Excess Transmission of the NAD(P)H:Quinone Oxidoreductase 1 (NQO1) C609T Polymorphism in Families of Children with Acute Lymphoblastic Leukemia

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Topoisomerase II is a DNA-processing enzyme, and secondary acute myeloid leukemia has been associated with exposure to drugs that inhibit its action. Hence, prenatal exposure to chemicals that inhibit topoisomerase II could plausibly contribute to the incidence of childhood leukemia. The NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme is involved in the metabolism of topoisomerase II-inhibiting chemicals. A functional polymorphism (C609T) associated with reduced activity has been identified on the NQO1 gene. To assess its role in the etiology of childhood acute lymphoblastic leukemia, the authors studied transmission of the variant T allele in the families (parents and grandparents) of 657 affected children in Québec, Canada (1980–2000). Log-linear models that stratified on parental or grandparental mating types were used. Prenatal exposure to potential topoisomerase II inhibitors such as benzene and maternal smoking was studied, as well as interactions between the variant and these exposures. The variant allele was transmitted to cases more frequently than expected (for one or two copies of the allele vs. none, relative risk = 1.39, 95% confidence interval: 1.07, 1.79). There was no evidence of a maternally mediated genetic effect on risk, based on a log-linear assessment of genetic symmetry between mothers and fathers, nor was there evidence of interaction between the studied maternal exposures and the child or maternal variant.

Abbreviations: ALL, acute lymphoblastic leukemia; CI, confidence interval; EDTA, ethylenediaminetetraacetic acid; MLL, mixed lineage leukemia; NQO1, NAD(P)H:quinone oxidoreductase 1; PCR, polymerase chain reaction.

Chromosomal translocations are commonly found in patients with acute lymphoblastic leukemia (ALL) (1). These translocations involve the fusion or juxtaposition of separate genes. The mixed lineage leukemia (MLL) gene is among the many involved in translocations related to childhood leukemia (1). MLL gene rearrangements are also common in secondary acute myeloid leukemia associated with exposure to drugs that inhibit the action of topoisomerase II (2), a DNA-processing enzyme. These observations and the fact that MLL gene fusions can originate in utero (3) have led investigators to hypothesize that prenatal exposure to topoisomerase II-inhibiting chemicals could be involved in childhood leukemia (4). Many of these chemicals have quinone rings (5), the metabolism of which is regulated by the NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme. A common polymorphism with a C→T change at position 609 on the NQO1 gene results in coding for proline instead of serine. This polymorphism is associated with decreased...
catalytic activity of the NQO1 protein and shows a phenotypic gene-dose effect (6, 7).

A few studies have evaluated the risk associated with possessing the variant allele T at the NQO1 C609T locus among patients with infant leukemia (usually immunophenotyped for the presence of MLL rearrangements) or childhood leukemia. For comparison, a convenience sample of controls was chosen or case subgroup comparisons were performed (8–14). Features and results of these studies are shown in table 1. Results were mixed. Earlier studies found an increased risk with the NQO1 C609T variant among MLL-positive infants (8, 9), but later studies did not (12, 14). On the contrary, Lanciotti et al. (13) reported an increased risk among MLL-negative infants. Finally, two studies of childhood leukemia not characterized for MLL gene rearrangements had contradictory results, one showing an association with the variant (10) while the other did not (11). Studies were generally small and limited to comparisons with unrelated controls, the selection of which was always ill-specified. Case-control or case-case studies of genetic factors are known to be vulnerable to population structure bias (15). One way to avoid these biases is to study the transmission of variants in families using case-parent trios (16) or, more efficiently, using cases, parents, and grandparents (17). Also worth considering in genetic studies for early-life diseases is the role played by the mother’s genes during pregnancy; Genetic effects due to maternally expressed phenotypes during pregnancy can produce causal mechanisms that are distinct from effects of the genes the mother transmits to the offspring (18). None of the above investigators considered such effects. Finally, there is good evidence linking the NQO1 polymorphism to benzene toxicity (19), benzene being a cause of adult leukemia (20), and its metabolites are potential topoisomerase II inhibitors (21). The previous studies (table 1) did not consider any relevant environmental exposures or gene-environment interaction between exposure to benzene and related components and the NQO1 C609T polymorphism.

We studied transmission of the NQO1 C609T polymorphism in families of children with ALL. We also evaluated interactions between the variant and maternal occupational exposure to mononuclear aromatic hydrocarbons (the chemical family for benzene) and smoking during pregnancy, both of which could potentially result in inhibition of topoisomerase II.

MATERIALS AND METHODS

Overall study design

We carried out a population-based case-control study targeting all childhood ALL cases diagnosed in the province of Québec, Canada, between 1980 and 2000. The primary purpose of the study was to evaluate the relations between prenatal and postnatal environmental exposures and the incidence of ALL (cases of acute myeloblastic leukemia were not studied); in a later phase of the study, we collected genetic material from cases and their parents and grandparents to study the transmission of variant alleles that were thought to be involved in the metabolism of these environmental contaminants. Details on the case-control study have been published elsewhere (22–28). We report here results from the case-parental-trio and case-parents-grandparents studies.

Case-parental-trio and case-parents-grandparents studies

Cases under age 15 years at diagnosis were recruited at the tertiary care centers in Québec that are mandated to treat children with cancer. A total of 790 cases were included in the study, representing 93.2 percent of all eligible cases diagnosed during the study period. Parents provided information on confounders and environmental exposures by telephone interview. Particular emphasis was placed on collecting and analyzing data on parental occupational exposures (28). The mother’s exposure to mononuclear aromatic hydrocarbons (i.e., benzene, toluene, and xylene) in the course of her work during pregnancy was assessed using a method whereby chemists and industrial hygienists code each job for exposure (28). Information on maternal smoking during pregnancy was also collected. Genetic material was collected from blood samples or through the mail by means of buccal swabs or saliva samples. Informed consent for the interview and the collection of genetic material was obtained from all participants. The project was approved by the respective institutional ethics committees.

DNA samples and genotyping

Cell pellets, buccal swabs, or saliva samples were obtained from study subjects. Genomic DNA from cell pellets was extracted with the QIAamp DNA blood kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. DNA was then quantified using PicoGreen (Molecular Probes, Invitrogen, Burlington, Ontario, Canada) and diluted to a concentration of 5 ng/μl with Tris-EDTA (10 mM Tris-hydrochloric acid, pH 7.5, and 0.05 mM ethylenediaminetetraacetic acid (EDTA)) containing 27 μM Rox (Molecular Probes, Invitrogen), which acts as an inert fluorochrome to trace the DNA solution.

Buccal swabs were transferred to a 2-ml-well extraction block, and DNA was extracted with the QIAamp DNA blood kit (QIAGEN) according to the manufacturer’s instructions, adapted for buccal swabs. The genomic DNA obtained was not diluted and was used directly as such, after the addition of Rox at a final concentration of 27 μM. DNA concentration ranged from 1 ng/μl to 15 ng/μl. Some samples did not yield DNA. A quantity of 5 μl was used for each genotyping reaction.

Some of these samples were amplified using a whole-genome amplification kit (GenomiPhi) from Amersham (GE Healthcare, Chalfont St. Giles, United Kingdom). We followed the manufacturer’s instructions but used 3 μl of starting material; after amplification, the volume was raised to 50 μl with Tris-EDTA, and a sample of 25 μl was drawn to dilute the solution to 200 μl with Tris-EDTA and Rox. A quantity of 5 μl of this dilution was used for genotyping.

Saliva was obtained in Oragene vials (DNA Genotek, Ottawa, Ontario, Canada). Upon receipt of saliva, the vial was incubated at 50°C in an air incubator for at least 2 hours or
TABLE 1. Characteristics and results of previous studies on the NAD(P)H:quinone oxidoreductase 1 (NQO1) C609T polymorphism in families of children with leukemia

<table>
<thead>
<tr>
<th>First author (reference number), year, and country(ies) of study</th>
<th>Cases</th>
<th>Healthy controls</th>
<th>Prevalence (%) of T allele in healthy controls</th>
<th>Results*</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wiemels (8), 1999, United Kingdom</td>
<td>Taken from ALL‡ patients (aged &lt;15 years) in the United Kingdom Childhood Cancer Epidemiology Study</td>
<td>“Healthy newborn infants” (n = 100)</td>
<td>17</td>
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<tr>
<td></td>
<td>36 MLL-positive cases (aged &lt;18 months; 30 ALL cases and six AML‡ cases)</td>
<td></td>
<td></td>
<td>2.54</td>
<td>1.08, 5.96</td>
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<tr>
<td></td>
<td>Subset of 21 MLL/AF4 cases</td>
<td></td>
<td></td>
<td>8.63</td>
<td>2.45, 33.2</td>
</tr>
<tr>
<td></td>
<td>50 TEL-AML1 cases</td>
<td></td>
<td></td>
<td>1.52</td>
<td>0.71, 3.25</td>
</tr>
<tr>
<td></td>
<td>29 hyperdiploid cases</td>
<td></td>
<td></td>
<td>0.91</td>
<td>0.33, 2.38</td>
</tr>
<tr>
<td>Smith (9), 2002, United States</td>
<td>39 cases with MLL de novo leukemia (aged birth to 18.5 years)</td>
<td>NA‡</td>
<td>NA</td>
<td>2.47</td>
<td>1.08, 5.68</td>
</tr>
<tr>
<td></td>
<td>18 cases with treatment-related MLL (aged 3.7–17.2 years)</td>
<td></td>
<td></td>
<td>0.59</td>
<td>0.19, 1.85</td>
</tr>
<tr>
<td></td>
<td>56 cases with de novo B-lineage without MLL (aged 1.4–19.1 years)</td>
<td>(reference group)</td>
<td></td>
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</tr>
<tr>
<td>Krajinovic (10), 2004, Quebec, Canada</td>
<td>174 ALL cases (median age, 5.2 years)</td>
<td>“Selected from a large institutional DNA bank”; not clear whether they were healthy (n = 323)</td>
<td>17.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genotype(s)</td>
<td>CT: 1.7</td>
<td>1.1, 2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT: 1.9</td>
<td>1.0, 3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT + TT: 1.8</td>
<td>1.2, 2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sirma (11), 2004, Turkey</td>
<td>“Blood collected from different geographic areas of the country” (aged 2–60 years) (n = 286)</td>
<td>24.8</td>
<td></td>
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<tr>
<td></td>
<td>189 ALL cases (aged 1–16 years)</td>
<td></td>
<td></td>
<td>0.79</td>
<td>0.58, 1.08</td>
</tr>
<tr>
<td></td>
<td>84 AML cases (aged 1–16 years)</td>
<td></td>
<td></td>
<td>0.71</td>
<td>0.46, 1.09</td>
</tr>
<tr>
<td></td>
<td>Total = 273</td>
<td></td>
<td></td>
<td>0.76</td>
<td>0.58, 1.01</td>
</tr>
<tr>
<td>Kracht (12), 2004, Germany, Austria, and the Czech Republic</td>
<td>35 MLL/AF4 fusion cases (aged &lt;20 years, of which 32 were aged &lt;18 months)</td>
<td>“Blood donors” aged 18–68 years (n = 190)</td>
<td>17.6</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Age &lt;20 years</td>
<td></td>
<td></td>
<td>0.79</td>
<td>0.36, 1.74</td>
</tr>
<tr>
<td></td>
<td>Age &lt;18 months</td>
<td></td>
<td></td>
<td>0.44</td>
<td>0.14, 1.35</td>
</tr>
<tr>
<td></td>
<td>31 BCR‡/ABL cases</td>
<td></td>
<td></td>
<td>1.42</td>
<td>0.38, 3.78</td>
</tr>
<tr>
<td></td>
<td>72 TEL-AML1 cases</td>
<td></td>
<td></td>
<td>0.92</td>
<td>0.52, 1.65</td>
</tr>
<tr>
<td>Lanciotti (13), 2005, Italy</td>
<td>Children admitted to the hospital for trauma, an acute infectious episode, or a minor surgical procedure (n = 147)</td>
<td>—§</td>
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<tr>
<td></td>
<td>18 MLL-negative ALL cases (aged &lt;12 months)</td>
<td></td>
<td></td>
<td>4.22</td>
<td>1.43, 12.5</td>
</tr>
<tr>
<td></td>
<td>32 MLL-positive ALL cases (aged &lt;12 months)</td>
<td></td>
<td></td>
<td>1.26</td>
<td>0.58, 2.74</td>
</tr>
<tr>
<td></td>
<td>Total = 50</td>
<td></td>
<td></td>
<td>1.91</td>
<td>1.00, 3.65</td>
</tr>
<tr>
<td></td>
<td>94 MLL-negative cases (pediatric age (≥12 months))</td>
<td>Infant MLL-negative cases vs. pediatric MLL-negative cases</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>5.55</td>
<td>1.81, 16.9</td>
<td></td>
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</tr>
<tr>
<td>Eguchi-Ishimae (14), 2005, Japan</td>
<td>All cases were aged &lt;18 months</td>
<td>“Umbilical cord blood samples” (n = 197)</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64 MLL-positive cases</td>
<td></td>
<td></td>
<td>1.44</td>
<td>0.80, 2.58</td>
</tr>
<tr>
<td></td>
<td>49 ALL</td>
<td></td>
<td></td>
<td>1.17</td>
<td>0.62, 2.21</td>
</tr>
<tr>
<td></td>
<td>25 MLL-AF4</td>
<td></td>
<td></td>
<td>1.03</td>
<td>0.44, 2.38</td>
</tr>
<tr>
<td></td>
<td>15 AML</td>
<td></td>
<td></td>
<td>3.23</td>
<td>0.88, 11.8</td>
</tr>
<tr>
<td></td>
<td>39 MLL-negative cases</td>
<td></td>
<td></td>
<td>1.16</td>
<td>0.58, 2.33</td>
</tr>
<tr>
<td></td>
<td>23 ALL</td>
<td></td>
<td></td>
<td>1.26</td>
<td>0.52, 3.04</td>
</tr>
<tr>
<td></td>
<td>16 AML</td>
<td></td>
<td></td>
<td>1.04</td>
<td>0.37, 2.9</td>
</tr>
</tbody>
</table>

* Controls were the reference group unless otherwise specified. Results for subgroups of cases described in the “Cases” column are aligned with those subgroups, except for the study by Krajinovic et al. (10), where all cases were compared with controls.
† Odds ratio for CT + TT unless otherwise specified.
‡ ALL, acute lymphoblastic leukemia; MLL, mixed lineage leukemia; AML, acute myeloblastic leukemia; NA, not applicable; BCR, breakpoint cluster region.
§ No information available.

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overnight. The next day, the entire contents were transferred to a cryovial for long-term storage. For isolation of genomic DNA, 500 µl of the Oragene/saliva sample was transferred to a 2-ml-well extraction block. DNA purification was conducted according to the manufacturer’s instructions. The final pellet was dissolved in 200 µl of Tris-EDTA, and a sample was drawn to be diluted 5 and 10 times with Tris-EDTA and Rox. Both dilutions were tested for each genotyping reaction. The concentration of DNA in the undiluted solution ranged from 10 ng/µl to 100 ng/µl.

Genotyping was performed using allele-specific polymerase chain reaction (PCR) with two sets of primers. The forward primers used were CTCTTGAGCCAGTCCGGA-TTT-common-NQO1*2F, GGCTTCCAAGTCTTTAGAAC-AS01-NQO1*2C, and GGCTTCCAAGTCTTTAGAAT-ASO2-NQO1*2T. The reverse primers used were AATGCTATGTCAGTTGAGG-AS01-NQO1*2revG, AATGCTATGTCAGTTGAGA-ASO2-NQO1*2revA, and GGAAGTGGAAGCGCAATGAG-common-NQO1*2revR. We used allele-specific PCR amplification of the target sequence followed by fluorometric detection of the PCR product using the intercalating dye SybrGreen I, as described by Moran et al. (29). More specifically, for each set of primers, PCR was performed in bar-coded 96-well microplates (Axogen Scientific, Union City, California) or 384-well microplates (Eppendorf, Mississauga, Ontario, Canada) with 5 µl (25 ng) of genomic DNA and 10 µl of PCR premix; the latter contains 1.5 µl 10X buffer (QIAGEN), 200 µM deoxyribonucleotide triphosphate, 3.75 pmol of each primer, and 0.375 units of HotStarTaq DNA polymerase (QIAGEN) per reaction.

Setup of PCR microplates was performed with a QIAGEN BioRobot 3.000 (QIAGEN) for distributing the PCR mix and a QIAGEN BioRobot Rapid-Plate (QIAGEN) for distributing the DNA. PCR was performed on MJ PTC-200 (MJ Research, Waltham, Massachusetts) at 95°C for 15 minutes, 35 cycles of 45 seconds at 95°C, 45 seconds at temperatures varying between 50°C and 65°C, depending on the single nucleotide polymorphism (55°C for NQO1 direct and 56°C for reverse), 45 seconds at 72°C, and a final extension at 72°C for 7 minutes. PCR products were detected by adding SybrGreen I (Molecular Probes, Invitrogen) to each reaction tube; fluorescence was then measured with a Fluoroskan Ascent fluorimeter (MTX Laboratory Systems, Vienna, Virginia). Raw genotyping results were analyzed with software developed in-house that uses the VC++ language (Microsoft Corporation, Redmond, Washington) and that calls genotypes directly from raw fluorescence measures for each (frequent and minor allele) allele-specific reaction. Genotypes were automatically linked to individual DNA samples using the DNA plate bar code and the two PCR reaction plate bar codes (one per allele). Bar codes were scanned at each step during the process to ensure correct tracking of samples, reagents, and results throughout the entire process. Case/control status was unknown to the laboratory.

We assessed the accuracy of genotyping data by analyzing at least 90 blinded duplicates per batch. The duplicate analysis was performed with a new primer set (to detect the polymorphism on the other DNA strand) in order to detect any other mutation(s) located on the primers. We also monitored quality by comparing the raw values and their dispersions within and between batches with the values and dispersions obtained during assay validation.

**Statistical analysis**

We used a log-linear model to analyze the family-trio data (16). In this model, likelihood ratio tests are used to compare larger models with reduced submodels and to test the null hypothesis of no within-family relation between the variant and the disease. Each model achieves robustness against genetic population structure through stratification on the possible parental “mating types” (16, 30). An expectation-maximization algorithm allows inclusion of incomplete families (31) and is fully implemented in LEM software (32). Whereas main effects for exposures cannot be tested with family trios, departures from a multiplicative joint effect of an exposure and the offspring genotype can be, by comparing transmission rates between exposed and unexposed cases (33). We also analyzed gene-environment interactions between maternal exposure to mononuclear aromatic hydrocarbons and smoking and NQO1 C609T in the trios (33).

A well-known problem with case-parent trios is that any family in which both parents are homozygous is noninformative. By contrast, many fewer families are noninformative when studying transmission from grandparents, because it will be rare for all four grandparents to be homozygous (17, 34). We also used a log-linear model (17) with an expectation-maximization algorithm to analyze the family data with grandparents, using a script developed for the case-parent-grandparents design in LEM (35).

**RESULTS**

We obtained genetic samples from at least one family member in 657 (83 percent) of the 790 families; 536 case children (68 percent), 615 mothers, and 566 fathers (1,181 of 1,580 parents, or 75 percent) provided samples. A total of 1,024 grandparents also provided DNA for genotyping. We included 121 families for which DNA from the case child was not available. The latter are useful in the context of the expectation-maximization algorithm, because they permit improved estimation of both maternal genetic effects and the stratification parameters related to the unordered parental genotypes.

The prevalence of the genotypes CC, CT, and TT among cases was 60.8 percent, 36.4 percent, and 2.8 percent, respectively. In the mothers, this distribution was 63.9 percent, 31.8 percent, and 4.2 percent, respectively; in the fathers, it was 65.5 percent, 30.7 percent, and 3.7 percent, respectively.

Analyses of trio data and the case-parent-grandparents data showed similar results, with excess transmission of the variant, suggesting that the child’s inheritance of the NQO1 C609T variant contributes to the risk of ALL. In the trio data, the estimated relative risk of ALL associated with inheriting one copy of the variant allele was 1.42 (95 percent confidence interval (CI): 1.09, 1.83). The relative risk associated with inheriting two copies of the variant was 0.78 (95 percent CI: 0.41, 1.47), and using a dominant model, the risk was 1.39 (95 percent CI: 1.07, 1.79) (table 2). Using the
To our knowledge, this is the first study to examine excess transmission of NQO1 C609T to children with leukemia. The advantage of using family data is that statistical deviation from the expected transmission probability (50 percent) of a variant allele provides strong causal evidence, because the distribution of alleles to gametes at meiosis should be random (“Mendelian randomization”) (37). Other advantages of the analyses we used are that no assumption of Hardy-Weinberg equilibrium is necessary and the potential for population structure bias is avoided.

In our study, the number of families with cases of infant leukemia (aged ≤12 months at diagnosis) that we could analyze using the present methods was small. Nevertheless, a large but non-statistically-significant increased risk was found for the variant allele among infant trios. This is compatible with some previous results (8, 9) but not all (12, 13).

Subjects with the variant NQO1 C609T polymorphism (and hence with assumed low or null NQO1 activity) have an increased risk of leukemia, supporting a role for NQO1 substrates (benzene and related compounds) and oxidative stress as determinants of ALL. NQO1 also has many other functions that may play a role in protecting against cancer: For example, it seems to stabilize the p53 protein (38).

Activation of p53 due to oxidative stress or DNA damage results in transcription of downstream genes that coordinate either growth arrest of the cell or apoptosis, preventing proliferation and clonal expansion of damaged cells. The identification of proteins such as NQO1 that increase p53 stability should result in protection against cancer progression (38). Another function of NQO1 that could contribute to protection against cancer is its pivotal role in signaling, activated by tumor necrosis factor and other inflammatory stimuli (39). The negligible NQO1 activity resulting from
the variant is likely to reduce or eliminate these advantages and thus contribute to the incidence of cancer.

Several agencies (e.g., the US Department of Health and Human Services, the US Environmental Protection Agency, and the International Agency for Research on Cancer) classify benzene as a confirmed human carcinogen. Despite our nonconclusive results for gene-environment interaction, this interpretation remains plausible, given that our method of assessing exposure to solvents, although considered the gold standard for community-based occupational studies (40), was probably imprecise. In addition to interaction of the variant with maternal smoking, we also considered paternal smoking before and during pregnancy. Results were negative. Diet is also a source of quinoids; however, diet was not assessed in this study. In conclusion, whereas we did not find indications of gene-environment interaction with the studied factors, more data are needed. To our knowledge, the studies cited in table 1 did not consider this aspect.

In this study, we considered only one single nucleotide polymorphism in a single candidate gene. Whereas the selection of this variant is based on reasonable evidence, this is a limited perspective in terms of genetic susceptibility. Nevertheless, we have shown here that the variant is linked and associated with ALL. While not all families of cases could be included in our study, 83 percent were. However, methods based on transmission distortion are not vulnerable to selection bias, except under an unlikely scenario where the decision to participate is influenced by which alleles the parents happen to have transmitted to their offspring. There were more DNA samples available from the parents than from the children; this happened because the genetic phase of the study was not initiated at the onset of the study and because we included deceased children in the study.

We observed that the risk estimate for two copies of the variant allele in this study was lower than that for a single copy, and although the confidence interval was wide, this observation seems to limit the biologic plausibility of the results. On the one hand, this may be due to small numbers in the TT genotype category. On the other hand, we could speculate about the possibility that homozygous cases simply develop their disease during fetal life and tend not to survive to birth, hence producing artifactual “protection.” Finally, contrary to our expectations, the analysis using two copies of the variant allele in this study was not initiated at the onset of the study and because we included deceased children in the study.

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REFERENCE

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Conflict of interest: none declared.

REFERENCES