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Evidence for associations between the purinergic receptor $P2X_7$ (P2RX7) and toxoplasmosis

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Abstract

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Conflict of Interest

The authors declare no conflict of interest.

Congenital *Toxoplasma gondii* infection can result in intracranial calcification, hydrocephalus, and retinochoroiditis. Acquired infection is commonly associated with ocular disease. Pathology is characterized by strong pro-inflammatory responses. Ligation of ATP by purinergic receptor $P2X_7$, encoded by P2RX7, stimulates pro-inflammatory cytokines and can lead directly to killing of intracellular pathogens. To determine whether $P2X_7$ plays a role in susceptibility to congenital toxoplasmosis, we examined polymorphisms at P2RX7 in 149 child/parent trios from North America. We found association (FBAT Z scores ±2.429; P= 0.015) between the derived C(+)G(−) allele (f= 0.68; OR= 2.06; 95% CI: 1.14–3.75) at SNP rs1718119 (1068T>C; Thr-348-Ala), and a second synonymous variant rs1621388 in linkage disequilibrium with it, and clinical signs of disease *per se*. Analysis of clinical sub-groups showed no association with hydrocephalus, with effect sizes for associations with retinal disease and brain calcifications enhanced (OR=3.0 to 4.25; 0.004<P<0.009) when hydrocephalus was removed from the analysis. Association with toxoplasmic retinochoroiditis was replicated (FBAT Z scores ±3.089; P= 0.002) in a small family-based study (60 families; 68 affected offspring) of acquired infection in Brazil, where the ancestral T(+) allele (f= 0.296) at SNP rs1718119 was strongly protective (OR= 0.27; 95% CI: 0.09–0.80). (Words 194)

Keywords

Toxoplasmosis; genetic polymorphisms; purinergic receptor $P2X_7$; North America; Brazil

Introduction

Toxoplasma gondii is a ubiquitous protozoan parasitic infection that, if acquired for the first time during pregnancy, can be transmitted to the fetus. At birth, infants infected *in utero* may have intracranial calcification, hydrocephalus, and ocular involvement.1–3 Severity of disease is known to be influenced by trimester in which infection is acquired by the mother, 4,5 but other factors including genetic predisposition contribute.2 For example, previous studies suggest that genes affecting immune response, including HLA,6 influence clinical outcome in congenitally infected children from North America. However, most but not all of the infants who have the most severe clinical signs in the brain and eye are those infected early in pregnancy.3,5,7,8 At this time fetal immunity is not well developed, leading us to consider whether genes that determine innate pro-inflammatory responses could contribute to clinical phenotype observed in congenitally infected children.

In the U.S. and in Brazil, *T. gondii* infection causes both severe congenital and recurrent, post-natally acquired ocular disease which threatens vision.9 Acute infection in humans is associated with production of pro-inflammatory cytokines such as interleukin (IL)-12, tumour necrosis factor (TNF)-α and interferon-γ, all of which can contribute to ocular pathology.10–12 Although acquired T cell immunity has been implicated in the response to post-natally acquired infection, 10–12 an interesting question is whether the innate triggers for this pro-inflammatory host response to the parasite are the same for both congenital and acquired disease.

Studies in mice (reviewed 13) demonstrate that interaction of the pathogen with the toll-like receptor (TLR)/MyD88 pathway is important, and we recently demonstrated an association between polymorphism at *TLR9* in humans and ocular disease caused by *T. gondii* infection and in the North American National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS) cohort (A.C. Hargrave, D. Melo, E.N. Miller, R. Gazinelli, J.M. Blackwell, R. McLeod, manuscript in preparation, 2010) and in Brazil.14 Ligation of pattern recognition receptors like TLRs leads to the expression of many inflammatory genes, including TNF-α, IL-12 and IL-1β.15 There is now compelling evidence that ligation of TLRs not only triggers induction of synthesis of pro-IL-1β, but also stimulates secretion of ATP that activates purinergic receptor $P2X_7$ through an autocrine loop.16 Activation of $P2X_7$ stimulates inflammasome activation and secretion of IL-1β.17 Intriguingly, the evidence from IL-1β converting enzyme (ICE)-deficient mice does not support a direct role for IL-1β in regulation of *T. gondii* infection,18 even though this cytokine commonly accompanies TNF-α production in TLR-activated macrophages.15 However, $P2X_7$ has also been implicated in the fusion of host cell phagosomes and lysosomes,19,20 the production of reactive oxygen species,21 and modulation of host cell apoptosis,22 each part of the host cell pathogen interactions during *T. gondii* infection.23–27 $P2X_7$ is expressed on T cells as well as macrophages, and has been implicated in a wide range of immunological pathways in controlling intracellular pathogens.28–30 It seems reasonable to propose that this receptor may also impact on innate immunity to *T. gondii* infection.

One way to query the role of $P2X_7$ in humans is to determine whether polymorphisms at the gene *P2RX7* that encodes $P2X_7$ are associated with *T. gondii*-mediated pathologies. Demonstration of genetic association between polymorphisms at *P2RX7* and clinical outcomes of congenital infection could provide unique insight into its possible role *in utero*. Here we report on two studies, the first a family-based study undertaken in the North American NCCCTS cohort which provides evidence for a role for *P2RX7* in congenital toxoplasmosis, and the second a small family-based study which confirms a role for *P2RX7* in retinochoroiditis caused predominantly by post-natally acquired infection in Brazil.

Results

Study samples and genotyping

As outlined previously,14,31 DNA was available (Table 1A) from 149 children with confirmed congenital infection plus available parents from the NCCCTS,1 and for 60 families from Brazil (Table 1B) comprising 30 families containing at least one offspring affected with ocular disease due to acquired toxoplasmosis plus parents, and 30 sibships comprising affected individuals plus unaffected sibs. For the NCCCTS study, children infected *in utero* had a range of clinical signs at birth or time of diagnosis, 124 (83%) infected children had eye or brain signs, 49 (33%) had hydrocephalus (with/without calcifications or retinal disease); 51 (34%) had intracranial calcifications (with/without retinal disease) without hydrocephalus; 20 (13%) had retinal lesions only, and 25 (17%) infected children were without these clinical findings but presented with other signs of pro-inflammatory disease (e.g. hepatosplenomegaly or thrombocytopenia). For the Brazilian study, all individuals classified as affected were *T. gondii*-seropositive and presented with

posterior retinal/retinochoroidal inactive lesions as described.12 All children and available parents for both studies were genotyped for the SNPs detailed in Table 2.

Family-based association test for the presence of clinical signs per se in congenital disease

We first considered the hypothesis that variants at *P2RX7* contribute to clinical signs *per se* following congenital infection with *T. gondii*. Table 3A presents the results of FBAT analysis when all children from the NCCCTS study were included in the analysis. Only data for the additive model are presented as this model provided the best fit for the observed associations, supported by the finding that the likelihood ratio test performed as part of the parallel conditional logistic regression analysis provided no evidence for dominance effects. SNPs rs28360457 and rs1653624 had insufficient power to contribute to the analysis (MAF<0.1; <10 families contributing to the FBAT analysis). The results across other SNPs indicate the strongest association (Z scores ±2.429; P=0.015) between the non-synonymous SNP rs1718119 and clinical signs associated with *T. gondii* infection in this population, with significance (Z scores ±2.309; P=0.021) also observed for the synonymous SNP rs1621388. In both cases, disease was associated with the common C(+)/G(−) allele. Pairwise analysis of linkage disequilibrium (LD) between the *P2RX7* SNPs in the parents of NCCCTS is presented in figure 1A. Both D' and r^2 statistics provide evidence with statistical confidence (LOD>2) for strong LD (D'=1; r^2=0.97) between these two associated SNPs rs1718119 and rs1621388. The difference in statistical significance for the two SNPs in the association data (Table 3A), and the reason why r^2 does not equal 1, is due to genotyping failures in some individuals for rs1621388 which reduced by one the number of families contributing to the analysis.

Family-based association test for ocular disease in acquired toxoplasmosis

We next considered whether polymorphisms at *P2RX7* also influenced susceptibility to ocular disease in (predominantly) acquired toxoplasmosis infection in Brazil. Table 3B presents the results of the FBAT analysis. SNPs rs208293, rs1718119 and rs1621388 provided sufficient power for this set of families in having >10 families contribute to the FBAT analysis and a MAF≥0.3. Only data for the additive model are presented as this model again provided the best fit for the observed associations and the likelihood ratio test provided no evidence for dominance effects. The results indicate the strongest association (Z scores ±3.089; P=0.002) between the non-synonymous SNP rs1718119 and ocular disease associated with *T. gondii* infection in this population, with significance (Z scores ±2.524; P=0.012) also observed for the synonymous SNP rs1621388. Pairwise analysis of LD between the *P2RX7* SNPs in family founders from Brazil (figure 1B) again provide evidence with statistical confidence (LOD>2) for complete LD (D'=1; r^2=1) between these two associated SNPs rs1718119 and rs1621388. The difference in statistical significance for the two SNPs in the association data (Table 3B) is due to genotyping failures in some individuals for rs1621388, which reduced the number of families contributing to the analysis. Although the known32–34 functional SNPs rs2239011, rs2239012 and rs3751143 are also in complete LD (D'=1) for the D' statistic, this is with low confidence (LOD<2) and is not supported by the r^2 statistic which takes allele frequency into account. This means that, where the minor alleles at these variants occur, the same alleles are always on the same

haplotype in the pairwise comparisons. However, LD between these known functional variants and rs1718119/rs1621388 cannot fully account for the associations with toxoplasmic retinochoroiditis observed in this population. The association at rs1718119 was robust to correction for multiple testing when multiplied by the number (n=5) of independent SNPs with MAF>0.1 ($P_{corrected}$=0.01).

Association testing for clinical subgroups of congenitally infected children

The associations observed for clinical signs *per se* in the NCCCTS study (Table 3A) were interesting in that they mimic quite precisely the associations observed for acquired toxoplasmosis in Brazil (Table 3B) where patients were ascertained on the basis of the single clinical phenotype of toxoplasmic retinochoroiditis. This led us to question whether all clinical phenotypes observed in the NCCCTS study of congenital toxoplasmosis contributed to the association observed. Teasing this apart was complicated by the fact that many children presented with more than one category of clinical signs. Nevertheless, as a first step we looked at transmission of alleles from *P2RX7* SNPs to children in 3 different (sometimes overlapping) clinical categories: intracranial calcifications, hydrocephalus, or eye disease. Data presented in Table 4A are results of the case-pseudocontrol conditional logistic regression analysis for rs1718119. These parallel the results of FBAT analysis (not shown) but allow for easy comparison of the effect size (odds ratio). Due to the almost complete LD between these two markers, results for rs1621388 (data not shown) were essentially the same as for rs1718119. These data were interesting in two ways: (i) the odds ratio for association between the C(+)/G(−) allele and disease remained positive for the broad categories of brain disease, eye disease, or both (affected) but with reduced statistical significance compared to analysis of clinical signs *per se*; and (ii) although the number of informative families was smaller, there was no evidence for bias in transmission of alleles to the hydrocephalus group. This suggested that the hydrocephalus group was in some way different, and prompted us to remove them from the analysis of the other groupings (Table 4A). Removal of the hydrocephalus group from the all infected children group increased the odds ratio from 2.06 to 3.00 (95% CI 1.41–6.38; *P*=0.004), from the eye disease group from 1.93 to 3.00 (95% CI 1.28–7.06; *P*=0.012), from the intracranial calcification disease group from 2.09 to 4.25 (95% CI 1.43–12.63; *P*=0.009), and from the combined grouping of brain calcifications and eye disease (AFF) from 2.07 to 3.28 (95% CI 1.41–7.66; *P*=0.006) for disease associated with the C(+)/G(−) allele. Overall, this analysis confirms a stronger association with all other clinical phenotypes of disease when the hydrocephalus group is removed from the analysis. Whilst association between eye disease caused by congenital infection with *T. gondii* and rs1718119 at *P2RX7* is consistent with the Brazilian data (Table 3B) for acquired ocular toxoplasmosis, the overlap in clinical phenotypes means that we cannot be certain that intracranial calcifications or other clinical signs (e.g. splenomegaly) are also associated with variation at *P2RX7*. The change in effect size observed with the intracranial calcification group certainly suggests that this phenotype is also influenced by polymorphism at *P2RX7*.

Clinical subgroup analysis uncovers apparent association with SNP rs2239012

Although none of the known32–34 functional SNPs rs2239011, rs2239012 and rs3751143 for which sufficient transmissions were available for analysis were associated with analysis

of clinical signs *per se* (Table 3A), in the process of analyzing data for the clinical sub-categories we uncovered apparent associations with SNP rs2230912 (Table 4B). As for rs1718119, the odd ratios for eye disease (OR=5.99), brain calcifications (OR=4.00) and the combined eye plus brain calcifications groups (OR=4.33) were high for disease associated with the common A(+)/T(−) allele at this SNP when the hydrocephalus group was removed from the analysis, but the confidence intervals were large.

Between group logistic regression analysis

Due to the presence of some mixed ethnicity present in the NCCCTS study sample (see legend to Table 1), the use of a family-based study meant that our association analysis was robust to ethnic mixture/admixture in the population. However, one disadvantage was the small number of informative transmissions from heterozygous parents to affected offspring, particularly for the hydrocephalus and eye only disease subgroups. We therefore used (Table 5) logistic regression in a case-control approach to compare clinical phenotypes within the study to see if we could derive more statistical power to distinguish the possible roles of rs1718119 and the known functional SNP rs2230912 in contributing to hydrocephalus versus other clinical signs of disease. This analysis suggested an apparent association between the minor T(+)/A(−) allele at rs1718119 and disease when hydrocephalus were set as the case group in comparisons with each of the other clinical subgroups. However, the likelihood ratio test provided no evidence for dominance effects, and when the other clinical phenotypes were set as the cases the corollary was for disease associated with the common C(+)/G(−) allele. Since there was no evidence for a bias in transmission of alleles to children with hydrocephalus in the family-based analysis, we interpret the logistic regression analysis as confirmation of the association of non-hydrocephalus clinical phenotypes with the common allele at rs1718119. Interestingly, the logistic regression analysis did not support an association with the known functional SNP rs2230912 (Table 5B).

Family-based haplotype analysis confirms the association with rs1718119

Examination of LD patterns between SNPs at *P2RX7* (Figure 1A) for the NCCCTS sample suggests the presence of haplotypes between rs1718119, rs2230912 and rs1621388 that could account for the associations at rs2230912 revealed in the family-based analysis of clinical sub-types. In particular, LD between rs2230912 and each of the other two SNPs (known to be in complete LD with each other) is associated with D'=1, but with low r^2, indicating that the common allele at rs2230912 does not always occur on the same rs1718119_rs1621388 haplotype even though the rare allele does. This is consistent with the variant rs2230912 arising more recently than variants at rs1718119 and rs1621388. Haplotype analysis performed in TRANSMIT (Table 6) using the children with intracranial calcification or eye disease but no hydrocephalus (AFF no HYD) confirmed over transmission (P=0.0006) of the haplotype C_A_C comprising the 3 common alleles at these 3 SNPs. Interestingly, both the common and the minor allele at rs2230912 occurred on significantly under-transmitted haplotypes (T_A_T and T_G_T) with the minor alleles at the other two SNPs, suggesting that this SNP does not itself account functionally for the association observed. Similar results were obtained for transmission of haplotypes to children with other clinical phenotypes without hydrocephalus (data not shown).

Discussion

The results of this study demonstrate that polymorphism at *P2RX7* influences susceptibility to toxoplasmosis in a North American family study cohort in which children presented with a range of clinical signs following congenital infection with *T. gondii*, and for retinochoroiditis caused by post-natal infection with *T. gondii* in Brazil. This is an interesting result in that it indicates that $P2X_7$ function influences the outcome of *T. gondii* infection independently of both the route of transmission and parasite genotype, which varies considerably amongst toxoplasmosis isolates from Brazil compared to parasites associated with congenital disease in North America.35 Clinical sub-group analysis in the North American cohort confirmed association with eye disease, but the overlap between retinal disease and other clinical signs in individual children made it difficult to determine definitively whether *P2XR7* also contributes to intracranial calcifications or other generalized signs of disease such as splenomegaly and thrombocytopenia. Comparison of effect sizes and significance levels suggested that this was the case. Interestingly, no evidence was found in the family-based analysis for association with hydrocephalus, and the effect size of SNP variants on other clinical phenotypes was markedly enhanced when hydrocephalus children were removed from the analysis. This suggests either that *P2RX7* is not expressed or functional at the gestational time when critical events that lead to hydrocephalus occur, or that the functional influence of *P2RX7* does not affect, or has opposing effects compared to other clinical phenotypes, on these events.

In both studies, protection was associated with the ancestral T(+)/A(−) (threonine) allele at rs1718119 which was the minor allele (frequencies 0.320 and 0.296) in these samples. This is more in line (Table 2) with the frequency of this allele observed in Asian (0.216) populations as compared to European (0.492) or Subsaharan Africa (0.517) populations.36 Whilst this is likely to reflect the mixed ethnicity present in both studies (see legend to Table 1), the use of a family-based study meant that our association analysis was robust to ethnic mixture/admixture in both study populations, as well as to pedigree clustering in Brazil. The corollary to protection associated with the ancestral T allele is that susceptibility to toxoplasmosis is associated with the derived C(+)/G(−) (alanine) allele, which was the common allele (frequencies 0.680 and 0.704) in both populations studied. The derived C allele at SNP rs1718119 encodes an alanine at position 348 in the amino acid sequence, as opposed to a threonine encoded by the ancestral primate T(+)/A(−) allele. Although there are published data32–34,37,38 demonstrating change of function with non-synonymous amino acid changes associated with allelic variants at SNPs rs2230911, rs2230912 and rs3751143, these were not statistically associated (Table 3B) with disease in Brazil. Haplotype analysis in the NCCCTS study demonstrated that association observed with the common allele at the known39 functional SNP rs2230912 was only observed when it was on a haplotype with the C(+)/G(−) allele at rs1718119. There are no reports to date for functional analysis of rs1718119 variants. We might expect that the ancestral T allele encodes the fully functional allele, and therefore that protection is associated with fully functional $P2X_7$ activity. Reduction in the pro-inflammatory response might therefore be secondary to enhanced reduction in parasite load due to a fully functional $P2X_7$ molecule. The alternative view is that the common derived allele is the fully functional allele, and that

pathologies associated with retinochoroiditis or other clinical signs are directly caused by enhanced pro-inflammatory responses mediated by a fully functional $P2X_7$ molecule.

Our analysis focused predominantly on SNPs that cause amino-acid substitutions, rather than a full analysis of haplotype-tagging SNPs across the gene. Hence, we cannot discount the possibility that rs1718119 is not the etiological variant, but is in LD with another functional variant. Although we did not find statistical association with the known functional variants in this study, our study samples were only powered to find large effect sizes for common alleles. However, large-scale re-sequencing studies of complex disease40 have now demonstrated that disease genes identified through initial analysis of common variants are also associated with rarer variants in the same gene when sufficiently large sample sizes are used. Hence, it is possible that a larger study of toxoplasmosis would find association with the rare functional variants studied here, which would help in determining what the pathological consequences of fully functional compared to loss-of-function variants might be. In addition, further work is required to determine the functional significance of the association with rs1718119, how this relates to expression and function of $P2X_7$ in the developing fetus and in post-natal infection, and which of the many pleiotropic effects of $P2X_7$ are responsible for mediating protection. At this time, the results presented here provide promising initial genetic support for the hypothesis that purinergic receptor $P2X_7$ may be functionally important in determining resistance and susceptibility to both congenital and post-natally acquired *T. gondii* infection.

Materials and methods

Family sample and clinical phenotypes for congenital toxoplasmosis from North America

Case-parent trios for the North American cohort were from the National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS).1 Ethical approval for the study was obtained from the local Institutional Review Boards of the University of Chicago and Michael Reese Hospital and Medical Center, and oversight was provided by an Internal Data Safety Monitoring Committee, the Data Safety Monitoring Board, and NIH. The diagnosis of congenital toxoplasmosis was confirmed on the basis of clinical findings and testing in the Toxoplasmosis Serology Laboratory (Palo Alto Medical Research Institute) as described. 1,5 At birth or time of diagnosis, each child was examined in the same center in Chicago with standardized ophthalmologic examination and review of all medical records and a brain CT scan 1. Samples for 176 clinically confirmed children were available for the genetic study, 138 from an ongoing treatment trial.1,2,5 Inclusion criteria for these 138 children were as follows: (1) age less than 2.5 months at diagnosis, (2) diagnosis of congenital toxoplasmosis highly likely as previously described2, (3) willingness to be periodically evaluated in Chicago, and (4) no concomitant immunosuppressive conditions. The additional 38 children presented after the first year of life and were therefore not treated during this time. However, their clinical evaluation was as described before.5 Peripheral blood cells were isolated and cryopreserved from all children and their mothers and some fathers. A small sample (10μl) of these cells in cryopreservation mix was placed in 100μl transport/lysis buffer (as above), and shipped to Cambridge at ambient temperature. A total of 149 children and available parents met the inclusion criteria to participate in the study,

which included successful preparation of DNA. Transmission disequilibrium test (TDT) power approximations41 showed that the ~100 full trio equivalents (Table 1) had ≥70% power to detect allelic association at an odds ratio of 2 (0.5 for protection) at P=0.01 for markers with minor allele frequencies ≥0.3. At birth or time of diagnosis, infected children presented with one or more of the following clinical signs: intracranial calcifications, hydrocephalus, retinal lesions, or with other signs of pro-inflammatory disease (e.g. hepatosplenomegaly or thrombocytopenia).

Family sample and clinical phenotypes for toxoplasmosis from Brazil

As outlined in our previous report,14 DNA from a total of 160 individuals from 60 families was available for the study (Table 1B); 30 families containing at least one offspring affected with ocular disease due to acquired toxoplasmosis plus parents, and 30 sibships comprising affected individuals plus unaffected sibs. TDT power approximations41 showed that the 60 families had ≥75% power to detect allelic association at an odds ratio of 3 (0.3 for protection) at P=0.01 for markers with minor allele frequencies ≥0.3. Families were from an area of the city of Campos dos Goytacazes, located in the northern region of the state of Rio de Janeiro.42 Ethical approval was obtained through the National Research Ethics Committee (Health Ministry of Brazil – n. 013/2007). All individuals classified as affected were *T. gondii*-seropositive and presented with posterior retinal/retinochoroidal inactive lesions as described.12 A total of 68 cases were included in the study, 39 females and 29 males. The age range across all cases was 9 to 48 years, with mean±SD of 27.01±10.02 years; for females 27.25±10.56, and for males 26.67±9.34 years. Most cases with ocular toxoplasmosis were known to be due to acquired toxoplasmosis from the time at which they became positive for *T. gondii* specific IgG, but at least one case was due to confirmed congenital infection.

Genotyping

Genotyping was performed using the Taqman™ technology for *P2RX7* SNPs at rs208293, rs28360457, rs1718119, rs2230911, rs2230912, rs3751143, rs1653624, rs1621388. Full details of these SNPs are in Table 2. All were in Hardy Weinberg Equilibrium in genetically unrelated founders of the NCCTS families, in genetically unrelated founders of the Brazilian families, and in a set of unrelated controls from the same region of Brazil (data not shown).

Statistical analyses

Family-based allelic association tests based on the TDT but generalized to allow analysis under additive and dominant models of inheritance were performed within FBAT under the null hypothesis of “no linkage and no association”.43,44 Case-pseudocontrol conditional logistic regression analysis was used to determine effect size (odds ratio) and 95% confidence interval for allelic association.45 A likelihood ratio test comparing the 1df and 2df tests was used to determine whether there were dominance effects. Logistic regression analysis was used for case-control comparisons of children with different clinical phenotypes within the NCCCTS cohort, with the likelihood ratio test again used to determine dominance effects. TRANSMIT46 was used to analyze haplotype transmission disequilibrium.

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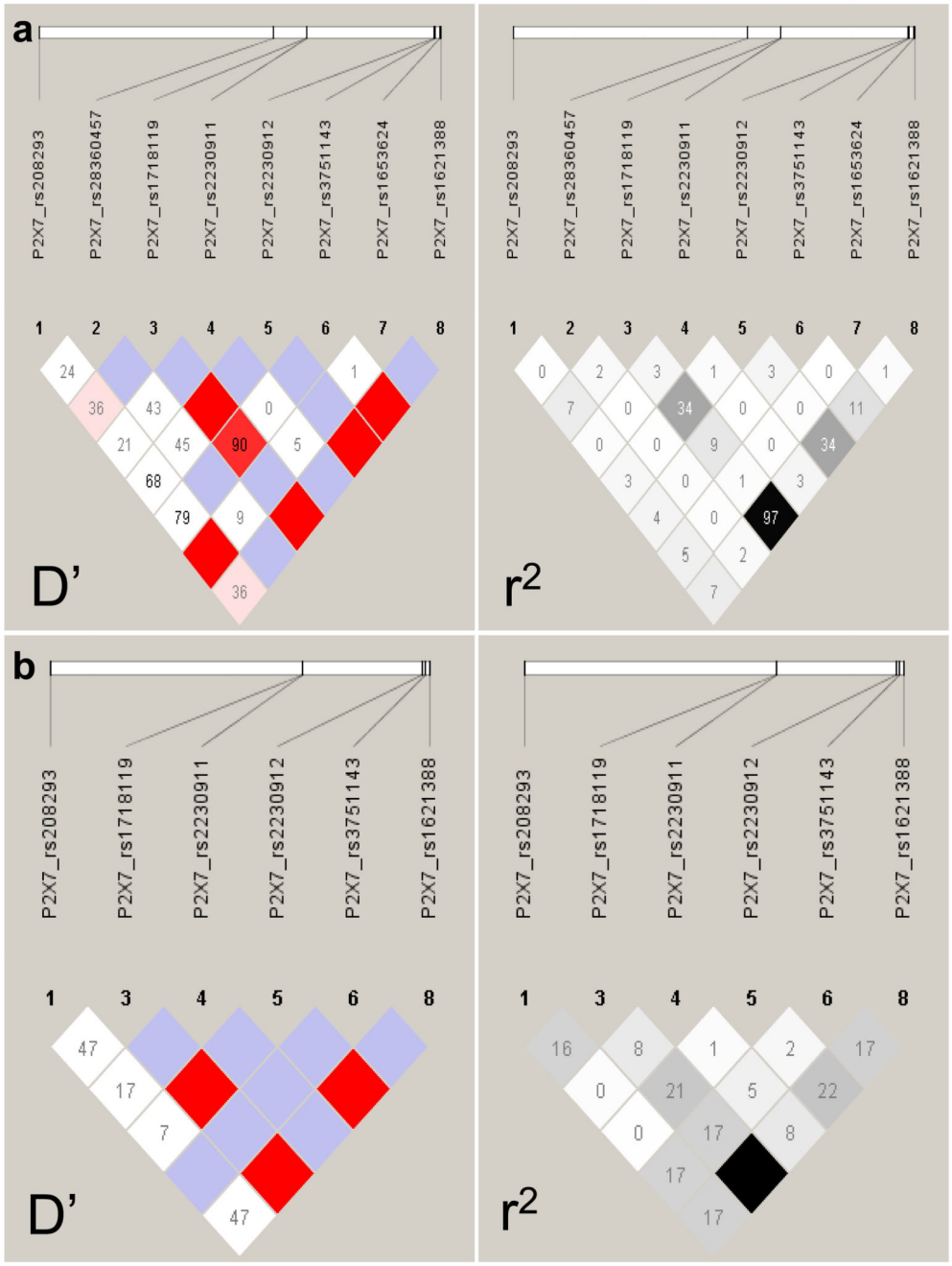

Figure 1.

Haploview analysis for D' and r^2 pairwise measures of LD between *P2RX7* SNPs in unrelated family founders for (a) NCCCTS and (b) Brazil. D' values and confidence levels (LOD) are represented as bright red for D'=1, LOD≥2; blue for D'=1, LOD<2; white for D'<1, LOD<2. r^2 values are represent as black for r^2=1, white for r^2=0, with intermediate values for $0<r^2<1$ indicated by shades of grey. The numbers within the squares represent the D' or r^2 scores for pairwise LD.

Table 1

Details of families used in the FBAT analysis for (A) the NCCCTS study and (B) the Brazilian study. In (A), the families comprised 69% Caucasian, 15% Hispanic, 8% Asian or Pacific Islander, 3% African American, 0.7% Native American, 4.7% mixed race. In Brazil, the population is also known to be admixed for Caucasian, African, native Brazilian and Asian ancestries.47 The use of a TDT-based association analysis was robust to this ethnic mixture/admixture in these cohorts.

(A)

Clinical Signs	Abbreviation	Affected Children	Parents	
			2 parents	1 parent
Nuclear families				
All signs = all infected children	INF	149	64	85
Eye and/or brain signs = affected	AFF	124	53	71
Eye signs (with/without brain signs)	EYE all	113	47	66
Eye signs (no brain signs)	EYE only	20	8	12
Brain signs (Hydrocephalus and/or calcifications)	BRAIN	100	44	56
Hydrocephalus (with/without calcifications)	HYD	49[b]	18	31
Intracranial Calcifications only	CALC	51	26	25
Neither eye or brain[a]		25	9	16
Total		149		

(B)

Family Type	No. of Families	Children		Parents	Total
		Affected	Unaffected		
Nuclear families					
1 affected offspring	28	28	0	56	84
2 affected offspring	2	4	0	4	8
Sibships					
1 affected offspring	25	25	31	0	56
2 affected offspring	4	8	1	0	9

(B)					
Family Type	**No. of Families**	**Children**		**Parents**	**Total**
		Affected	**Unaffected**		
3 affected offspring	1	3	0	0	3
Total					**160**

[a] These children were confirmed antibody positive for toxoplasmosis at birth and had other clinical signs including hepatosplenomegaly.

[b] Only one child had hydrocephalus without brain calcifications.

Table 2

Details of the genotyped SNPs including allele frequencies for Caucasian (CEPH), Asian (JPT) and Subsaharan (YRI) populations as recorded in the NCBI Entrez SNP database.36

Gene/SNP	bp Alias	Position in Gene	Amino Acid Change	Allele (Strand)[a]	Caucasian Allele F	Asian Allele F	Subsaharan African Allele F
rs208293	G>A	Intron 4	-	G (+)[a]	0.650	0.557	0.150
				A (+)	0.350	0.443	0.850
rs28360457	946G>A	Exon 9	Arg-307-Gln	G (+)[a]	-	-	-
				A (+)	-	-	-
rs1718119	1068T>C	Exon 11	Thr-348-Ala	T (+)[a]	0.492	0.216	0.517
				C (+)	0.508	0.784	0.483
rs2230911	1096C>G	Exon 11	Thr-357-Ser	C (+)[a]	0.900	0.864	0.808
				G (+)	0.100	0.136	0.192
rs2230912	1405A>G	Exon 13	Gln-460-Arg	A (+)[a]	0.783	1	0.983
				G (+)	0.217	0	0.017
rs3751143	1513T>G	Exon 13	Glu-496-Ala	T (+)[a]	0.864	0.761	0.933
				G (+)	0.136	0.239	0.067
rs1653624	1729T>A	Exon 13	Ile-568-Asn	T (+)[a]	0.983	1	1
				A (+)	0.017	0	0
rs1621388[b]	1772C>T	Exon 13	Pro-582-Pro	C (+)[b]	0.625	0.875	0.652
				T (+)	0.375	0.125	0.348

[a] Ancestral primate allele

[b] Ancestral allele not known; data for CEU, JPT, YRI not available; data presented for AFD_EUR_PANEL, AFD_CHN_PANEL, AFD_AFR_PANEL.36

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Table 3

FBAT analysis under additive model of inheritance for associations between *P2RX7* SNPs and (A) clinical signs of congenital toxoplasmosis in NCCCTS, and (B) ocular disease caused by infection with toxoplasmosis in Brazil. # Fam = number of families informative for the FBAT analysis; S and E(S) represent the observed and expected transmissions for that allele, V(S) is the variance. A positive Z score indicates association with disease; a negative Z score indicates the non-associated or protective allele. Bold indicates significant associations at $P<0.05$.

(A)

Gene/SNP	Allele (Strand) [a]	Allele F	# Fam	S	E(S)	Var(S)	Z score	P value
P2RX7_rs208293	G (+)	0.769	25	32	30	8	+0.707	0.479
	A (+)	0.231	25	18	20	8	−0.707	0.479
P2RX7_rs28360457	G (+)	0.980	5[b]	-	-	-	-	-
	A (+)	0.020	5[b]	-	-	-	-	-
P2RX7_rs1718119	C (+)	0.680	39	56	47.5	12.25	**+2.429**	**0.015**
	T (+)	0.320	39	22	30.5	12.25	**−2.429**	**0.015**
P2RX7_rs2230911	C (+)	0.907	19	25	25.5	5.25	−0.218	0.827
	G (+)	0.093	19	13	12.5	5.25	+0.218	0.827
P2RX7_rs2230912	A (+)	0.859	22	33	30.5	6.75	+0.962	0.336
	G (+)	0.141	22	11	13.5	6.75	−0.962	0.336
P2RX7_rs3751143	T (+)	0.812	17	26	24	5	+0.894	0.371
	G (+)	0.188	17	8	10	5	−0.894	0.371
P2RX7_rs1653624	T (+)	0.977	5[b]	-	-	-	-	-
	A (+)	0.023	5[b]	-	-	-	-	-
P2RX7_rs1621388	C (+)	0.674	38	55	47	12	**+2.309**	**0.021**
	T (+)	0.326	38	21	29	12	**−2.309**	**0.021**

(B)

Gene/SNP	Allele (Strand)[a]	Allele F	# Fam	S	E(S)	Var(S)	Z score	P value
P2RX7_rs208293	G (+)	0.623	25	35	29.25	9.049	+1.912	0.056
	A (+)	0.377	25	17	22.75	9.049	−1.912	0.056

(B)								
Gene/SNP	**Allele (Strand)[a]**	**Allele F**	**# Fam**	**S**	**E(S)**	**Var(S)**	**Z score**	**P value**
P2RX7_rs28360457	G (+)	0.994	1[b]	-	-	-	-	-
	A (+)	0.006	1[b]	-	-	-	-	-
P2RX7_rs1718119	C (+)	0.704	26	46	37.75	7.132	**+3.089**	**0.002**
	T (+)	0.296	26	10	18.25	7.132	**−3.089**	**0.002**
P2RX7_rs2230911	C (+)	0.842	16	26	23.83	4.417	+1.031	0.303
	G (+)	0.158	16	8	10.17	4.417	−1.031	0.303
P2RX7_rs2230912	A (+)	0.942	10	18	17.25	2.382	+0.486	0.627
	G (+)	0.058	10	4	4.75	2.382	−0.486	0.627
P2RX7_rs3751143	T (+)	0.726	29	41	40.83	8.861	+0.056	0.955
	G (+)	0.274	29	19	19.17	8.861	−0.056	0.955
P2RX7_rs1653624	T (+)	1.0	0[b]	-	-	-	-	-
	A (+)	0	0[b]	-	-	-	-	-
P2RX7_rs1621388	C (+)	0.691	23	38	31.75	6.132	**+2.524**	**0.012**
	T (+)	0.309	23	8	14.25	6.132	**−2.524**	**0.012**

[a] Major allele for this population shown first;

[b] Too few families contributing to the analysis

Table 4

Case-pseudocontrol conditional logistic regression analysis under an additive model of inheritance for associations between (A) *P2RX7* rs1718119 and (B) rs2230912 SNPs and different clinical phenotypes. Odds ratios, 95% confidence intervals (CI) and *P*-values are for association with the common allele at each SNP. Abbreviations: NV = not valid less than 10 informative families; INF = all infected children; AFF = children with brain and eye signs; BRAIN = any brain signs; CALC = intracranial calcifications; HYD = hydrocephalus; EYE = eye signs

(A)

Gene/SNP	Clinical Phenotype	Allele	N Informative Trios	Odds Ratio	95% CI	P-value
P2RX7_rs1718119	INF	C	58	2.06	1.13–3.74	**0.017**
	INF no HYD	C	42	3.00	1.41–6.38	**0.004**
	AFF	C	50	2.07	1.09 – 3.91	**0.025**
	AFF no HYD	C	34	3.28	1.41–7.66	**0.006**
	BRAIN	C	41	2.09	1.01 – 4.29	**0.044**
	CALC no HYD	C	25	4.25	1.43–12.63	**0.009**
	EYE (+/− brain)	C	44	1.93	1.01 – 3.68	**0.046**
	EYE no HYD	C	29	3.00	1.28–7.06	**0.012**
	EYE no BRAIN	C	8	-	-	NV
	HYD	C	16	0.86	0.29–2.55	0.782

(B)

Gene/SNP	Clinical Phenotype	Allele	N Informative Trios	Odds Ratio	95% CI	P-value
P2RX7_rs2230912	INF	A	61	1.45	0.68–3.13	0.339
	INF no HYD	A	43	3.00	1.09–8.25	**0.033**
	AFF	A	52	1.56	0.67–3.59	0.301
	AFF no HYD	A	34	4.33	1.23–15.21	**0.022**
	BRAIN	A	43	1.13	0.43–2.92	0.808
	CALC no HYD	A	25	4.00	0.85–18.84	0.080
	EYE (+/− brain)	A	47	1.85	0.74–4.65	0.187
	EYE no HYD	A	30	5.99	1.34–26.81	**0.019**

(B)

Gene/SNP	Clinical Phenotype	Allele	N Informative Trios	Odds Ratio	95% CI	P-value
	EYE no BRAIN	A	8	-	-	NV
	HYD	A	18	0.17	0.02–1.38	0.097

Table 5

Results of logistic regression “case-control” analysis in which children with hydrocephalus (HYD) were compared with other clinical phenotypes.

(A)						
Gene/SNP	**Case-Control Comparison**	**Allele**	**N Case/ Control**	**Odds Ratio**	**95% CI**	**P-value**
P2RX7_rs1718119	HYD vs INF no HYD	T	48/96	2.04	1.18–3.52	**0.010**
	HYD vs AFF no HYD	T	47/74	2.38	1.33 – 4.25	**0.003**
	HYD vs CALC no HYD	T	47/51	2.32	1.23–4.40	**0.009**
	HYD vs EYE all no HYD	T	46/64	2.39	1.31–4.38	**0.005**
	HYD vs EYE only	T	47/20	2.32	0.96 – 5.61	0.061

(B)						
Gene/SNP	**Clinical Phenotype**	**Allele**	**N Case/ Control**	**Odds Ratio**	**95% CI**	**P-value**
P2RX7_rs2230912	HYD vs INF no HYD	G	48/96	1.11	0.54–2.29	0.780
	HYD vs AFF no HYD	G	47/71	1.27	0.59–2.73	0.550
	HYD vs CALC no HYD	G	47/51	1.08	0.49–2.41	0.842
	HYD vs EYE all no HYD	G	46/64	1.36	0.60–3.07	0.455
	HYD vs EYE only	G	47/20	2.22	0.52–9.59	0.808

Table 6

Results of the family-based haplotype analysis undertaken in TRANSMIT.

Phenotype	Over/Under Transmitted	Allele/ Haplotype	Frequency	Markers (χ^2_{1df}; *P*)		
				rs1718119	rs2230912	rs1621388
AFFnoHYD	Over	C	0.67	8.24; **0.004**		
		A	0.86		4.39; **0.036**	
		C	0.68			8.23; **0.004**
		C.A	0.67	10.47; **0.001**		
		A.C	0.67		10.69; **0.001**	
		C.A.C	0.66	11.69; **0.0006**		
	Under	T	0.33	8.24; **0.004**		
		G	0.14		4.38; **0.036**	
		T	0.32			8.23; **0.004**
		T.G	0.14	3.78; **0.052**		
		T.A	0.19	4.96; **0.026**		
		G.T	0.14		4.00; **0.046**	
		A.T	0.19		5.01; **0.025**	
		T.G.T	0.14	3.72; **0.054**		
		T.A.T	0.20	4.65; **0.031**		