation of gene expression involving affected organ systems in trisomy 18. However, further investigation is needed to determine how different genes within a specific pathway or organ system interact (e.g. if gene expression levels are affected by feedback mechanisms).

Cardiovascular system—The IPA network of 35 up-regulated genes highlighted pathways related to cardiovascular disease. Specifically, Rho-associated kinase 1 (*ROCK1*) was both significantly up-regulated and located on chromosome 18. ROCKs, the immediate downstream targets of RhoA, consist of two isoforms, and are ubiquitously expressed serine-threonine protein kinases involved in diverse cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, and gene expression (Ichizaki et al. 1996; Leung et al. 1985; Matsui et al. 1996). During early heart development, endothelial cells in the outflow tract and atrioventricular regions transform into mesenchyme that ultimately becomes endocardial cushion tissue. *ROCK1* plays an important role in the regulation of endocardial cell differentiation and migration (Zhao and Rivkees 2004; Sakabe et al. 2006). Specifically, *ROCK1* is up-regulated at the onset of the epithelial-mesenchyme transition phase in the heart (Sakabe et al. 2008). The fact that *ROCK1* is up-regulated in both trisomies 18 and 21, and that both valvular and endocardial cushion abnormalities are found in these conditions, is intriguing and suggests a common mechanism. Furthermore, a PubMed search indicated that *ROCK1* has never previously been associated with trisomy 18 in the literature. This highlights one of the benefits of our discovery-driven approach.

Lipid metabolism—Given that fetuses and neonates with trisomy 18 have been demonstrated to have abnormally low amniotic fluid and plasma cholesterol levels (Lam et al. 2006), it was surprising to find that a developmental gene set involved in lipid, fatty acid, and steroid metabolism was up-regulated in the aneuploid fetuses. We speculate that there may be a feedback mechanism that relates the low cholesterol levels to up-regulation of specific genes. Hypocholesterolemia is a feature of Smith-Lemli-Opitz syndrome (Irons et

al. 1993), a condition that shares phenotypic features with trisomy 18 such as growth restriction, developmental delay, and under-masculinization of male fetuses.

Immune regulation—Gene sets implicated in T-cell mediated immunity were shown to be highly differentially expressed between the trisomy 18 samples and the controls. The immunological consequences of trisomy 18 are substantial, as infection is a common cause of morbidity in long-term survivors (Van Dyke and Allen 1990). Trisomy 18 fetuses have significantly lower mean T-cell and natural killer cell counts than euploid fetuses, although the mean B-cell count is not significantly affected (Makrydimas et al. 1994). The identification of specific differentially expressed gene sets related to immune function in this population might facilitate discovery of the proximate causes of the reduced immune function associated with trisomy 18. In time this might lead to better clinical management of infections and pneumonia in surviving patients.

Central nervous system—Long-term survivors with trisomy 18 have severe developmental delay (Baty et al. 1994). A number of neurological developmental pathways were implicated in our analysis, suggesting possible mechanisms for the disruption of neurological development. One possibility is that dysregulation of ion transport proteins due to the downstream effects of increased gene dosage on chromosome 18 interferes with the cells' ability to manage oxidative stress, with consequences for neurological development (Kourie 1998).

CONCLUSIONS

In summary, the present study contributes novel information regarding developmental gene expression differences between second trimester fetuses affected with trisomy 18 and matched euploid controls. Most of the specific transcriptional effects of trisomy 18 identified were due to dysregulated genes on chromosomes other than 18. This “downstream effect” is consistent with prior studies of transcriptional differences in both chorionic villi in fetuses with trisomy 18 and cell-free amniotic fluid supernatant in fetuses with trisomy 21. Further analyses of these results may lead to a new understanding of the molecular aspects of both the trisomy 18 phenotype as well the isolated congenital anomalies that are observed in trisomy 18 but can also present in euploid fetuses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Heatmap showing hierarchical clustering of control (C1–C6; solid red bar) and trisomic (T1–T5; solid blue bar) samples. Clustering is based on data from 345 probe sets: the 352 from the Individual Gene Set, minus the 7 from chromosome 18. If these 7 are not removed, the dendrogram looks quite similar; in particular, it similarly separates the control and trisomic samples. Expression values in each row are z-score normalized.

Figure 2.

A Venn diagram depicting the number of differentially regulated genes that overlap between trisomies 18 and 21.

Figure 3.

Network associated with adrenal gland development as generated by Ingenuity® Pathways Analysis (IPA). Focus genes found to be down-regulated in trisomy 18 are shaded gray. Genes related to canonical pathways whose functions include corticotropin releasing hormone signaling and EGF, FGF, and IGF-1 signaling are outlined in orange.

Table 1

Pathways identified by DAVID as significantly over-represented in trisomy 18

Category	Term	Number of probes	p-value ^a
PANTHER_BP_ALL	BP00142:Ion transport	53	< 0.0001
PANTHER_BP_ALL	BP00143:Cation transport	74	0.00049
PANTHER_BP_ALL	BP00036:DNA repair	33	0.0011
PANTHER_BP_ALL	BP00033:Pyrimidine metabolism	14	0.0014
PANTHER_MF_ALL	MF00239:Phosphatase inhibitor	17	0.0023
PANTHER_BP_ALL	BP00064:Protein phosphorylation	43	0.0086
PANTHER_BP_ALL	BP00063:Protein modification	50	0.0093
PANTHER_BP_ALL	BP00286:Cell structure	56	0.010
PANTHER_BP_ALL	BP00151:MHCII-mediated immunity	50	0.011
PANTHER_BP_ALL	BP00044:mRNA transcription regulation	131	0.011
PANTHER_BP_ALL	BP00040:mRNA transcription	91	0.012
PANTHER_BP_ALL	BP00104:G-protein mediated signaling	61	0.013
PANTHER_BP_ALL	BP00102:Signal transduction	64	0.013
PANTHER_BP_ALL	BP00079:Sulfur redox metabolism	8	0.013
PANTHER_BP_ALL	BP00144:Anion transport	18	0.013
PANTHER_BP_ALL	BP00132:Receptor mediated endocytosis	14	0.014
PANTHER_MF_ALL	MF00108:Protein kinase	35	0.014
PANTHER_MF_ALL	MF00107:Kinase	19	0.015
PANTHER_BP_ALL	BP00246:Ectoderm development	15	0.017
PANTHER_BP_ALL	BP00193:Developmental processes	36	0.017
PANTHER_BP_ALL	BP00119:Other intracellular signaling cascade	20	0.018
PANTHER_BP_ALL	BP00149:T-cell mediated immunity	60	0.020
PANTHER_BP_ALL	BP00067:Protein glycosylation	32	0.023
PANTHER_BP_ALL	BP00014:Amino acid biosynthesis	15	0.027
PANTHER_BP_ALL	BP00034:DNA metabolism	19	0.029
PANTHER_BP_ALL	BP00273:Chromatin packaging and remodeling	20	0.031
SP_PIR_KEYWORDS	alternative splicing	107	0.011
SP_PIR_KEYWORDS	phosphoprotein	91	0.048

^aBenjamini-Hochberg adjusted probability values

DAVID: Database for Annotation, Visualization, and Integrated Discovery

Table 2

Ingenuity® Pathways Analysis (IPA) networks associated with down-regulated genes in trisomy 18

Network	Genes in Network ^a	Focus Genes	Top Functions
1	Akt, ASF1B , ATP5G2 , CASQ1, CD9 , Ck2, CSNK2A1 , CSPG5, CTBP2 , CYBB (includes EG:1536), CYP2C9 , DLC1 , EMP1 , FSH, GNAI3 , H3F3A (includes EG:3020), HEMC (includes EG:7909), IF2, IgG, Insulin, KEL, KHDRBS1 , KNCN, LDL, LDLR , MYH13, NEO1 , NRF1 , PI3K, PLC, SEC14L2, STON1-GTF2A1L, SULF1 , TLK1 , XK	17	Organismal Functions, Organismal Injury and Abnormalities, Cardiovascular Disease
2	ABHD6, ADSS, AGT, APEH, ATF6B, C3ORF15 , C8ORF4, CKAP4 , CLDN2 , CLDN3, CLDN12, COX7A2, COX7C (includes EG:1350), CPA1, GAP43, GNAO1, HNF4A, ID3, IKG, KIF26A , MIPEP, NEUROD2 , NKTR , NLN, NME7, Pka, PKLR , PSMD7 , REXO2, RGS11 , SPCS3, TCF7L2 (includes EG:6934), VPS29, ZFHX3 , ZNF3	14	Cancer, Respiratory Disease, Amino Acid Metabolism
3	ATF2-JUN, BCL2, beta-estradiol, CASP3, CSF2, CTTN, dihydrotestosterone, EGFBP2, EIF4G3 , GPD1 , ITPR3 , Jnk, KIAA1219, KLK15, MAPK9, MINK1 , MIR296, MIRHG2, MKK1/4, MKP2/5, NCR1 , NMT2, NUMA1, PADI1 , PANK2 , PGLYRP1, PLXDC1, PTPRD , RET, RNF40 , SDK1 , SRC, TIAM1 , TMCC1 , YWHAZ	12	Cell Death, Gene Expression, Cancer
4	AFF4 , AP1P, ARL17P1 , C11ORF30 , C11ORF54 , C9ORF5 , CBX5, CHD1L , DCLRE1B, DSN1, FCAMR , FEM1C, GCKR, HNF4A, HOOK3 , HSDL2 , INCENP, LZTS2, MAP2K5, MIR214, MIS12, MTRF1 , NAV1 , NSL1, PRR14, SPATA2, SRP68, TAOK3, TCEB3B, TRAF2, TRPC4AP, TTRAP, UBP1, USP2, ZNF516	12	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair
5	ALCAM, APBB1, ATF7IP, ATP5B, C10ORF140, CAMKK2 , CBLC, EGFR, EIF4E, EPHA3, EPHA7, EREG, HOXC10, INO80, KCNAB1, LAMA4, MACF1 , MIR17 (includes EG:406952), MIRLET7A1, NFIB , OTX2, PABPN1, PARP6 , PHYHIP, PLXNC1, PTPN2, REPS2, SENP3 , SLC25A5, SOCS5, TUSC2 , WAP, ZNF259, ZNF462, ZNF644	6	Tissue Development, Cancer, Cell Morphology

^aDown-regulated genes in trisomy 18 (focus genes) within each pathway in bold

Table 3

Ingenuity® Pathways Analysis (IPA) networks associated with up-regulated genes in trisomy 18

Network	Genes in Network ^a	Focus Genes	Top Functions
1	ADRA2B , ADRBK1 , Caspase, EPO , ERK, ESR1 , G alpha1, GLI2 , GNAS , ITPKB , KCNC2 , LZTS1 , MAML3 , MAP4K2 , Mapk, MAPKAPK3 , MKNK1 , MKNK2 , NCRNA00162 , NFkB (complex), NOTCH3 , NUMA1, P38 MAPK, PIAS4 , Pka, PP2A, Rac, Raf, ROCK1 , Sapk, SH3BP1 , SLCO1B1 , TMOD1 , TUBB1 , Tubulin	22	Endocrine System Development and Function, Lipid Metabolism, Molecular Transport
2	ABTB2 , BCL6, beta-estradiol, CHCHD8, CLCN4, CREB1, CRYGS , D-galactose, DNAH9 , IL28RA , INS1, LMX1A , LSM11 , MARCKS (includes EG:25603), MIDN, MIRLET7B, NFRKB, ONECUT1, PLEKHA3, PLEKHA4 , PLXNB2 , SEMA6A , SERPINA12, SEZ6, SLC26A6 , SLC28A1, SLC28A2, SLC8A1 , STAT5a/b, TBX2 , TNF, TXN2, UGT8 , UGT2B11, ZNF267	14	Drug Metabolism, Molecular Transport, Nucleic Acid Metabolism
3	ABCC2, ACTR5 , BRIP1, C2 , C2ORF43 , C9ORF86 , CATSPER1, CHIC2, EXO1, FKBP10, GSTCD, HNF4A, ILF3 , LRRC40 , MDFI, MOV10, MTMR2, NR1I3 , OGFOD2 , PARP4, PDZD3 , PMS1, PP1L1, PP1L2 , REPIN1 , RNF31, SBF2 , SLC35A2, SLC01A2, SULT1A1, UPF3B, USP5, WDR42A, XPR1 , ZNF764	13	Molecular Transport, Small Molecule Biochemistry, Drug Metabolism
4	Akt, Akt-Calmodulin-Hsp90-Nos3, Ap1, APOC4, ARAF , ATP2B2 , ATP2B3, CABP7 , Calmodulin, Calmodulin-Ca2+-CaMKII+Calmodulin-Ca2, Calmodulin-CaMKII-Ca2+, CAMK1G, CAMK2G , CAMK2N2, CLDND1, CREBZF , DYRK3, E2F4 , FSH, G3BP1 , GAS2L1, Histone h3, Interferon alpha, Jnk, MAN2A1 , MAP6, MIR124, MYO1B , NAP1L4, PCP4, PRKX, Ras, RGL4 , SIM1 , WIPF1	12	Cancer, Cellular Assembly and Organization, Immunological Disease
5	CCDC120 , CCDC85B , DUB, FN1, FOXA2, heparin, HNF1A, ITGA2B (includes EG:3674), MMP11 , ONECUT2 , PHF2 , SLC7A2 , SPEG (includes EG:10290), TGFBI, TSPB2 , USP17, USP25, USP26, USP29, USP30, USP32, USP35, USP36 , USP38, USP40, USP41, USP42, USP43, USP44, USP45, USP50, USP54, USP27X, USP37 (includes EG:57695), USP51 (includes EG:158880)	10	Endocrine System Development and Function, Organ Development, Amino Acid Metabolism
6	ACTB, ANAPC2 , APIP, ARRB1, BBS4 , CASP3, CHRN3 , DGKE, DGKG , DGKH, DHRS2 (includes EG:10202), dopamine, EMILIN2 , FTCD , GAS2, GPM6A , IL17A (includes EG:3605), IL17a dimer, IL17a-IL17f, IL17RA, IL17RC , LRP4, MICALL2 , MKL1, MKL2 , MT1A, OPHN1, PKP1, PLS1, SEPT4, SERPIND1, SRF, STK38L (includes EG:23012), UBB, VIM	10	Cellular Compromise, Skeletal and Muscular Disorders, Behavior
7	Adaptor protein 2, AP2S1, ARID2, CASKIN1 , CD320 , CDK4/6, GABAR-A, GABRA3, GABRA5, GABRE, GABRG1, GABRG3 , GABRP, GABRQ, GABRR1, GABRR2, GRPEL1, HSPB11 , IFITM3, KHK , LYZ, MYC, MYO9A, NPM1 (includes EG:4869), PBRM1 , PERP, RBL1, SCPEP1 , SIGLEC8 , SMARCA4, ST6GALNAC4 , STON2 , STRA13, UBR2, VHL	10	Neurological Disease, Developmental Disorder, Psychological Disorders
8	APLP1, APLP2, ATP1A2, ATP5J, CD96 , CDON, CHKA, CKAP5 , COX4NB , DAB1, DNM, ERI3 , FNTA, Has, HINT1, HRAS, Integrin $\alpha 5$ - β , Integrin alpha 4 beta 1, KIAA1219 , MIR188, NLGN2 , PCDH18 , PRNP, RALB, RASAL1, RASGRP3, RICS, RPL38 (includes EG:6169), SCTR , SHC2, SPON1 , SRC, TOM1, TSC22D1, TYRO3	9	Cancer, Developmental Disorder, Neurological Disease
9	CaMKII, CAV1, CCND3, CDK5, CDK5R1, CDK5RAP2 , EIF2AK2, EMP2 , GRK5, Gsk3, ITPR1, ITPR3, JARID1B, LMTK2, LRCH4 , LRRC68 (includes EG:284352), NEK2, PP1, PP1/PP2A, PPP1CA, PPP1CB, PPP1CC, PPP1R7 , PPP1R10, PPP1R11, PPP1R1A, PPP1R1B, PPP1R3A, PPP1R3C, PPP2CA, SAP25, STAU1, STX1A, TTL12	6	Carbohydrate Metabolism, Molecular Transport, Nervous System Development and Function

^aUp-regulated genes in trisomy 18 (focus genes) within each pathway in bold