Trophoblast Inclusions Are Significantly Increased in the Placentas of Children in Families at Risk for Autism

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Background: Gestation is a critical window for neurodevelopmental vulnerability. This study examined whether the presence of trophoblast inclusions (TIs) in the placenta could serve as a predictor for children at elevated risk for autism spectrum disorder (ASD).

Methods: Placentas were obtained from 117 births in the MARBLES (Markers of Autism Risk in Babies—Learning Early Signs) cohort of families who have one or more previous biological children with ASD, placing their newborn at elevated risk for neurodevelopmental compromise. Control samples were obtained from 100 uncomplicated term pregnancies of multiparous women with one or more typically developing biological children. Frequency of TIs was compared across the two groups.

Results: Placentas from at-risk pregnancies had an eightfold increased odds of having two or more TIs compared with control samples (odds ratio: 8.0, 95% confidence interval: 3.6–18.0). The presence of $\geq$2 TIs yielded a sensitivity of 41% and a specificity of 92% for predicting ASD risk status, whereas $\geq$4 TIs yielded a sensitivity of 19%, a specificity of 99.9%, and a positive likelihood ratio of 242 and conservatively predicted an infant with a 74% probability of being at risk for ASD.

Conclusions: Our findings suggest that the placentas from women whose fetuses are at elevated risk for autism are markedly different from control placentas. These differences are manifested histologically as TIs. Their identification has the possibility of identifying newborns at risk for ASD who might benefit from targeted early interventions aimed at preventing or ameliorating behavioral symptoms and optimizing developmental outcomes.

Key Words: ASD, autism, genetics, pathology, placenta, trophoblast inclusions

Autism spectrum disorders (ASDs) are increasingly common, with the Centers for Disease Control and Prevention estimating the prevalence to be approximately 1 in 88 and the diagnosis five times more common among boys (1 in 54) than among girls (1 in 252) (1). Most scientists consider gene $\times$ environment interaction to play a prominent role in the etiology of ASDs, with genetic vulnerability (2) setting the stage for environmental influences (3,4). The recurrence risk of ASDs among children with an older affected sibling was recently estimated to be 18.7%, with an almost threefold increased risk for male subjects and an additional twofold increased risk if there was $>$1 older affected sibling (5). Neurobiological findings suggest that the pathophysiology of ASD originates during fetal development (6,7).

The human placenta mediates interactions between mother and fetus throughout gestation and provides a historical record of maternal physiologic influences on the fetus (8). Its genetic composition is fetal, and thus it provides a proxy for evaluation of morphological patterns in the fetus. Moreover, through its role in regulating the passage of substances between maternal and fetal compartments and production of bioactive proteins and small molecules, the placenta is central to fetal health, survival, and programming (9).

The serendipitous observation of an increased frequency of trophoblast inclusions (TIs) in two placenta consult cases by the corresponding author (H.J.K.), which coincidentally were associated with children with ASD, led to the hypothesis that TIs might be a marker of ASD—much as TIs are a marker for a number of other genetic abnormalities. Trophoblast inclusions are the result of abnormal infoldings of the trophoblast bilayer, either due to an increased number of cytotrophoblasts or a decreased fusion rate of cytotrophoblasts into the overlying syncytial trophoblast layer (10–12). They were first described as a marker of triploid gestations (13) but are now recognized to be common in a wide range of karyotypically abnormal gestations (14) and spontaneous pregnancy losses (15–17). They have not been described in common placental pathologies, including chorioamnionitis, decreased maternal placental perfusion, or chronic villitis. As TIs age, they become calcified and are referred to as calcified TIs (18). Thus, uncalcified TIs are by convention simply referred to as TIs. Our initial retrospective case-control study in 2007 validated the hypothesis and identified a threefold increase in the rate of TIs among children with ASD in comparison with children from the general population (18).

The goal of this study was to determine in a blinded case-control study whether TIs are more common among placentas

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Received Oct 15, 2012; revised Feb 23, 2013; accepted Mar 10, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.03.006

from newborns at elevated risk for developing ASDs in comparison with those from children born from the general population without a familial predisposition to ASD.

Methods and Materials

Subjects and Study Design

For this analysis, our exposure was defined as presence or absence of enhanced risk for ASD in the offspring of a multiparous woman on the basis of the neurodevelopmental status of one or more previous births. High-risk women were drawn from the MARBLES (Markers of Autism Risk in Babies – Learning Early Signs) Study, a prospective cohort that enrolls mothers of children with ASD in a subsequent pregnancy who are thus at high risk for delivering a child who develops ASD (http://marbles.ucdavis.edu). The MARBLES families are recruited from lists of children receiving services for autism through the California Department of Developmental Services, from other studies at the Medical Investigations of Neurodevelopmental Disorders Institute, and by self-referral. Inclusion criteria for the MARBLES Study included: 1) maternal age at least 18 years; 2) planning a pregnancy or pregnant; 3) one or both parents of the current or planned pregnancy being the biological parent of a child with ASD; and 4) residence within the specified catchment area in California. All 117 archived MARBLES placental specimens (39 $\pm$ 1.5 weeks gestation; range 36 + 1 to 41 + 6 weeks) of 146 births between January 11, 2008 and January 10, 2011 were processed. We did not have placental samples from 29 MARBLES births, because either study staff were not able to attend the delivery or placentas were retained by the hospital. Women in the low-risk control group were drawn from a convenience sample of 100 multiparous women delivering at the University of California Davis Medical Center between December 6, 2010 and January 29, 2011 with the following inclusion criteria: 1) at least 18 years of age; 2) a singleton gestation; 3) at term between 39 and 40 6/7 weeks; 4) without medical complications of pregnancy; and 5) with one or more other biological children who were developing typically. Due to limitations imposed by the University of California Davis Medical Center institutional review board, no additional clinical information was permitted to be collected from the control group mothers. The institutional review board of the University of California at Davis and the State of California Committee for the Protection of Human Subjects approved the study, and informed consent was obtained at enrollment.

Placentas were obtained shortly after birth, chilled, and maintained until processing, with a time interval between birth and fixation of 1–24 hours. Randomly selected 3 $\times$ 3 cm full-thickness samples were taken, de-identified, and placed in 10% phosphate-buffered formalin for between a few days to 3 years before they were batched and sent for histopathologic evaluation at the Yale Reproductive and Placental Research Unit. This aspect of the study was approved by the Yale University Human Investigation Committee (HIC protocol #1003006495). The pathology team was blinded to risk status. Each placental sample was cut into five equal slices, four of which were placed into 25 $\times$ 30 mm cassettes, processed in the Yale Dermatopathology Histology laboratory, embedded in paraffin, microtomed at 5 μm, placed on routine glass slides, and stained with hematoxylin and eosin. The final observable cross-sectional area averaged 315 $\pm$ 90 mm^2. The 868 resulting slides were randomized (http://randomizer.org) and read in numeric order by an experienced placental pathologist (H.J.K.).

The outcomes of interest were the frequencies of TIs, calcified TIs, and total TIs. As described previously (11,12,18,19), TIs were identified by the presence of central syncytiotrophoblast nuclei surrounded by one or more cytotrophoblasts, always away from the villus edge (Figure 1A–D), whereas TIs with a calcified core were considered calcified TIs (Figure 1E–H). Calcification is a proxy for TI age, because calcified TIs are likely older than TIs. All the chorionic villi on each slide were scanned systematically in a row $\times$ row (raster) pattern. The TIs and calcified TIs were counted in each slide and added together to obtain a total TI count. Poor fixation was defined both in a binary fashion as zero versus one or more poorly fixed slides and also as the number of slides (zero to four) that were poorly fixed.

Statistical Analysis

We examined the frequency of TIs in the MARBLES group compared with the control group. The TIs were counted and summed across all four slides and analyzed both as a continuous variable and a dichotomous one, with zero to one total TIs being considered a negative placenta and two or more total TIs being considered a positive placenta. This dichotomous cut-point was set a priori on the basis of previous research (18).

Proportions of positive placentas in the at-risk versus control groups were compared with two-tailed Fisher exact probability test. Additional exploratory analyses examined different TI cutoffs, the use of TIs only, calcified TIs only, and total TIs—which were then used to estimate sensitivity and specificity of alternative cutoffs for discriminating between MARBLES and control placentas.

Intra-rater test-retest reliability was established by rereading all four slides from a 10% random subset of the samples (22 of 217 cases, yielding 88 of the original 868 slides) and analyzing for two or more TIs only, calcified TIs only, and total TIs/sample as well as poor fixation between reads. The observed percent agreement across all slides with two or more total TIs/slide was 95% (84 of 88) and per sample was 82% (18 of 22), with per slide and per sample κ values of .58 and .54, respectively, indicating good agreement. The Spearman correlation coefficient for total TI counts between the two reads in a slide-to-slide comparison was .64, indicating that they were moderately to strongly correlated. The intra-rater test-retest reliability for poor fixation was good (κ .59).

Poisson distribution data fitting was performed initially with the population means of TIs in the control and MARBLES groups. The data were fit to a mixture of two Poisson distributions (20), to improve fitting for the MARBLES group. Bayes theorem was then used to update the probability of being in the at-risk population (21).

Logistic regression was performed to determine the level of association between autism risk status and total TIs/placenta, controlling for fixation quality and duration. A mixed-effects multinomial logistic regression model was used to assess whether autism risk status was associated with the relative timing of TI formation, as judged by the presence or absence of calcification, while adjusting for fixation quality for individual slides (up to four/placenta). The three-level categorical dependent variable classified the slide as containing inclusions, calcified inclusions, or no inclusions. Confidence intervals (CIs) for the mixed-effects model were based on the robust sandwich covariance estimator, with a small sample adjustment to ensure accurate coverage (22).

Analyses were carried out with SAS (version 9.3; SAS Institute, Cary, North Carolina) and R software (version 2.14.2; R Foundation for Statistical Computing, Vienna, Austria).