

Use of Routinely Collected Amniotic Fluid for Whole-Genome Expression Analysis of Polygenic Disorders

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Background: Neural tube defects related to polygenic disorders are the second most common birth defects in the world, but no molecular biologic tests are available to analyze the genes involved in the pathomechanism of these disorders. We explored the use of routinely collected amniotic fluid to characterize the differential gene expression profiles of polygenic disorders.

Methods: We used oligonucleotide microarrays to analyze amniotic fluid samples obtained from pregnant women carrying fetuses with neural tube defects diagnosed during ultrasound examination. The control samples were obtained from pregnant women who underwent routine genetic amniocentesis because of advanced maternal age (>35 years). We also investigated specific folate-related genes because maternal periconceptional folic acid supplementation has been found to have a protective effect with respect to neural tube defects.

Results: Fetal mRNA from amniocytes was successfully isolated, amplified, labeled, and hybridized to whole-genome transcript arrays. We detected differential gene expression profiles between cases and controls. Highlighted genes such as *SLA*, *LST1*, and *BENE* might be important in the development of neural tube defects. None of the specific folate-related genes were in the top 100 associated transcripts.

Conclusions: This pilot study demonstrated that a routinely collected amount of amniotic fluid (as small as 6 mL) can provide sufficient RNA to successfully hybridize to expression arrays. Analysis of the differences in fetal gene expressions might help us decipher the complex genetic background of polygenic disorders.

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Neural tube defect (NTD)⁴, the second most common birth defect in the world (1 in 1000 live births), can be easily diagnosed prenatally with fetal sonography combined with screening of maternal serum for increased α -fetoprotein concentration. Genetic analysis of this disorder is lacking, however, because genetic diagnostic methods including invasive techniques such as genetic amniocentesis, chorion villus sampling, and cordocentesis and noninvasive techniques such as analysis of fetal cells and cell-free fetal DNA in maternal blood focus on monogenic disorders or chromosomal anomalies. We do not know exactly how many genes are involved in the polygenic disorders such as NTD, which has a multifactorial etiology (1), but their pathomechanism may be elucidated by analysis of genome-wide fetal gene expression. Research on fetal gene expression has, in large part, been limited to animal models and examination of tissues from aborted human fetuses. Genetic amniocentesis can be used as a routine technique in which analysis can be performed on amniocytes, which typically require in vitro expansion. Currently, cell-free fetal DNA and RNA in amniotic fluid are also the focus of many research studies (2).

There is great interest in risk-free alternatives to amniocentesis, particularly prenatal diagnostic techniques

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⁴ Nonstandard abbreviations: NTD, neural tube defect; MAS, Microarray Analysis Suite; PAM, Prediction Analysis for Microarrays; GEM, glycolipid- and cholesterol-enriched membrane.

that use fetal cells isolated from maternal blood (3-5). Because the low number of fetal cells found in most maternal samples (4) make this technique unsuitable for gene expression analysis, we used amniocytes from amniotic fluid samples, in which all of the cells are thought to be of fetal origin.

Development of gene expression microarrays to analyze the presence and quantity of tens of thousands of gene transcripts simultaneously is made possible by the information from the Human Genome Project combined with recent technologic advances (6) such as the increased feature density of the HG-U133 Plus 2.0 Array (Affymetrix Inc.), which enables measurement of transcription over the entire human genome in a single hybridization. More than 54 000 probe sets are used to analyze the expression of 47 000 transcripts and variants, including ~38 500 well-characterized human genes.

We investigated the use of fetal mRNA extracted from amniocytes to detect the presence and quantity of fetal gene transcripts in the hope of identifying gene expression differences related to the pathogenesis of NTD.

Materials and Methods

AMNIOTIC FLUID COLLECTION

We obtained approval from the Semmelweis University institutional review board to collect amniotic fluid samples for this study. After informed consent was obtained from participants, we collected amniotic fluid samples by amniocentesis and centrifuged the samples for 15 min at 600g. We immediately placed the cell pellets into 3 mL RNeasy RNA Stabilization Reagent (Qiagen GmbH). These samples were stored at -80°C until the RNA isolation occurred.

CASES

We have had 7204 live births in our department in the last 2 years. During that time, we had 7 pregnant women carrying fetuses with NTD, diagnosed during ultrasound examination. We obtained samples of amniotic fluid (7–17 mL) from each of these patients. One woman had a fetus with anencephaly and spina bifida in the lumbosacral region (sample MV1, gestational age 13 weeks and 2 days), 1 woman had a fetus with spina bifida in the lumbosacral region (MV2, gestational age 17 weeks and 1 day), 4 had fetuses with ventriculomegaly, “lemon-shaped” head, and lack of closure of the neural tube in the lumbosacral region (MV3, MV4, MV5, MV13, gestational ages of 19 weeks 1 day, 18 weeks 4 days, 19 weeks 6 days, and 19 weeks), and 1 had a fetus with ventriculomegaly and lumbosacral spina bifida (MV14, gestational age 20 weeks 3 days).

CONTROLS

We obtained the control samples (identified as MV6, MV7, MV8, MV11, MV12) from 10 pregnant women who underwent routine genetic amniocentesis because of advanced maternal age (>35 years). The fetal gesta-

tional ages were 17 weeks 1 day to 19 weeks 5 days. For each of these cases we obtained only 3 mL of amniotic fluid for analysis because larger amounts were needed for routine examinations. As a result, before RNA isolation, we pooled control samples by forming pairs randomly, because preliminary experiments (unpublished data) showed that 5–7 mL of amniotic fluid from a healthy singleton pregnancy was needed to acquire a sufficient quality and quantity of mRNA for microarray analysis.

TOTAL RNA ISOLATION

We isolated total RNA with the RNeasy Mini Kit (Qiagen GmbH). The frozen stabilized cell fractions in RNeasy later were centrifuged for 10 min at 400g, and the cell pellets were lysed and homogenized in a mixture of 300 μL 2,3,4,6-tetra-O-acetyl-beta-d-glucopyranosyl isothiocyanate-containing lysis buffer and 3 μL β -mercaptoethanol. The lysed samples were digested in proteinase K solution at 55°C for 10 min. After silica membrane cleaning and DNase I treatment (to completely remove genomic DNA), we eluted the total RNAs in 50 μL RNeasy-free water.

QC

We tested the quantity and quality of the isolated RNA with the Agilent 2100 Bioanalyzer. The high-quality, intact RNA samples that showed regular 18S and 28S ribosomal RNA band pattern during Bioanalyzer analysis were used for microarray analysis.

LABELED PROBE SYNTHESIS

Biotinylated cRNA probes were synthesized from 10 ng total RNA and fragmented according to the Affymetrix protocols using GeneChip Two-Cycle Target Labeling and Control Reagents (https://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf).

HYBRIDIZATION, WASHING, STAINING, AND SCANNING

We hybridized 10 μg of each fragmented cRNA sample into a preheated prewet GeneChip U133 Plus 2.0 (Affymetrix) whole-genome transcript array at 45°C for 16 h, with rotation at 0.5g. The slides were washed and stained with Fluidics Station 450 (Affymetrix) and antibody amplification staining method according to the manufacturer's instructions (sandwich phycoerythrin staining, protocol EukGE-WS2v5). The fluorescent signals were detected by a GeneChip Scanner 3000 (Affymetrix).

STATISTICAL ANALYSIS

Preprocessing and QC. Preprocessing was performed in the R statistical environment (7). QC analyses of microarrays were performed according to the recent suggestions of “Expression Profiling: Best Practices for Data Generation and Interpretation in Clinical Trials” (8). Scanned images were inspected for artifacts, and percentage of present calls ($>30\%$) and control of the RNA degradation were

evaluated, and all cell-line measurements fulfilled the minimal quality requirements. According to the recommendations, we applied 2 different processing methods: Microarray Analysis Suite, version 5.0 (www.affymetrix.com), and robust multichip average analysis (9). MAS 5.0 applied calibration to an individual chip with excellent specificity and good sensitivity. Robust multichip average analysis applied cross-project normalization, which had good specificity and excellent sensitivity (www.R-project.org). Further data analysis and interpretation carried out with both of these preprocessing methods yielded the best comparison and calibration properties across all measurements.

Feature selection. We arranged the complete dataset consisting of 9 expression measurements into 2 classes according to the diagnosis of the samples (see *Results*). To obtain characteristic signal profiles with high predictive/discriminative power, we applied the Prediction Analysis for Microarrays (PAM) (10), which uses soft thresholding to produce a shrunken centroid that allows the selection of genes with high discriminative potential. With only 4 samples in the diseased group, however, the search for a minimum number of genes with maximum predictive accuracy was not promising, because we could distinguish the 2 different groups with a very short gene list. Furthermore, the individual cross-validation led to zero misclassification error with only the very first discriminating gene (graph not shown). Therefore we selected the top 100 discriminating genes for each condition. The PAM score is presented in Table 1 to show the relative significance of the selected gene.⁵ Finally, the overlap of the 2

lists—based on the 2 different processing methods—was further analyzed (Fig. 1). To control the false discovery rate, we performed a significance analysis of the microarrays.

Additional analyses. We performed hierarchical clustering with Genesis software (11) and used the mean linkage of Euclidean distances to compute clustering dendrograms for both samples and genes. We performed annotation with the Affymetrix Netaffx analysis center (<http://www.affymetrix.com/analysis/index.affx>) and used the 2-tailed Student *t*-test to test the differential expression of single selected genes. Statistical significance was set at $P < 0.05$.

VALIDATION OF MICROARRAYS

Because of the limited sample size, all RNA had to be used for the cRNA chips. Real-time quantitative reverse-transcriptase PCR, which would have been the optimal method, could not be performed. However, in 1 case, we had enough material to perform a 2nd run to independently process the same sample from the same pregnancy to validate the accuracy of the microarray measurements. Using this sample, we performed an independent hybridization to a 2nd HG-U133 Plus 2.0 Array. After hybridization, the corresponding transcripts on the 2 slides representing the same sample were spotted against each other.

⁵ Human genes: *SLA*, Src-like-adaptor; *LST1*, leukocyte-specific transcript 1; *BENE*, BENE protein; *KRT24*, keratin 24; *C9orf3*, chromosome 9 open reading frame 3; *KIAA0779*, KIAA0779 protein; *TMCC1*, transmembrane and coiled-coil domain family 1; *ZNF292*, zinc finger protein 292; *LOC153222*, adult retina

protein; *CBEP4*, cytoplasmic polyadenylation element binding protein4; *TIGD2*, tigger transposable element derived 2; *UBAP2*, ubiquitin associated protein 2; *AVPI1*, arginine vasopressin-induced 1; *KIAA0669*, the same as *TSC22D2*; *TSC22D2*, TSC22 domain family, member 2; *DHFR*, dihydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide; *KA36*, type I hair keratin; *KRT4*, Keratin 4; *KRT9*, Keratin 9.

Table 1. Genes detected in both statistical processing methods as having substantially different expression states in fetuses with NTD compared with controls.

Probe Set ID	UniGene ID	Gene Symbol	Gene Title	Fold Difference	PAM score
220267_at	Hs0.87383	<i>KRT24</i>	Keratin 24	-18.09	-1.35
203760_s_at	Hs0.75367	<i>SLA</i>	Src-like-adaptor	2.72	0.97
232270_at	Hs0.15806	<i>C9orf3</i>	Chromosome 9 open reading frame 3	-3.49	-0.72
213351_s_at	Hs0.362996	<i>KIAA0779 is TMCC1</i>	KIAA0779 protein is transmembrane and coiled-coil domain family 1	-1.98	-0.71
214181_x_at	Hs0.436066	<i>LST1</i>	Leukocyte specific transcript 1	3.30	0.64
243918_at				-6.87	-2.12
212368_at	Hs0.448341	<i>ZNF292</i>	Zinc finger protein 292	-2.58	-0.73
225956_at	Hs0.163725	<i>LOC153222</i>	Adult retina protein	-4.21	-0.64
224828_at	Hs0.108159	<i>CPEB4</i>	Cytoplasmic polyadenylation element binding protein 4	-5.34	-0.57
229983_at	Hs0.58924	<i>TIGD2</i>	Tigger transposable element Derived 2	-2.82	-1.13
219192_at	Hs0.14953	<i>UBAP2</i>	Ubiquitin associated protein 2	-2.18	-0.51
218631_at	Hs0.23918	<i>AVPI1</i>	Arginine vasopressin-induced 1	-2.97	-0.70
204094_s_at	Hs0.52526	<i>KIAA0669 is TSC22D2</i>	KIAA0669 gene product is TSC22 domain family, member 2	-4.10	-1.17
209373_at	Hs0.185055	<i>BENE</i>	BENE protein	-3.52	-0.46

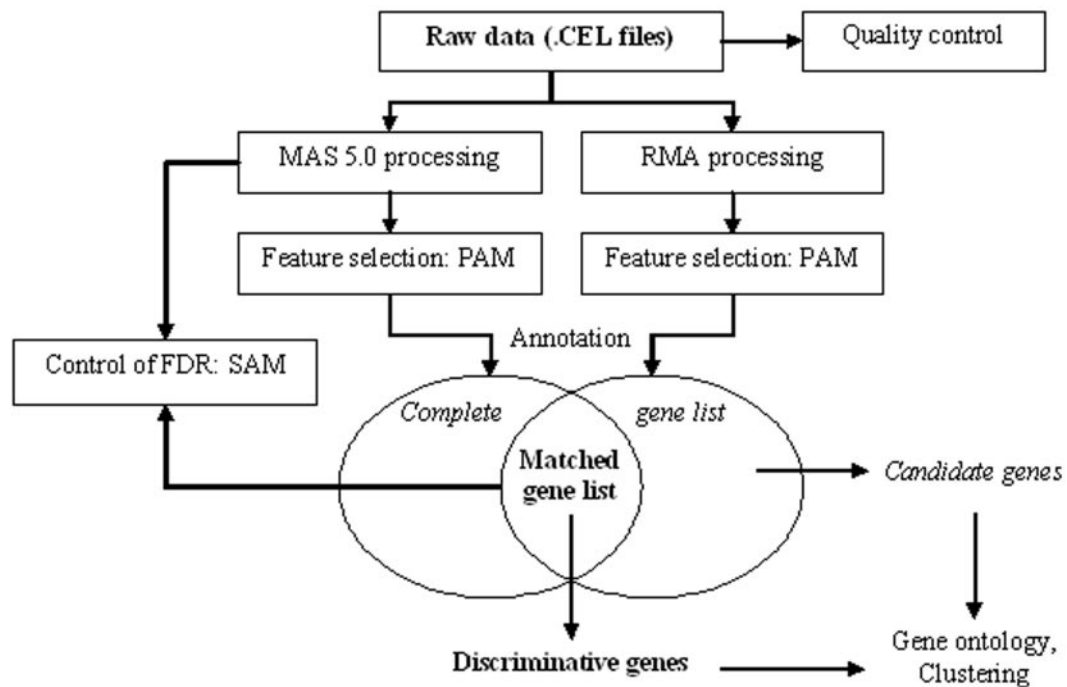


Fig. 1. Overview of the applied statistical processing methods.

RMA, robust multichip average analysis; FDR, false discovery rate; SAM, significance analysis of microarrays.

ANALYSIS OF EXPRESSION OF GENES ASSOCIATED WITH FOLIC ACID

Administration of folic acid decreases the risk of NTD. Therefore, to find out whether genes in connection with folic acid showed differential expression in NTD fetuses, specific genes were investigated and compared with control samples.

Results

TOTAL RNA ISOLATION, LABELED CRNA SYNTHESIS, AND HYBRIDIZATION TO MICROARRAYS

The volumes of obtained amniotic fluid, amount of total RNA eluted, QC results of the isolated RNA, quantities of

biotinylated cRNA after amplification, and present calls on whole-genome transcript arrays are shown in Table 2. We excluded samples MV3 and MV5 from further analysis because the eluted total RNA was found to be not intact. Nine of the remaining 10 samples (5 samples from the NTD group and 5 control samples), were well hybridized to the arrays. In these cases, the percentage of present calls was >30%. Data from MV14 were not used in the analysis because, in this case, the percentage of present calls was only 16%.

Oligonucleotide whole genomic microarray analyses of amniotic fluid cell fractions were of good quality considering the background and noise, array sensitivity, present

Table 2. Quantities of total RNA, isolated and amplified from amniocytes, and percentage of present calls on microarrays.

	Amniotic fluid volume, mL	Total RNA eluted, ng	Quality control (Bioanalyzer)	Biotinylated cRNA after amplification, μg	Present calls on microarray, %
Cases with NTD					
MV1	7	105	Intact	62.04	39.3
MV2	17	88	Intact	51.58	37.8
MV3	16	84.6	Not intact		
MV4	16	96	Intact	10	32.9
MV5	16	36	Not intact		
MV13	17	62.4	Intact	83.98	37.2
MV14	16	35	Intact	29.85	16.3
Controls					
MV6	6	60	Intact	85.05	41.4
MV7	6	72	Intact	44.8	38.1
MV8	6	95	Intact	77.96	41.4
MV11	6	89.3	Intact	54.25	38.4
MV12	6	70.5	Intact	45.28	37.3

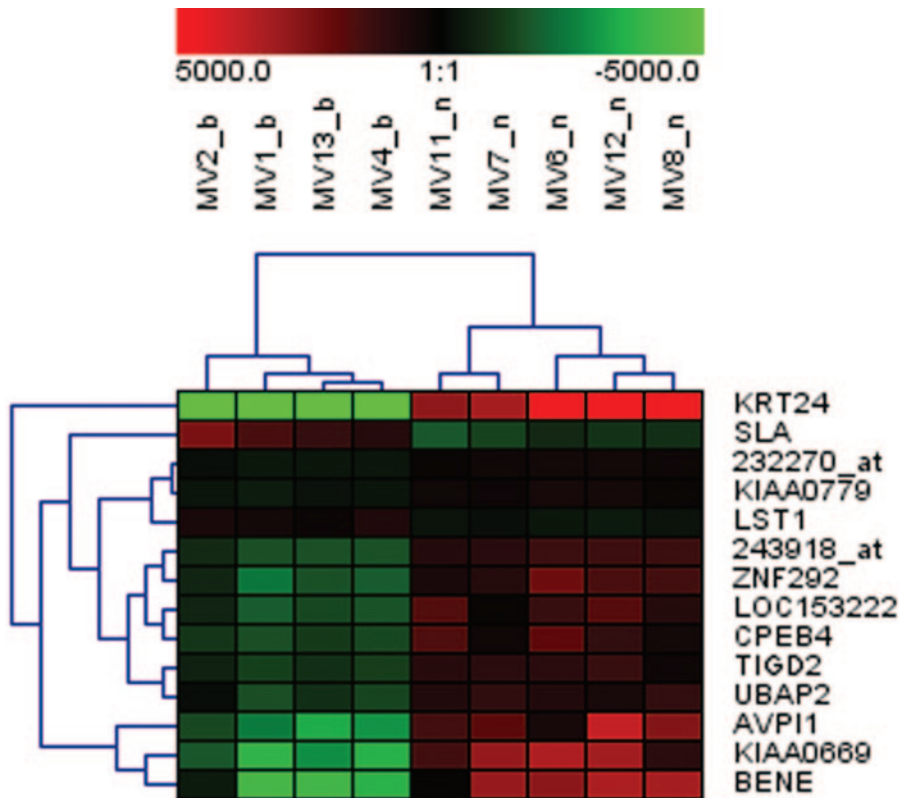


Fig. 2. Hierarchical cluster image of transcripts selected after both processing techniques (top discriminative genes, listed in Table 1).

Up-regulated genes are marked with *red*, down-regulated genes are marked with *green*. The NTD and normal cases are indicated with "b" and "n" at the end, respectively. Gene symbols or Affymetrix IDs are indicated.

percentage (signal intensity), and GAPDH 3'/5' ratio. The mean (SD) background [67.5 (12.8)] and noise [3.82 (0.72)] values were low. The mean (SD) of percentages (the signals that were considered present according to the Affymetrix software) was 38.2 (2.54)%. The mean (SD) GAPDH3'/5' ratio was 5.24 (2.31), only slightly >3. Because of the extra manipulations involved, higher 3'/5' ratios in the 2-cycle method than the 1-cycle method were not unusual. The 2-cycle T7 method was suitable for appropriate linear amplification of small amniotic fluid samples that contained only 10–20 ng total RNA.

GENE EXPRESSION DIFFERENCES, DISCRIMINATIVE GENES

Before finding top genes that had substantial differences in expression in the diseased and control groups, we applied 2 different processing methods. All genes selected in at least 1 statistical analysis, with annotation, are shown in Supplemental Table 1 of the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol52/issue11>. To find validated discriminative genes, we used the overlap of the 2 lists based on the 2 different processing methods. A hierarchical cluster image of the transcripts selected after using both processing techniques (top discriminative genes, depicted in Table 1) is presented in Fig. 2. In the NTD group compared with the control group, the genes *KRT24*, *C9orf3*, *KIAA0779* (*TMCC1*), *ZNF292*, *LOC153222*, *CBEP4*, *TIGD2*, *UBPP2*, *AVPI1*, *KIAA0669* (*TSC22D2*), and

BENE showed decreased expression and the genes *SLA* and *LST1* showed increased expression.

A list of the genes associated with folic acid can be found as Supplemental Table 2 in the online Data Supplement. The genes *DHFR*, *MTR*, and *ATIC* showed increased expression in the NTD group compared with the control group (Table 3).

According to the Significance Analysis of Microarray, all genes detected by PAM, listed in Table 1, were significant at a false discovery rate <8%. The Pearson correlation for validating the accuracy of the microarray measurements was 0.997. Furthermore, the presence of Y chromosome probe sets in male samples but not in female samples provided physiologic validation of our data.

PUBLIC DATA ACCESS

All microarray data, including MAS 5.0 processed and raw data, are accessible in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) using the accession number GSE4182. The array information required for Minimum Information about a Microarray Experiment compliance can be accessed using the GEO platform ID GPL570.

Discussion

This in vivo study of whole-genome expression by oligonucleotide microarray analysis of fetal mRNA isolated from amniocytes demonstrates that global gene expression array analysis can be performed with an amniotic fluid sample as small as 6 mL, allowing the examination

Table 3. Genes in connection with folic acid with substantially different expression states in NTD cases compared with controls. All 3 genes showed a higher expression in the diseased group.

Gene Symbol	P Value	Fold Change	Probe Set ID	UniGene ID	Gene Title
<i>ATIC</i>	0.041	1.58	208758_at	Hs0.90280	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
<i>DHFR</i>	0.075	1.59	202532_s_at	Hs0.83765	Dihydrofolate reductase
	0.022	5.18	202533_s_at	Hs0.83765	Dihydrofolate reductase
	0.030	1.52	202534_x_at	Hs0.83765	Dihydrofolate reductase
	0.049	1.62	48808_at	Hs0.83765	Dihydrofolate reductase
<i>MTR</i>	0.034	1.43	203774_at	Hs0.82283	5-Methyltetrahydrofolate-homocysteine methyltransferase

of individual fetuses by analysis of routinely collected amniotic fluid samples. Total RNA was successfully isolated from amniotic fluid cells, amplified, labeled, and hybridized to whole-genome transcript arrays. The mean of the signals considered present according to the Affymetrix software was as high as that observed in higher volume samples such as surgical tissue samples or biopsy specimens.

The majority of nucleated cells in amniocentesis fluids are derivatives of fetal epidermal cells. Exfoliation of such cells from the fetal epidermis has been directly observed. The hypothesis of epithelial origin was supported by results of immunofluorescent studies with antibodies against epidermal keratins (12). Furthermore, Greenebaum and colleagues (13) demonstrated the presence of neuroepithelial cells in amniotic fluids in cases with open NTDs. Tsai and colleagues (14) found that clonal amniotic fluid-derived stem cells from 2nd trimester amniocentesis expressed characteristics of neural progenitor cells. Therefore, amniotic fluid cells are good candidates for examination of this malformation process. Because it is not possible to obtain samples from the living human fetus at the expected time of neural tube closure (the 26th day after conception), we used amniotic fluid samples from amniocentesis performed in the 2nd trimesters to study the expression profiles of amniocytes.

At this time, it is not possible to discriminate between causality and downstream effects in regard to the differences in gene expression profiles we observed for NTD and control groups. The kidney, intestine, and skin are probable sources of cells that produce keratins. Therefore the observed decrease in keratin expression (*KTR24* in the discriminative genes, *KA36*, *KRT4*, *KRT9*, in the top 100 associated transcripts) may be attributable to the presence of fewer keratin-producing cells in direct contact with the amniotic fluid in NTD cases. We also found decreased expression of adult retina protein that could be related to the defect of the neural tube. Expression was lowest in the anencephalic case (MV1); higher in the 2 cases with ventriculomegaly, "lemon-shaped head", and missing closure of the neural tube in the lumbosacral region (MV4 and MV13). Expression was highest in the case of spina bifida alone (MV2) but was still lower than the gene expression in the controls. *BENE*, *243918_at*, and *TSC22D2*

showed the same profile. *TSC-22* protein was expressed in astrocytes (15). In the NTD group, we saw decreased expression of *TSC22D2*. We also found decreased expression of *CPEB4*. Cytoplasmic polyadenylation element binding proteins are important in the hippocampus, where these proteins are thought to regulate local protein synthesis and synaptic plasticity (16).

The compartmentalization of cellular membranes into microdomains or rafts is an important concept in cell biology. Unlike most membranes, which are enriched in phospholipids and packed in a disordered state, rafts have a high glycosphingolipid and cholesterol content and appear to be packed in a liquid-ordered structure. In polarized epithelial cells, the segregation and transport of apical proteins during biosynthetic transport was initially explained by the recruitment of specific proteins into glycolipid- and cholesterol-enriched membrane (GEM) rafts. *MAL* is an integral membrane proteolipid protein expressed in polarized epithelial cells, oligodendrocytes, and T lymphocytes. The *BENE* gene is a member of the *MAL* gene family, which was identified in the GEM fraction of endothelial cells. So *BENE* protein is an essential component of the GEM raft machinery for apical sorting of membrane proteins (17). *BENE* proteins could build a connection with epithelial cell polarity genes required for neural tube closure (18). This theory is supported with our finding of decreased expression of *BENE* gene in the NTD group compared with the control group.

Another difference in the gene expression profiles of cases and controls could be attributed to the pathomechanism of the disease. Monocytic cells can be found (12) in amniotic fluid cell cultures from NTD pregnancies. The Src-like adaptor protein (*SLAP*) is a negative regulator of T-cell antigen receptor signaling (19) and also regulates B-cell antigen receptor concentrations and signal strength during lymphocyte development (20). *SLAP* is a negative regulator of signaling initiated by growth factors (21). In the NTD group, we found increased expression in the *SLA* gene and decreased expression in the *EGFR* gene.

The *LST1* gene is expressed in leukocytes and dendritic cells. The expression of *LST1* was increased in autoimmune-induced inflammation and in response to stimulation with inflammatory mediators and bacterial agents,

suggesting that *LST1* may play a role in inflammatory and infectious diseases (22). We found increased expression of *LST1* gene in samples from the NTD group.

Taken together, the findings of higher expression of *SLA* and *LST1* genes in NTD samples highlights the immunological aspect of this polygenic disease. Hatta and colleagues (23) found that a persistent viral infection could cause infected epithelial cells to lose cellular polarity, leading to cell transformation. Therefore, if we hypothesize that these changes may be connected with a viral infection, we can make a putative correlation with the decreased expression of *BENE* protein as well. The idea of a viral infection in the pathogenesis of NTD is not new; significant differences with the control group for anencephaly and spina bifida in relation to maternal viral upper respiratory infections was reported (24), and a sudden increase in the incidence of NTD was also found in newborns whose 1st trimester coincided with an epidemic of dengue fever (25). Maternal hyperthermia or maternal fever in the 1st trimester was also found to increase the risk of NTD (26, 27). Such data must be interpreted with caution at this stage, and further investigation is necessary.

Unquestionably, the most significant epidemiological finding with respect to NTD is the protective effect of maternal periconceptional folic acid supplementation. Reports from countries that have implemented fortification programs indicate that the population prevalence of NTD has declined. All pregnant women involved in our study were taking a prenatal multivitamin, which is routinely advised in Hungary. It is not surprising that there has been substantial interest in the relationship between genes involved in folate-related metabolic pathways and NTDs (28). Although the folate pathway has been explored extensively, only the methylenetetrahydrofolate reductase 677C>T variant has been found to have an association, and only in an Irish population (29). Theories regarding the relationship between NTD and variants of specific folate-related genes are largely unproven at this time (28).

None of the folate-related genes we investigated (see Table 2 in the online Data Supplement) were in the top 100 associated transcripts. Genes of dihydrofolate reductase (1st enzyme of folate metabolism), 5-methyltetrahydrofolate-homocysteine methyltransferase (enzyme playing a role in the methylation cycle), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (important enzyme in purine nucleotide biosynthesis) enzymes showed an increased expression in NTD cases compared with controls, a finding that suggests a compensating effect for the developed NTD. Although identification of the relationship between folic acid and NTD is valuable, the discovery of other risk factors is essential. Our data showed that aspirant genes playing an important role in the pathogenesis of NTD were not specific folate-related genes.

We attempted to create a data warehouse for genes involved in the pathomechanism of NTD. The major

difficulty of collecting cases is the low incidence of the disease (1 in 1000 live births), although it is the second most common birth defect in the world. Because of the small sample size, data must be interpreted with caution at this stage, and further investigation is necessary.

In conclusion, we found that the technique of RNA extraction from amniocytes and hybridization to expression arrays could be used with routinely collected amniotic fluid samples. The observed differences in gene expression in NTD suggest that this technology might be useful for elucidating the complex genetic background of polygenic disorders in the living human fetus.

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