Risk of Autistic Disorder in Affected Offspring of Mothers With a Glutathione S-Transferase P1 Haplotype

Tanishia A. Williams, PhD; Audrey E. Mars, MD; Steven G. Buyske, PhD; Edward S. Stenroos, BS; Rong Wang, MS; Marivic F. Factura-Santiago, MD; George H. Lambert, MD; William G. Johnson, MD

Objective: To test whether polymorphisms of the glutathione S-transferase P1 gene (*GSTP1*) act in the mother during pregnancy to contribute to the phenotype of autistic disorder (AD) in her fetus.

Design: Transmission disequilibrium testing (TDT) in case mothers and maternal grandparents.

Setting: Autistic disorder may result from multiple genes and environmental factors acting during pregnancy and afterward. Teratogenic alleles act in mothers during pregnancy to contribute to neurodevelopmental disorders in their offspring; however, only a handful have been identified. *GSTP1* is a candidate susceptibility gene for AD because of its tissue distribution and its role in oxidative stress, xenobiotic metabolism, and JNK regulation.

Participants: We genotyped *GSTP1*^*G313A* and *GSTP1*^*C341T* polymorphisms in 137 members of 49 families with AD. All probands received a clinical diagnosis of AD by Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule–Generic testing.

Main Outcome Measures: Association of haplotypes with AD was tested by the TDT-Phase program, using the expectation-maximization (EM) algorithm for uncertain haplotypes and for incomplete parental genotypes, with standard measures of statistical significance.

Results: The *GSTP1*^*A* haplotype was overtransmitted to case mothers (*P* = .01 [95% confidence interval, 1.39-5.13]). Results of the combined haplotype and genotype analyses suggest that the *GSTP1*-313 genotype alone determined the observed haplotype effect.

Conclusions: Overtransmission of the *GSTP1*^*A* haplotype to case mothers suggests that action in the mother during pregnancy likely increases the likelihood of AD in her fetus. If this is confirmed and is a result of a gene-environment interaction occurring during pregnancy, these findings could lead to the design of strategies for prevention or treatment.

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In most cases in which a gene has been associated with a disorder, the disease allele acts in the affected individual. Alternately, a disease allele of a gene may act in the mother during pregnancy to contribute to the phenotype of her affected child. So far, there is evidence for such maternally acting alleles, so-called teratogenic alleles, for only a handful of genes.

Examples of teratogenic alleles include the following: (1) in spina bifida, the G allele of *MTR* A2756G and *MTRR* A66G polymorphisms and the deletion allele of the *DHFR* 19-base pair (bp) deletion polymorphism; (2) in Down syndrome, the G allele of *MTRR* A66G and the T allele of *MTHFR* C677T polymorphisms; and (3) in orofacial clefting, the *GSTT1*-null allele homozygotes. Demonstrating increased frequency of a putative teratogenic allele in mothers but not fathers of affected individuals is evidence for a teratogenic allele and is the method that has been used in most articles. Strong evidence of a teratogenic allele (eg, by maternal transmission disequilibrium testing [TDT] as in the present study) has rarely been achieved, and there is no strong evidence for any so far in autism.

Children with autistic disorder (AD) show deviation from the normal developmental pattern with impaired social interactions and communication, restricted interests, and repetitive stereotyped patterns of behavior that are evident before 36 months of age. Findings from clinical genetic studies and modeling studies suggest that AD may be caused by multiple
interacting gene loci, while environmental and epigenetic factors may contribute to variable expressivity possibly through interaction with genetic susceptibility factors. Environmental factors contributing to AD could include toxic endogenous metabolites or exogenous toxins or teratogens.

Neuropathological studies, cytoarchitectonic studies, minicolumn studies, and neonatal blood studies of neurotrophins and neuropeptides support the prenatal origin of certain brain abnormalities in autism. Consequently, it is possible that maternal genes, acting during pregnancy, could contribute to the autism phenotype in the fetus.

Several maternal effects have been described for autism, but none reported so far gives strong evidence of a teratogenic allele. For some of these, no involvement of specific maternal genes has yet been demonstrated, e.g., autism associated with maternal ingestion during pregnancy of thalidomide or valproic acid and increased risk of autism spectrum disorder in children of mothers with diabetes mellitus or epilepsy. There is some evidence that maternal alleles at the MAOA locus and possibly the DBH locus may modify IQ in children with autism. Diminished IQ is often seen in autism but not as a cardinal feature. Although mental retardation is not part of the diagnostic criteria for autism, the 2 diagnoses could be interacting through diagnostic substitution in the population. In addition, some alleles of the glutamate receptor 6 gene (GLUR6, GRIK2) reportedly showed increased maternal transmission to male children with autism; these findings were ascribed to meiotic drive or imprinting. There is evidence that the major histocompatibility complex extended haplotype, HLA B44-SC30-DR4, may act as a teratogenic allele for autism because the frequency of this haplotype was increased in mothers of children with autism compared with controls. So far, this has not been confirmed using a stronger study design such as maternal TDT. This haplotype frequency was also increased in autism cases compared with controls, suggesting action in the cases as well.

Some recent investigations in humans have linked oxidative stress to autism. For example, significantly decreased levels of glutathione, a significantly lower ratio of reduced glutathione to oxidized glutathione, and other metabolic abnormalities in individuals with autism were interpreted as evidence of oxidative stress. Glutathione is the most important endogenous antioxidant and is the most abundant nonprotein thiol. Recently, increased urinary excretion of 8-isoprostane-F2α, a biomarker of lipid peroxidation and oxidative stress, was found in autism, a finding that has been confirmed. Accumulating data support the importance of the glutathione S-transferase (GST) supergene family as a protective factor against reactive oxygen species and the products of oxidative stress. Glutathione S-transferases, a category of phase 2 enzymes, catalyze the conjugation of glutathione to different toxic electrophiles that have been activated by phase 1 enzymes. Glutathione S-transferases conjugate and detoxify products of oxidative stress. They also conjugate toxins that produce oxidative stress. Conjugation of glutathione to a compound by GST can sometimes increase its toxicity or even create toxicity.

Seven cytosolic families of GSTs are known in humans, including at least 16 cytosolic GST subunits (most of them polymorphic), with some alleles causing functional alteration. For alleles with diminished function, their specific substrates might accumulate and contribute to oxidative damage; increased enzyme activity could also lead to oxidative damage if the product is toxic. The pi class of GSTs, represented by a single GST (variably known as GSTP1, GSTP1-1, GSTP, and GSTpi) coded for by a gene on chromosome 11q13, is expressed at the highest levels in most extrahepatic tissues.

GSTP1 has 4 polymorphic alleles resulting from 2 amino acid changes, Ile105Val (A313G) and Ala114Val (C341T). There is evidence that these polymorphic variants are functional, affecting enzyme activity and substrate specificity. For example, variation at position 105 affects thermostability of the GSTP1 enzyme and its catalytic efficiency for some substrates and correlates with oxidative DNA damage in breast cancer tissues.

GSTP1 was recently studied in autism using a family-based association study design. That study included only case trios (affected individual and parents) and had negative results. In the present study, we genotyped 137 individuals in 49 families with AD for the GSTP1*G313A and GSTP1*C341T polymorphisms using maternal trios (mother of individual with AD and her parents) to look for a teratogenic allele. We analyzed these single nucleotide polymorphisms and the resulting haplotypes using a haplotype-based statistical approach.

METHODS

The study was approved by the Institutional Review Board of University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, and informed consent was obtained from the participants. Families with a child diagnosed as having autism were invited to participate through advertisements in the newsletter of the New Jersey Center for Outreach and Services for the Autism Community. A few families were recruited through the Department of Pediatrics. Selection criteria for families were as follows: (1) participation of a proband with the clinical diagnosis of autism by his or her neuropsychologist as assessed by telephone interview with the primary caregiver, (2) clinical diagnosis of AD confirmed for each proband by Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule–Generic testing, and (3) blood sampling from the mother and at least 1 maternal grandparent. All probands were tested using the Autism Diagnostic Interview–Revised and the Autism Diagnostic Observation Schedule–Generic by one of us (A.E.M.), a trained and certified examiner, and all received the clinical diagnosis of AD by results of these tests.

Venous blood was collected in specimen tubes (Vacutainers; Becton Dickinson, Mountainview, Calif) containing EDTA. The samples were frozen immediately in cryovials at −70°C or were frozen briefly (2-3 days) at −20°C for transport to the laboratory and prepared there. Genomic DNA was obtained from whole blood or white blood cells isolated by centrifugation. DNA extraction was performed using commercially available blood kits (QIAamp DNA; Qiagen, Valencia, Calif). Genomic DNA was stored at 4°C.

Genotyping of the GSTP1*A313G (Ile105Val) and GSTP1*C341T (Ala114Val) polymorphisms was carried out by polymerase chain reaction (PCR)–restriction fragment length

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polymorphism methods as previously described.43 Polymerase chain reaction was carried out in a thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, Conn). For GSTP1*313G (Ile105Val), the PCR product was restricted using Alw26I; products were separated on 8% polyacrylamide gels and were visualized using ethidium bromide. Digestion produced 110- and 90-bp fragments for the G allele and a 200-bp band consistent with the PCR amplimer for the A allele, which had no Alw26I restriction site. For GSTP1*C341T (Ala114Val), the PCR product was restricted with AccI; products were separated on 8% polyacrylamide gels using 1X Tris-borate-EDTA buffer and were visualized using ethidium bromide. Digestion produced approximate 120- and 90-bp fragments for the C allele and an approximate 210-bp band consistent with the PCR amplimer for the T allele, which had no AccI restriction site. The GSTP1*A haplotype comprises 313A/341C, the GSTP1*B haplotype comprises 313G/341T, and the GSTP1*C haplotype comprises 313G/341C. The GSTP1*D haplotype (313A/341T) is rare and was not found in the present data set.

The 2-locus haplotypes were analyzed, with the individual genotypes examined in a secondary analysis. Haplotype frequencies were determined by means of maximum likelihood estimation using the expectation-maximization (EM) algorithm.46 Association of haplotypes with AD was tested by means of the TDT-Phase program,43 using the EM algorithm for uncertain haplotypes and for incomplete parental genotypes. The program fits an unconditional logistic regression analysis using the EM algorithm to loop over uncertain parental genotypes.

RESULTS

We genotyped the GSTP1*G313A and GSTP1*C341T single nucleotide polymorphisms in 137 members of 49 families with an AD proband. There were 49 mothers, 49 maternal grandmothers, and 39 maternal grandfathers in 39 complete maternal trios and 10 incomplete maternal trios.

The distribution of GSTP1 haplotype frequencies (Table 1) was comparable with published frequencies.3 The haplotype analysis (Table 2) demonstrated significant overtransmission (P = .01 [P = .03 using permutation testing]). The GSTP1*A haplotype was overtransmitted to case mothers, while the GSTP1*B and GSTP1*C haplotypes were undertransmitted at almost the same rates. When those 2 rates are constrained to be equal, the odds ratio for the GSTP1*A haplotype is 1 divided by 0.375 (odds ratio, 2.67 [95% confidence interval, 1.39-5.13]).

To better understand the haplotype effect, we examined the individual genotypes. The individual genotypes (Table 3 and Table 4) were not significantly overtransmitted using TDT (P = .06 for GSTP1-313 and P = .36 for GSTP1-341), although the A allele at GSTP1-313 was close to being significantly overtransmitted. Testing by means of maximum likelihood estimation using the EM algorithm for missing data gave a highly significant overtransmission for GSTP1-313*G (P = .005 [P = .004 using permutation testing]), while GSTP1-341 was not significantly overtransmitted (P = .10). Both loci were in Hardy-Weinberg equilibrium among maternal grandparents (P = .31 for GSTP1-313 and P = .60 for GSTP1-341) and among mothers of individuals with AD (P = .90 for GSTP1-313 and P = .71 for GSTP1-341).

Findings from the combined haplotype and genotype analyses suggest that the GSTP1-313 genotype alone determined the observed haplotype effect. The haplotype data enabled some matings that were uninformative for classic TDT to be resolved and enabled greater power for testing.

The GSTP1*A haplotype was significantly more frequently transmitted to mothers of individuals with AD in maternal trios (Table 2), suggesting that it may be acting in mothers during pregnancy to contribute to the phenotype of autism in the fetus. Findings from the combined haplotype and genotype analyses suggest that the GSTP1-313 genotype alone determined the observed haplotype effect. Therefore, the GSTP1-313*A allele may be acting as a teratogenic allele.2,3,30

The action of a GSTP1 polymorphic variant in the mother (possibly during pregnancy) to affect her offspring may not be unprecedented. Suggesting action during pregnancy, a recent study found evidence that the same GSTP1 polymorphisms in mothers were significantly correlated with lung function in their children with asthma, an effect that was independent of transmission of alleles to the child.31 Asthma is a disorder in which oxidative stress plays a role31 and in which isoprostanes have been implicated,2 as in autism.29,30 Although we did not have information about maternal asthma in our study, a recent study32 reported a greater than 2-fold increased risk of autism spectrum disorder in offspring if maternal diagnoses of asthma and allergies were present during the second trimester of pregnancy.

At least 15 other examples of the action of teratogenic alleles have been reported involving at least 12 different alleles.23,30 One of these was a GST, the GSTT1*0 allele, implicated as a possible teratogenic allele for orofacial clefting.2 The interaction of a maternal deletion of GSTT1 (which detoxifies products of cigarette smoke) with a maternal environmental effect (ie, smoking) was associated with increased risk of having a child with oral clefting.2
To our knowledge, the present study is only the second to document action of a teratogenic allele or haplotype using a stringent case-parent study design and is the first for autism. Although TDT is robust against population stratification, the unconditional logistic regression analysis used in this study can be affected by stratification. The permutation testing included herein is considered to give results more robust to population stratification.

GSTP1 has a role in preventing and controlling oxidative stress, and oxidative stress has been linked to AD, as discussed earlier. Thalidomide and valproic acid are teratogens associated with AD, and both induce oxidative stress. Both have been linked to depletion of glutathione, with thalidomide associated with glutathione depletion in a sensitive species (rabbit) but not a resistant species (rat) and with valproic acid associated with glutathione depletion in humans. GSTP1 not only inhibits JNK but also physically binds to it. Four domains of GSTP1 were implicated in its regulation of JNK activation through binding or inhibition. Both alleles of the GSTP1 haplotype that were associated herein with autism occur within the region contributing to binding of GST to the Jun-JNK complex; GSTP1 residue 105, which contributed most or all of the haplotype effect observed, lies within the H site, the region where electrophilic toxins, xenobiotics, or metabolites bind to GST for conjugation with glutathione.

MA PKs are well situated to affect brain development and differentiation, and some genes in this pathway could be candidates for autism susceptibility genes. The stress kinases are transiently activated in response to various environmental or metabolic stimuli, including UV or x-irradiation, heat shock, osmotic shock, or inflammatory cytokines. JNK is regulated by factors besides GSTP1, including reactive oxygen species and changes in the redox potential. The selective and potent regulation by GSTP1 is independent of other functions of GSTP1 such as detoxification and excretion of toxins and xenobiotics by conjugating them with glutathione.

### Table 2. Transmission Disequilibrium Testing (TDT-Phase) Haplotype Analysis Results

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Transmitted</th>
<th>Not Transmitted</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1*A</td>
<td>80</td>
<td>61.25</td>
<td>0.82 (0.63)</td>
</tr>
<tr>
<td>GSTP1*B</td>
<td>13</td>
<td>25.61</td>
<td>0.13 (0.26)</td>
</tr>
<tr>
<td>GSTP1*C</td>
<td>5</td>
<td>11.14</td>
<td>0.05 (0.11)</td>
</tr>
</tbody>
</table>

*The expectation-maximization (EM) algorithm was used for uncertain phase and missing genotype information.

### Table 3. Transmission Disequilibrium Testing (TDT-Phase) of Individual Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transmitted</th>
<th>Not Transmitted</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1-313*A</td>
<td>23</td>
<td>12</td>
<td>0.66 (0.34)</td>
</tr>
<tr>
<td>GSTP1-313*G</td>
<td>12</td>
<td>23</td>
<td>0.34 (0.66)</td>
</tr>
<tr>
<td>GSTP1-341*C</td>
<td>7</td>
<td>4</td>
<td>0.64 (0.36)</td>
</tr>
<tr>
<td>GSTP1-341*T</td>
<td>4</td>
<td>7</td>
<td>0.36 (0.64)</td>
</tr>
</tbody>
</table>

*Using 1 marker at a time and without the expectation-maximization (EM) algorithm, yielding classic TDT.

### Table 4. Transmission Disequilibrium Testing (TDT-Phase) Results of Individual Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transmitted</th>
<th>Not Transmitted</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1-313*A</td>
<td>80</td>
<td>61.95</td>
<td>0.82 (0.63)</td>
</tr>
<tr>
<td>GSTP1-313*G</td>
<td>18</td>
<td>36.05</td>
<td>0.18 (0.37)</td>
</tr>
<tr>
<td>GSTP1-341*C</td>
<td>93</td>
<td>86.60</td>
<td>0.95 (0.88)</td>
</tr>
<tr>
<td>GSTP1-341*T</td>
<td>5</td>
<td>11.40</td>
<td>0.05 (0.12)</td>
</tr>
</tbody>
</table>

*Using maximum likelihood estimation and the expectation-maximization (EM) algorithm for missing genotype information.
tion, were reported in Crohn disease. A panenteric inflammatory bowel–like disease has been reported in regressive AD. Therefore, a therapeutic target for the inflammatory bowel–like disease in AD might be relevant if the present results linking GSTP1 alleles and autism are confirmed.

Several genes reported to be associated with autism are related to MAPKs or to MAPK pathways, as is GSTP1. For example, the reelin gene and the apolipoprotein E (APOE) gene have been associated with autism, and both proteins competitively bind the same receptor, ApoER2. A direct molecular link between ApoER2 and the JNK signaling pathway has been demonstrated. The finding is strengthened by a previous study in which homozygosity for the GSTM1 deletion allele was associated with AD. GSTM1 also detoxifies xenobiotics and independently regulates 2 MAPKs, ASK1 and MEKK1, by binding to them.

If further studies confirm the association of GSTP1 haplotypes with AD, the following questions will be raised: (1) Does a possible GSTP1 effect occur through conjugation of glutathione to a toxin or a xenobiotic for excretion as a mercapturic acid through GSTP1 regulation of JNK activation or through another pathway (eg, selenium sulfide–independent glutathione peroxidase activity)? (2) If the conjugation function of GSTP1 is involved, is an effect in AD due to accumulation of a toxic GSTP1 substrate or a toxic glutathione conjugate? (3) How might GSTP1 act in the mother during pregnancy to contribute to AD? Because of the complexity and possible genetic heterogeneity of autism, these results (if confirmed) may not apply to all families with autism, even in the presence of the risk allele in mothers. If this apparent GSTP1 effect on AD is confirmed and is a result of a gene-environment interaction occurring during pregnancy, these findings could lead to the design of strategies for prevention or treatment.

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Correspondence: William G. Johnson, MD, Department of Neurology and Center for Childhood Neurotoxicology and Exposure Assessment, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, UBHC Room D-431, 671 Hoes Ln, Piscataway, NJ 08854 (wjohnson@umdnj.edu).

Author Contributions: Dr Johnson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis for this study. Study concept and design: Williams, Stenroos, Lambert, and Johnson. Acquisition of data: Williams, Mars, Stenroos, Factura-Santiago, Lambert, and Johnson. Analysis and interpretation of data: Williams, Buyske, Stenroos, Wang, Lambert, and Johnson. Drafting of the manuscript: Williams, Buyske, Stenroos, and Johnson. Critical revision of the manuscript for important intellectual content: Williams, Mars, Buyske, Stenroos, Wang, Factura-Santiago, Lambert, and Johnson. Statistical analysis: Buyske. Obtained funding: Lambert and Johnson. Administrative, technical, and material support: Williams, Mars, Stenroos, Wang, Factura-Santiago, and Lambert. Study supervision: Williams, Stenroos, Lambert, and Johnson.

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REFERENCES

23. Daniels WW, Warren RP, Odell JD, et al. Increased frequency of the extended or

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