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Genetic variants in Major Histocompatibility Complex-linked genes Associate with Pediatric Liver Transplant Rejection

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Abstract

Background/Aims—Limited access to large samples and independent replication cohorts precludes genome-wide association (GWA) studies of rare but complex traits. To localize candidate genes with family-based GWA, a novel exploratory analysis was first tested on 1,774 major histocompatibility complex single nucleotide polymorphisms (SNPs) in 240 DNA samples from 80 children with primary liver transplantation (LTx), and their biological parents.

Methods/Results—Initially, 57 SNPs with large differences ($p < 0.05$) in minor allele frequencies were selected, when parents of children with early rejection (Rejectors) were compared with parents of Non-Rejectors. In hypothesis-testing of selected SNPs, the gamete competition statistic identified the minor allele G (ancestral allele T) of the SNP rs9296068, near HLA-DOA, as being significantly different ($p = 0.018$) in parent-to-child transmission between outcome groups. Subsequent simple association testing confirmed over- and under-transmission of rs9296068 based on 1) the most significant differences between outcome groups, of 1,774 SNPs tested ($p = 0.002$), and 2) allele (G) frequencies that were greater among Rejectors (51.4 vs. 36.8%, $p = 0.015$), and lower among Non-Rejectors (26.8 vs. 36.8%, $p = 0.074$), compared with 400 normal control Caucasian children. In early functional validation, a) Rejectors demonstrated significant repression of the first HLA-DOA exon closest to rs9296068, and b) Rejectors with the risk allele showed 3-fold greater intra-graft content of B-lymphocytes, whose antigen-presenting function is inhibited selectively by HLA-DOA, compared with Rejectors without the allele.

Conclusions—The minor allele of the SNP rs9296068 is significantly associated with LTx rejection, and with enhanced B-lymphocyte participation in rejection, likely due to a dysfunctional HLA-DOA gene product.

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Introduction

In children, liver transplantation (LTx) is usually performed for multiple *congenital* liver diseases, and results in highly *variable* outcomes (1). Acute cellular rejection (ACR) occurs in 50% and post-LTx lymphoma-like malignancy affects 2-10% (2). Genetic variants, exemplified most commonly by single nucleotide polymorphisms (SNPs) are a significant basis for individual variation (3). However, the majority of the >10 million catalogued SNPs do not alter gene function (4). Also, promising associations involving discrete SNPs are rarely reproduced (5-7). *Population stratification*, or over-representation of one ethnicity within an outcome group may allow a SNP representing this ethnicity to be viewed as the outcome-specific SNP (8). Family-based association studies can minimize stratification (9). In this design, parental genotype serves as the control, and transmission frequency of a SNP from heterozygous parents to affected offspring is compared with the expected 50% transmission frequency. Significant deviations reflect transmission disequilibrium, and are more likely to represent disease-specific variation than variations due to ethnicity. The known location of such a SNP is then used to identify a potential candidate gene, and a causal variant in proximity. Increasingly, genome-wide association (GWA) studies are being conducted, recognizing that most disease traits are complex, and likely originate from an interaction between multiple causal variants within multiple genes, and the environment.

Several limitations preclude application of GWA in transplantation, especially among pediatric LTx recipients. First, simultaneous testing of large numbers of SNPs necessitates stringent multiple-testing correction. Under these conditions, statistical power requirements mandate a large subject sample, or a two-step study in which candidate SNPs identified in a screening sample are replicated in a second step. Accruing a homogeneous validation cohort can take several years, because approximately 550 pediatric LTx are performed annually in the United States, and are distributed among >50 transplant centers (10). Secondly, the numbers of candidate SNPs in an association study may not exceed false-discovery thresholds. From such a limited list of candidates, any true-positive association may at best account for large effects.

We report for the first time, a novel, multi-step approach to candidate gene localization, which incorporates preliminary functional validation in the same test cohort. First, a two-tier, family-based association method identifies the candidate gene or locus. Next, transmission distortion in that locus is confirmed by allele frequency comparisons with a large control group. Because SNPs within non-coding regions can influence gene expression and splicing at distances of up to 100 kb, differential regulation and alternative splice variants of candidate genes is evaluated with probes specific to exons of whole gene transcripts in the first of two preliminary functional validation tests (11,12). In the second, the candidate is characterized in diseased tissue with immunohistochemistry. Because our sample size is small, and consists of 80 case-parent-parent trios, our association method is based on the following biologic assumptions: A) If a SNP is strongly associated with an inherited trait, it should demonstrate differences in parental allele frequencies (PAF) between the two groups, B) In family-based association testing, a genetic variant that is associated with rejection/non-rejection should show over-transmission in one outcome group and under-transmission in the other in the gamete competition test (GC). The GC statistic evaluates transmission disequilibrium using full pedigree data, similar to the well-known Transmission Disequilibrium Test (TDT) (8), but is based on a likelihood ratio test (13). The GC statistic also handles missing data and allows haplotype-based analysis. C) In a confirmatory step, potential candidates should also demonstrate i) significant differences in allele frequency when the two outcome groups are compared with each other, and ii) evidence of overtransmission in one group and undertransmission in the other when compared individually, with a large cohort of 400 normal Caucasian children.

We asked whether rejection/non-rejection outcomes are associated with genetic variants in the major histocompatibility complex (MHC) region. The 1,774 MHC-SNPs evaluated here are a subset of 550,000 genome-wide SNPs recently characterized in our test population. MHC-SNPs have been analyzed separately at first, to explore the utility of our statistical method, which is based in part on screening/testing approaches for quantitative traits proposed by others, by applying it to a candidate region with known impact in organ transplantation (14-17). The results show that the minor allele G at the SNP locus rs9296068, which is significantly associated with rejection outcomes, localizes to the 5' UTR (untranslated) region of the HLA-DOA gene. Differential regulation of HLA-DOA, which is selectively expressed in B-lymphocytes, is suggested by associated repression of the first HLA-DOA exon, and significantly higher B-lymphocyte content in allograft biopsies from Rejectors (18,19).

Methods

Patient Population

All studies were performed with approval from the University of Pittsburgh's Institutional Review Board. DNA was extracted from 3 ml whole blood samples from 80 primary pediatric LTx recipients ages 0-22 years, and their biological parents (240 DNA samples). All children received previously described Tacrolimus monotherapy after steroid-free induction with rabbit, anti-human thymocyte globulin (rATG, Genzyme, Cambridge, MA) (20). Children who experienced biopsy-proven ACR within the first 60 days after LTx were termed Rejectors. Normal controls consisted of 400 disease-free Caucasian children with no prior or ongoing use of immunosuppressants, or transplantation. In addition, all 400 samples were selected for the absence of large copy number variants that could suggest a disease association. This normal control population was recruited at the Center for Applied Genomics at the Children's Hospital of Philadelphia (CAG-CHOP).

Genotyping

was performed with 550,000 genome-wide SNP loci with the HumHap550k SNP bead array (Illumina, San Diego, CA.). Analysis from this experiment was restricted to 1,813 SNPs covering 168 MHC genes on chromosome 6p, of which 1,774 passed our quality control criteria.

DNA extracted by the Gentra Purigene system (Minneapolis, MN) was activated, and SNPs were characterized using manufacturer's protocol. The Illumina Beadstation GX software was used to extract genotype calls (Illumina, San Diego, CA.) (21).

Statistical Methods

A total of 1,813 SNPs from the MHC region of the HumHap550k SNP array (Illumina, San Diego, CA.) were identified for analysis. Genotype data from the array were merged with pedigree and phenotype data and quality controlled for the following: Mendelian errors (recoded to missing), Hardy-Weinberg equilibrium (SNPs with deviations at $p < .001$ removed), missing rate across patients ($>10\%$ removed), minimal minor allele frequency (SNPs with MAF $<1\%$ removed), and low genotyping rate for an individual ($<10\%$ removed in the simple association test) using the PLINK v0.99r software package (22). This filtering reduced the number of SNPs to 1,774 total. A two-sample proportions statistic (two-sided) was used to determine PAF differences (p -value cutoff <0.05) between parents of Rejectors and Non-Rejectors. This screening step yielded a subset of MHC-SNPs for subsequent hypothesis testing using the Gamete Competition (GC) statistic, as implemented in the software package Mendel v7.0.0 (23). In this approach, a segregation parameter τ is estimated by maximum likelihood from the family data, and a likelihood ratio test is then applied. For a two-allele locus with alleles 1 and 2, the probability that a heterozygous parent with genotype 1/2 transmits allele 1

is defined as $\Pr(1/2 \rightarrow 1) = \tau_1 / (\tau_1 + \tau_2)$; the null hypothesis of Mendelian transmission corresponds to $\tau_1 = \tau_2 = 1$ (14). The transmission parameter τ_k is set to 1 for the most frequent allele k , while the remaining τ_k values (for all k not equal to the most frequent allele) are estimated by maximum likelihood. In our application, we assumed symmetric transmission: that is, an allele that is over-transmitted to Rejectors is assumed to be similarly under-transmitted to Non-Rejectors. Results are summarized as the p-value and chi-squared statistic (with 95% confidence bounds) for the PAF comparison, and the estimated segregation parameter (τ), the allele frequency, and the p-value of the GC statistic (see Appendix 1). Note that this screening/testing approach markedly reduces multiple testing problems because the PAF test (which only uses parental genotypes) is independent of the gamete competition test (which depends on how often specific alleles are transmitted to the children). Thus, since the PAF test alone is used for SNP selection, we only have to adjust for the actual number of GC tests conducted on the much smaller number of selected SNPs.

Confirmatory association testing with 400 normal Caucasian controls—

Comparisons of MAF for significant MHC-SNPs were conducted, between Rejectors and normal controls, and between Non-Rejectors and normal controls (chi-square test).

Validating candidacy of the HLA-DOA locus with differential gene splicing patterns—

The Affymetrix Human Exon 1.0 ST array was used to measure differential splicing patterns in archived RNA isolated from 29 of 80 children. Probe summaries for both the genes and exons were computed using the Affymetrix Power Tools (APT) software and ‘rma-sketch’ normalization method. The gene-level normalized intensities (NI) were computed with the MiDAS algorithm and both the splicing index (SI) (24) and Student’s t-test p-values (two-sided) were computed on the NI values in R (25). Principal components analysis (PCA) was calculated separately on exon-level and gene-level intensities and was used to remove 3 outlier samples, for a remaining total of 26 samples-11 Rejectors and 15 Non-Rejectors. To remove low expressed gene-level probes, those with values less than 3.5 ($\log_2$ scale) in >50% of the samples in either group were filtered out leaving 17,242 of the original 22,011 probes. Those gene-level probes that were highly differentially expressed between groups were also removed using a fold change threshold of $\log_2$ (10). This step accounts for the tendency for the gene-level probe set intensities in each group to be “disproportionately affected by background noise or saturation” (Affymetrix Technical notes). Exon-level probes were filtered based on APT’s detection above background (DABG) p-value greater than 0.05 in n-1 samples leaving 218,402 of the original 287,329 exon-level probes.

After gene and exon probe filtering, 7 gene-level probes (and 67 corresponding exon-level probes for the genes) within a window size of 200 kb that included the HLA-DOA gene on chromosome 6 were extracted and examined for differential splicing (positions 33,000,000-33,200,000). This was conducted to restrict the analysis to only the immediate region surrounding the HLA-DOA gene.

Relating intra-graft content of B-lymphocytes, to rs9296068—Because the HLA-DOA gene inhibits class II antigen presentation selectively in B-cells, we asked whether B-cells were present in rejecting allograft biopsies, and whether the B-cell content of allograft biopsies varied with the presence or absence of the risk allele of rs9296068. Slides were re-cut from stored tissue blocks for 36 of 80 children, with 4 μ m thick tissue sections, stained in batches on a Ventana immunostainer using mouse monoclonal anti CD79a (DAKO M7050 at 1:100, Ventana CCI mild, 32 minutes at 32C, iView DAB detection), as well as their positive and negative biological staining controls for each batch. Hematoxylin counterstain was applied after DAB detection. The surface marker CD79a identifies cells of B-cell/plasmacytoid lineage.

Cell counting was done blinded to outcome, and prior to histologic re-review of the tissues. Duplicate counts were done on each 10th case to assess variance. Portal and lobular regions of allografts were optically scored separately, at a magnification of $\times 400$ and $\times 200$, respectively. B-cell counts were expressed as total number per portal area, and total number per lobule (Figure 3).

Results

Demographics

Early ACR occurred at mean $\pm$ SD 32.5 $\pm$ 4.2 days after LTx in 37 children who were termed Rejectors. Rejectors and Non-Rejectors (n=43) were similar with respect to age (median $\pm$ SEM 6 $\pm$ 1.1 vs 4.6 $\pm$ 0.9 years, p=NS), male: female gender distribution (16:21 vs 21:22, p=NS), and etiology of liver failure requiring LTx (Table 1). The racial distribution in the Rejector and Non-Rejector groups was (Caucasian: African-American: Other=31:2:4 vs 42:0:1, respectively). Also, there were no differences between Rejectors and Non-Rejectors, in donor-recipient matching at the HLA-A, HLA-B, and HLA-DR loci (1.4 $\pm$ 1.2 vs 1.8 $\pm$ 1.2 antigens, p=NS), or disease severity as reflected in the Pediatric End-Stage Liver Disease score (PELD, 25.5 $\pm$ 13.2 vs 24 $\pm$ 14.2, P=NS). The sample size was too small to evaluate the effect of disease, age at transplant, and immunosuppression on rejection outcomes, or whether the highly associated SNP(s) were independent predictors of rejection outcome.

Fifty-seven SNPs passed our screening test due to large differences in MAF, when parents of Rejectors were compared with parents of Non-Rejectors (Appendix 1). When the GC statistic was applied to these 57 SNPs, only one SNP, rs9296068, in the 5' flanking UTR of HLA-DOA demonstrated differences between groups with p<0.05 in parent-to-child transmission (p=0.0183, Table 2). Specifically, the minor allele G was transmitted more frequently to Rejectors, and less so to Non-Rejectors from biological parents. The physical position of the implicated SNP is 33096673 (Build 35). Figure 1 summarizes the results of the PAF comparison for the entire set of 1,774 MHC SNPs (upper panel), and the results of the GC test applied to the 57 selected SNPs for Rejectors and Non-Rejectors (lower panel).

In the confirmatory association testing step, 77 SNPs showed significant differences in allele frequencies when Rejectors were compared directly with Non-Rejectors (p $\leq$ 0.05). Among these 77 SNPs, additional between-group comparisons showed 39 SNPs to be significantly different among Rejectors, and 19 SNPs to be significantly different among Non-Rejectors, when each group was compared separately with 400 normal controls (Appendix 2). In direct comparisons, the differences between Rejectors and Non-Rejectors were most significant (p=0.002) for the SNP rs9296068 (Table 3). When compared with normal controls, the minor allele (G) of rs9296068 was more commonly seen among Rejectors (36.7% vs 51.4%, p=0.015), but less commonly among Non-Rejectors (36.7% vs 26.8%, p=0.074). Only one other SNP rs9276994 shows similar differences in distribution, when Rejectors are compared with Non-Rejectors (48.6% vs 28%, p=0.009), and when normal controls are compared either with Rejectors (37.7% vs 48.6%, p=0.074) or with Non-Rejectors (37.7% vs 28%, p=0.083) (Table 4). In all, five of 14 top-ranked discriminatory SNPs localized to the 5' flanking UTR of HLA-DOA (Table 4).

The first HLA-DOA exon is repressed in Rejectors—Following probe filtering (explained above) and retention of only those genes with at least one exon with p<0.05, two genes remained, HLA-DOA and HLA-DPA1 (Appendix 3). The first HLA-DOA exon between physical positions 33,085,305-33,085,362 was repressed among Rejectors, as suggested by -1.53 -fold decreased expression, compared with Non-Rejectors (Figure 2). This repressed exon lies immediately adjacent to the 5' flanking UTR of HLA-DOA, which contains the risk allele rs9296068 at position 33,096,673, and is ≈ 11.3 kb removed from the risk allele. In

contrast, ≈ 47.8 kb, and three HLA-DPA exon transcripts separate rs9296068 from the discriminatory HLA-DPA exon at position 33,144,437-33,144,544, whose expression is ~ 1.30 -fold lower among Rejectors, compared with Non-Rejectors.

Intragraft B-cell content is higher in Rejectors with the risk allele of rs9296068 (Figure 3)—The risk allele (G) of rs9296068 was present in seven of 10 liver allograft biopsies from Non-Rejectors (allele frequency=0.4), and 19 of 26 Rejectors (allele frequency=0.5). B-cell content (median $\pm$ SEM) was low at 1.1 cell per portal area in Non-Rejectors. Among Rejectors, the risk allele (G) was absent in seven, heterozygous in 12, and homozygous in seven. B-cell content in respective allograft biopsies from these Rejectors was 4 ± 2.6 cells, 11 ± 3.3 cells, and 16 ± 4.7 cell per portal area, and showed significant correlation with the risk allele ($r^2=0.194$, $p=0.021$, Figure 3). Also, differences between B-cell content were significant when Rejectors without the risk allele ($n=7$, 4 ± 2.6 B-cells/portal area) were compared with Rejectors who were either homozygous or heterozygous for the risk allele of rs9296068 ($n=17$, 11.54 ± 2.4 cells/portal area, $p=0.022$). The lobular areas contained an average of 0-3 B-cells per lobule in both Rejectors and Non-Rejectors, and showed no differences.

Subanalysis in Caucasian children to evaluate population stratification—The HapMap database shows rs9296068 (G) allele frequencies of 31% in Caucasians (CEU), and 67.8% in Yoruba (YRI) populations. To avoid false positive association in case-control comparisons, trios for two African-Americans and all six Non-Caucasians were excluded, five new trios from Caucasians children with LTx added, and both, the PAF comparison and simple case/control association were recalculated for 35 Rejectors, and 42 Non-Rejectors. Four SNPs adjacent to HLA-DOA remain discriminatory ($p<0.05$) in comparison of Rejectors with Non-Rejectors. Among them, rs6457699, rs9276994, and rs9296068 are ranked 3, 8 and 9 among the top ten SNPs, with all showing greater MAF among Rejectors and less among Non-Rejectors, compared with 400 normal Caucasian children (Appendix 4). In the PAF test, higher MAF among parents of Rejectors approached significance for rs6457699 ($p=0.055$) and rs9276994 ($p=0.077$) but not for rs9296068 (40 vs 34.7%, $p=0.43$). This observation suggests that the Rejector group is relatively underpowered to detect MAF differences for the rs9296068 SNP, even though the GC test confirms significant differences in parent-to child transmission between groups for its risk allele ($p=0.004$). Finally, both rs6457699, and rs9276994 are situated ≈ 4 kb and ≈ 6 kb upstream of the first HLA-DOA exon, and the GC test was significant for rs6457699 ($p=0.04$), implicating HLA-DOA once again (Appendix 4).

The GC test, unlike a case/control association, is based on distortions in allele transmission from parent to child, and so should be less sensitive to population substructure. It is of interest to assay whether or not transmission distortion occurs at the SNP of interest in the HapMap non-disease reference population (26). Therefore, the GC statistic was calculated for both alleles of rs9296068 for 30 YRI families (90 individuals), and 20 CEU families (90 individuals) using genotype and pedigree files from the Hapmap database. In both populations, the risk allele failed to show preferential transmission compared with its complementary allele, as suggested by GC p -values >0.05 . Therefore, the null hypothesis of Mendelian transmission cannot be rejected. This sub-analysis supports our conclusions from GC testing of 72 Caucasian and 8 Non-Caucasian trios.

Discussion

Our multi-step approach identifies the HLA-DOA gene, and the B-lymphocyte in which it is exclusively expressed, as plausible candidates contributing to pediatric LTx rejection. Adding early functional validation to family-based association in the same dataset is especially useful, because it obviates the need for immediate replication in an independent cohort, whose accrual may take several years in the case of rare disease traits. This novel approach was motivated by

failure when we first performed genome-wide association with the unmodified TDT applied to all 550,000 SNPs (on-going study and data not shown). All SNPs showing significant p-values failed to remain significant after Bonferroni correction for multiple hypothesis testing.

Because SNP reduction with PAF comparisons and the GC test are independent, the combined p-value for rs9296068 is a multiple of values from both procedures ($0.041 \times 0.0183 = 0.00075$). However, this would not be significant after multiple-testing correction for 1,774 SNPs. Therefore, transmission characteristics of rs9296068 were confirmed by showing that allele frequencies in Rejectors and Non-Rejectors were respectively, significantly greater (51.4 vs 36.8%, $p=0.015$), and less (26.8 vs 36.8%, $p=0.074$) than those in the normal control population of 400 Caucasian children (Table 3). Finally, in direct comparisons of allele frequencies between Rejectors and Non-Rejectors, the 5' flanking UTR of HLA-DOA was represented by five SNPs among 14 top-ranked SNPs ($p \leq 0.01$) (Table 4), of which rs9296068 achieved the highest p-value (0.002), and another, rs9276994, also showed evidence of over-transmission in Rejectors ($p=0.074$), and under-transmission in Non-Rejectors ($p=0.082$), even though parental allele frequencies were similar. Significantly, all five highly-ranked SNPs, beginning with rs6457699 and ending with rs9277015, were present upstream, in an ≈ 11.6 kb segment, at a distance of ≈ 4 -15 kb from the first HLA-DOA exon (Figures 2 and 3). Promoter function has been predicted for a highly conserved ≈ 10 kb region immediately upstream of the first HLA-DOA exon, with Caucasians demonstrating a linkage disequilibrium (LD) block encompassing the rs9296068 locus (Figure 4) (27). Because 3 of 5 discriminatory SNPs localize to this putative promoter, altered transcription factor binding, and differential regulation of HLA-DOA gene expression can be postulated. Significant repression of the first HLA DOA exon among Rejectors in our study supports this view. Decreased HLA-DOA gene expression is also seen with increasing numbers of the risk allele of rs9296068 for both Caucasian and Yoruba populations in public databases (28).

We interpret our observed associations as suggestive of a causal role for the HLA-DOA gene, and for the B-lymphocyte, rather than a causal role for the SNP itself; however causality remains unproven until definitive studies identify a true causal variant, and the mechanism by which it might alter HLA-DOA gene expression and B-cell function. For the association itself, all statistical tests used sequentially in the current study are necessary because neither simple association testing nor family-based association testing generates, by itself, a list of SNPs larger than the expected false positive prediction ($p=0.05$ times 1,774 SNPs=89 false-positives). The HLA-DOA gene, and its adjacent region, is of interest for several reasons. Compared with one of its 3' neighbors, HLA-DPB1, which is highly polymorphic, and can influence immunological outcomes in bone-marrow, corneal and renal transplantation, the HLA-DOA gene is relatively non-polymorphic, and inhibits class II antigen presentation in mature B-cells; but its role in organ transplantation is unknown (29-31,19,20). Our findings lead us to speculate that a missing exon transcript may produce a dysfunctional HLA-DOA gene product, which facilitates rejection by failing to inhibit antigen presentation by B-lymphocytes. The resulting increased B-lymphocyte participation in the rejection process is seen as nearly three-fold higher intra-graft B-lymphocyte content in rejecting liver grafts in our study, when the minor allele G of rs9296068 was present, compared with rejecting allografts from children without this allele. Among pediatric LTx, prior supportive evidence also includes greater resistance of activated B-lymphocytes to immunosuppression, as well as a relative excess of B-lymphocytes during the risk period for early rejection (18,32). B-lymphocyte-rich infiltrates, as well as B-lymphocyte-specific gene expression products have already been demonstrated in renal allografts with steroid-resistant acute cellular rejection (33). While our observations are preliminary, they suggest that the HLA-DOA gene and its vicinity be mapped further to identify novel causal variants.

We acknowledge several limitations. The heterogeneous diagnoses precipitating LTx are potential sources of stratification, although no single disease dominated either outcome group. For example, all 5 children with PSC carried the risk allele, with four experiencing rejection, despite statistically similar proportions of PSC in either group (4/37 Rejectors vs 1/43 Non-Rejectors, $p=NS$). Second, our SNP reduction step which relies on PAF comparisons, is biased by inclusion of eight Non-Caucasians trios, seven in the Rejector group. In accepting rs9296068 as a candidate, an illustrative subanalysis shows that our conclusions would be unchanged if an adequately powered Caucasian sample was available (Appendix 5). For these reasons, we have also relied on functional studies, coupled with allele-specific decrease in HLA-DOA expression in public databases for both CEU and YRI, to suggest biological relevance for the risk allele. We hope to relate differences in HLA-DOA exon repression to allelic variations at rs9296068 in a larger future sample. The small numbers of Rejectors ($n=11$) in whom HLA-DOA exon repression was shown, could not be divided further for adequately powered correlations with allelic variants in this study.

We conclude that our combination of methods can identify biologically relevant associations in small populations. We propose to validate our conclusions and address the primary power limitations of this pilot study by extending it to multicenter LTx populations.

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Appendix 1

Appendix 1
p-values for parental allele frequency comparison, and
for the GC statistic comparing prent-to-child
transmission of SNPs between Rejectors and Non-
Rejectors

The minor allele for each snp is identified in the allele column, and the minor allele frequencies (MAF)s in both the parents and offspring for each group (NR and R) are provided for this allele.

For each SNP in the GC model, the transmission parameter τ_k is set to 1 for the most frequent allele k , while the remaining τ_k values (for all k not equal to the most frequent allele) are estimated by maximum likelihood.

SNP	Closest Gene	Distance to gene	Position	SNP type	Parental allele frequency comparison, p-value	Lower conf. Interval	Higher conf. Interval	Par
rs4713213	OR5V1	0	29446941	INTRONIC	0.046	0.0027	0.2463	
rs4598109	OR5V1	0	29452683	INTRONIC	0.028	0.0146	0.2546	
rs238883	OR5V1	0	29454205	INTRONIC	0.033	-0.2547	-0.0115	
rs238882	OR5V1	0	29454342	INTRONIC	0.012	0.0324	0.2711	
rs238872	OR5V1	0	29459852	INTRONIC	0.040	0.0066	0.251	
rs3094556	OR5V1	0	29461387	INTRONIC	0.004	0.0571	0.2963	
rs3094551	OR5V1	0	29462778	INTRONIC	0.010	-0.2759	-0.0379	
rs3094550	OR5V1	0	29462788	INTRONIC	0.009	-0.28	-0.0399	

SNP	Closest Gene	Distance to gene	Position	SNP type	Parental allele frequency comparison, p-value	Lower conf. Interval	Higher conf. Interval	Par
rs2517817	HLAH_HUMAN	671	29967496	DOWNSTREAM	0.032	-0.1501	-0.009	
rs7758512	Q6ZU40_HUMAN	0	30078568	INTRONIC	0.034	0.0049	0.1686	
rs9261129	Q6ZU40_HUMAN	0	30087558	INTRONIC	0.042	0.0024	0.1605	
rs9261277	ZNRD1	0	30139070	INTRONIC	0.044	0.0014	0.16	
rs1264701	TRIM31	-4318	30174337	DOWNSTREAM	0.027	-0.2051	-0.0144	
rs3734838	TRIM31	0	30188210	NON_SYNONYMOUS_CODING	0.042	0.0005	0.1417	
rs9261441	TRIM31	10891	30199737	INTERGENIC	0.012	0.019	0.1779	
rs2022065	TRIM10	0	30229439	3PRIME_UTR	0.045	0.0021	0.224	
rs1345229	TRIM26	1191	30290374	UPSTREAM	0.008	0.0226	0.1713	
rs9357097	TRIM39	-9500	30393100	INTERGENIC	0.015	-0.2417	-0.0275	
rs2516649	GNL1	-16814	30604861	INTERGENIC	0.040	-0.2333	-0.0069	
rs2844713	GNL1	0	30627237	INTRONIC	0.043	-0.2307	-0.0052	
rs4248154	NM_001010909	44941	31110595	INTERGENIC	0.017	-0.1853	-0.02	
rs2523849	C6orf15	-53952	31133030	INTERGENIC	0.031	0.0079	0.1963	
rs2428514	C6orf15	-51487	31135495	INTERGENIC	0.044	0.0015	0.1804	
rs2517403	C6orf15	-11994	31174988	INTERGENIC	0.048	0.0013	0.2352	
rs2844635	C6orf15	-3522	31183460	DOWNSTREAM	0.040	0.0055	0.2384	
rs9295957	Q6H1K9_HUMAN	11917	31265572	INTERGENIC	0.046	-0.1814	-0.0039	
rs7745906	1C07_HUMAN	-32518	31311987	INTERGENIC	0.037	-0.1749	-0.0076	
rs2074488	1C07_HUMAN	524	31348410	UPSTREAM	0.038	-0.156	-0.0066	
rs9366778	1C07_HUMAN	29266	31377152	INTERGENIC	0.039	-0.2425	-0.0071	
rs406936	NP_008860.4	0	32041140	INTRONIC	0.048	-0.1643	-0.0031	
rs454212	NP_008860.4	0	32042351	INTRONIC	0.028	-0.1715	-0.0123	
rs387608	STK19	0	32049536	INTRONIC	0.048	-0.1643	-0.0031	
rs2269429	TNXB	0	32137161	NON_SYNONYMOUS_CODING	0.044	-0.1475	-0.0044	
rs204899	TNXB	0	32165605	INTRONIC	0.012	-0.1571	-0.0214	
rs3115553	C6orf10	-14648	32353805	INTERGENIC	0.049	0.0004	0.2025	
rs9268384	C6orf10	0	32444564	NON_SYNONYMOUS_CODING	0.045	-0.2416	-0.004	
rs4424066	C6orf10	2096	32462406	UPSTREAM	0.039	-0.2411	-0.0078	
rs3817973	BTNL2	-635	32469089	DOWNSTREAM	0.022	-0.2518	-0.0207	
rs3793126	BTNL2	0	32479597	INTRONIC	0.044	-0.2128	-0.0048	
rs86567	HLA-DOA	0	33084737	INTRONIC	0.037	-0.2434	-0.0089	
rs6457699	HLA-DOA	4258	33089625	UPSTREAM	0.031	0.0123	0.2547	
rs6933994	HLA-DOA	9731	33095098	INTERGENIC	0.042	0.0053	0.2485	
rs6457702	HLA-DOA	10660	33096027	INTERGENIC	0.009	-0.2802	-0.0399	
rs9296068	HLA-DOA	11306	33096673	INTERGENIC	0.041	0.0053	0.2447	
rs986521	COL11A2	0	33244123	INTRONIC	0.038	-0.2075	-0.0074	
rs9277932	COL11A2	0	33249231	INTRONIC	0.028	-0.25	-0.0154	
rs2855425	COL11A2	0	33252351	INTRONIC	0.040	-0.221	-0.0067	
rs6531	RXRB	0	33271429	SYNONYMOUS_CODING	0.036	-0.2241	-0.0088	

SNP	Closest Gene	Distance to gene	Position	SNP type	Parental allele frequency comparison, p-value	Lower conf. Interval	Higher conf. Interval	Par
rs211474	DAXX	29822	33428591	INTERGENIC	0.045	0.0029	0.2401	
rs211455	KIFC1	-31102	33436496	INTERGENIC	0.019	0.023	0.2572	
rs211452	KIFC1	-28639	33438959	INTERGENIC	0.020	0.0223	0.263	
rs211457	KIFC1	0	33473618	INTRONIC	0.025	-0.1556	-0.0122	
rs3116713	PHF1	0	33490266	NON_SYNONYMOUS_CODING	0.025	-0.1445	-0.0116	
rs211456	ZBTB9	0	33497359	INTRONIC	0.041	0.006	0.2481	
rs396746	NP_997380.1	0	33665023	INTRONIC	0.032	-0.1845	-0.0101	
rs169737	ITPR3	-12767	33684555	INTERGENIC	0.024	-0.2185	-0.0165	
rs12529825	ITPR3	0	33725381	INTRONIC	0.012	-0.1571	-0.0214	

Appendix 2

Appendix 2

List of 77 SNPs with significant differences in allele frequencies between Rejectors (R, n=37) and Non-Rejectors (NR, n=43), between Rejectors and 400 normal control Caucasian children, and Non-Rejectors vs 400 normal control Caucasian children. When compared with normal controls, only rs9296068 shows allele frequencies which are greater in Rejectors, and less among Non-Rejectors.

rs926068 also shows the greatest differences in allele frequencies between Rejectors and Non-Rejectors, and is one of five SNPs representing the HLA-DOA gene for which allele frequency differences between groups (R vs NR) are ≤ 0.01 .

SNP	Closest Gene	Distance to gene	SNP type	Position	Allele frequencies		
					Rejectors	Non-Rejectors	Normal Controls
rs9296068	HLA-DOA	11306	INTERGENIC	33096673	51.40%	26.80%	36.70%
rs6457699	HLA-DOA	4258	UPSTREAM	33089625	54.30%	31.70%	46.70%
rs602875	HLA-DRB1	16048	INTERGENIC	32681607	42.90%	21.90%	23.80%
rs1264583	HLAH_HUMAN	10138	INTERGENIC	30401462	14.30%	2.40%	5.30%
rs3094097	Q6ZU40_HUMAN	0	INTRONIC	30741854	14.30%	2.40%	9.70%
rs1345229	TRIM26	1191	UPSTREAM	30290374	14.30%	2.40%	11.90%
rs3134879	TRIM39	-1138	UPSTREAM	30123884	26.60%	48.70%	41.10%
rs2517904	DHX16	0	INTRONIC	29976963	2.90%	15.80%	7.50%
rs9366752	C6orf12	0	3PRIME_UTR	30132656	34.30%	15.80%	20.30%
rs1264581	TRIM39	0	SYNONYMOUS_CODING	30405484	16.20%	3.70%	5.30%
rs9276994	HLA-DOA	6866	INTERGENIC	33092233	48.60%	28.10%	37.70%
rs9277015	HLA-DOA	15877	INTERGENIC	33101244	44.30%	24.40%	37.70%
rs6933994	TRIM31	10891	INTERGENIC	33095098	57.10%	36.30%	46.70%
rs9261441	HLA-DOA	9731	INTERGENIC	30199737	15.70%	3.70%	13.60%
rs2394255	HLA-A	9079	INTERGENIC	30057800	58.60%	37.80%	48.60%
rs532098	HCG9	3625	DOWNSTREAM	32686030	30.90%	51.20%	45.60%

							Allele frequency
SNP	Closest Gene	Distance to gene	SNP type	Position	Rejectors	Non-Rejectors	Normal Controls
rs9468829	NP_003888.2	36906	INTERGENIC	30857212	21.40%	7.30%	12.10%
rs16896742	HLA-DRB1	20471	INTERGENIC	30030719	46.90%	26.80%	34.20%
rs2516684	RPP21	48325	INTERGENIC	30470974	17.10%	4.90%	13.10%
rs12206499	HCG9	-5765	INTERGENIC	30045106	37.10%	19.50%	25.70%
rs6904029	HCG9	-809	UPSTREAM	30051046	37.10%	19.50%	25.70%
rs3823355	HCG9	0	NON_SYNONYMOUS_CODING	30050062	37.10%	19.50%	25.90%
rs6933546	HLAH_HUMAN	671	DOWNSTREAM	33103992	42.90%	24.40%	36.60%
rs2517817	HLA-DOA	18625	INTERGENIC	29967496	2.90%	14.50%	7.90%
rs3869070	Q6ZU40_HUMAN	0	INTRONIC	30131847	55.90%	36.60%	44.70%
rs2647044	HLA-G	23348	INTERGENIC	32775888	22.70%	8.70%	9.90%
rs2975033	BAT2	0	INTRONIC	29930240	35.70%	18.70%	25.60%
rs9267522	BAT2	0	NON_SYNONYMOUS_CODING	31711749	27.10%	12.20%	13.10%
rs3117583	BAT3	0	INTRONIC	31727555	27.10%	12.20%	13.10%
rs3130618	BAT4	0	NON_SYNONYMOUS_CODING	31740113	27.10%	12.20%	13.10%
rs3115663	HB25_human	21592	INTERGENIC	31709822	27.10%	12.20%	13.10%
rs4711207	Q6ZU40_HUMAN	0	INTRONIC	30113733	34.40%	17.50%	22.80%
rs2040450	RPP21	19669	INTERGENIC	30442318	16.20%	4.90%	12.70%
rs4259245	ITPR3	0	INTRONIC	33732199	27.10%	45.10%	35.70%
rs2107202	TRIM40	0	INTRONIC	30213722	20	36.60%	25.90%
rs915664	DDR1	-54188	INTRONIC	30902596	35.70%	19.50%	30.60%
rs3129763	HA25_HUMAN	-14209	INTERGENIC	32698903	38.60%	21.90%	22.90%
rs3893464	HCG9	-7642	INTERGENIC	30043229	37.10%	54.90%	50.60%
rs9261301	RNF39	0	INTRONIC	30149538	52.90%	35.40%	44.40%
rs9267546	LY6G6D	-1245	UPSTREAM	31781415	17.60%	6.20%	9.30%
rs387608	HCG9	0	INTRONIC	32049536	5.70%	17.10%	15.10%
rs406936	TRIM39	-9500	INTERGENIC	32041140	5.70%	17.10%	14.70%
rs9357097	NP_008860.4	0	INTRONIC	30393100	17.10%	32.50%	30.90%
rs2394250	STK19	0	INTRONIC	30051635	51.40%	34.10%	41.60%
rs2257914	TRIM40	0	5PRIME_UTR	30228542	10%	23.20%	17.50%
rs2021723	TRIM10	0	3PRIME_UTR	30211902	10%	23.20%	17.30%
rs2844776	TRIM26	0	INTRONIC	30279806	15.70%	30.50%	20.10%
rs2395175	HLA-DRA	-2621	UPSTREAM	32513004	4.30%	14.60%	14.20%
rs2187668	HA25_HUMAN	0	INTRONIC	32713862	20.60%	8.50%	8.60%
rs9267911	TRIM31	0	INTRONIC	32313088	58.60%	41.50%	47.30%
rs2523990	NOTCH4	13266	INTERGENIC	30185208	58.60%	41.50%	47.40%
rs12212092	HCG9	-9631	INTERGENIC	30236421	12.90%	3.70%	6.30%
rs6457109	TRIM10	0	NON_SYNONYMOUS_CODING	30041240	18.60%	7.30%	9.90%
rs9277027	HLAH_HUMAN	-5770	INTERGENIC	33106216	30%	15.80%	30.40%
rs2523809	HLA-DOA	20849	INTERGENIC	29957598	8.60%	20.70%	11.80%
rs375912	NR_002139.1	-14455	INTERGENIC	33124706	32.90%	18.30%	36.50%

SNP	Closest Gene	Distance to gene	SNP type	Position	Allele frequency		
					Rejectors	Non-Rejectors	Normal Controls
rs2517930	HLA-DPA1	-15618	INTERGENIC	29853054	37.10%	21.90%	30.80%
rs4713411	NM_001010909	29501	INTERGENIC	31095155	28.10%	44.90%	43.40%
rs1264567	TRIM40	-11178	INTERGENIC	30474079	20%	8.50%	18.90%
rs213213	RPP21	51430	INTERGENIC	33291708	21.40%	36.60%	26.70%
rs1419675	RING1	3232	DOWNSTREAM	30200686	21.40%	36.60%	25.90%
rs6910071	C6orf10	0	INTRONIC	32390832	7.14%	18.30%	19.10%
rs86567	HLA-DOA	0	INTRONIC	33084737	31.40%	47.60%	38.10%
rs3734838	RNF39	12015	INTERGENIC	30188210	10.30%	2.40%	8.50%
rs6909253	TRIM31	-6114	INTERGENIC	30163622	52.90%	36.60%	37.30%
rs9261394	TRIM31	0	NON_SYNONYMOUS_CODING	30172541	52.90%	36.60%	38.10%
rs1003581	GABBR1	0	INTRONIC	29648183	9.10%	21.20%	19.90%
rs594223	C6orf125	0	INTRONIC	33775943	21.40%	9.80%	17.90%
rs9468692	RNF39	1571	UPSTREAM	30227869	14.30%	4.90%	7.10%
rs1150735	TRIM10	0	3PRIME_UTR	30153178	27.10%	42.70%	40.10%
rs1052486	BAT3	0	NON_SYNONYMOUS_CODING	31718665	40%	56.40%	43.70%
rs1264701	TRIM31	-4318	DOWNSTREAM	30174337	10%	21.90%	22.40%
rs1077393	BAT3	0	INTRONIC	31718508	40%	56.10%	44.10%
rs986521	COL11A2	0	INTRONIC	33244123	15.70%	29.30%	20.10%
rs9262138	DHX16	0	NON_SYNONYMOUS_CODING	30735846	10%	2.40%	5.90%
rs9267665	ZBTB12	1087	UPSTREAM	31978835	10%	2.40%	4.30%
rs7745906	1C07_HUMAN	-32518	INTERGENIC	31311987	7.35%	18.30%	13.10%

Appendix 3

Appendix 3

Summary statistics for differentially expressed exons between Rejectors and Non-rejectors

Exon ID	Transcript ID	Exon fold change [†]	Exon p-value [†]	Transcript fold change [‡]	Transcript p-value [‡]	Transcript/SNP name
2950312	2950307	1.063	0.555	-1.083	0.326	HLA-DOA
2950313	2950307	1.122	0.397	-1.083	0.326	HLA-DOA
2950314	2950307	1.069	0.372	-1.083	0.326	HLA-DOA
2950317	2950307	-1.065	0.638	-1.083	0.326	HLA-DOA
2950322	2950307	-1.060	0.395	-1.083	0.326	HLA-DOA
2950323	2950307	-1.011	0.905	-1.083	0.326	HLA-DOA
2950324	2950307	1.017	0.84	-1.083	0.326	HLA-DOA
2950325	2950307	1.127	0.383	-1.083	0.326	HLA-DOA
2950326	2950307	-1.048	0.748	-1.083	0.326	HLA-DOA
2950327	2950307	-1.532	0.0153	-1.083	0.326	HLA-DOA
	untranslated					rs6457699
	untranslated					rs9276994

Exon ID	Transcript ID	Exon fold change [†]	Exon p-value [†]	Transcript fold change [‡]	Transcript p-value [‡]	Transcript/SNP name
	untranslated					rs6933994
	untranslated					rs9296068
	untranslated					rs9277015
2950331	2950329	1.129	0.545	-1.166	0.419	HLA-DPA1
2950332	2950329	-1.161	0.517	-1.166	0.419	HLA-DPA1
2950333	2950329	-1.246	0.204	-1.166	0.419	HLA-DPA1
2950338	2950329	-1.302	0.0128	-1.166	0.419	HLA-DPA1
2950340	2950329	-1.114	0.31	-1.166	0.419	HLA-DPA1
2950341	2950329	-1.014	0.875	-1.166	0.419	HLA-DPA1
2950342	2950329	1.030	0.792	-1.166	0.419	HLA-DPA1
2950343	2950329	-1.114	0.479	-1.166	0.419	HLA-DPA1
2950345	2950329	1.057	0.565	-1.166	0.419	HLA-DPA1
2950346	2950329	1.346	0.113	-1.166	0.419	HLA-DPA1
2950348	2950329	1.035	0.723	-1.166	0.419	HLA-DPA1

[†] Exon fold change, or splicing index, is calculated as the ratio of mean gene-level normalized intensities between rejectors and non-rejectors. Negative values indicate exon skipping or repression, whereas positive values indicate exon enrichment. The p-values are calculated using a Student's two-sample *t*-test on gene-level normalized intensities. Orange cells indicate $p < .05$.

[‡] Transcript fold change is calculated as the ratio of the mean gene-level intensities between rejectors and non-rejectors. All exons within a transcript have identical gene-level intensities, but different exon-level intensities. The p-values are calculated using a Student's two-sample *t*-test on gene-level intensities.

Appendix 4

Appendix 4

Top-ranked SNPs with $p < 0.01$ from simple association testing with Rejectors (n=35) vs Non-Rejectors (n=42), all of whom are Caucasians

						MAF	MAF
SNP	rank	physical position	type	Closest gene	Distance to gene	Rejectors	Controls
rs2975033	4	29930240	INTERGENIC	HLA-G	23348	36.36	25.62
rs12206499	10	30045106	INTERGENIC	HCG9	-5765	36.36	25.69
rs3823355	11	30050062	UPSTREAM	HCG9	-809	36.36	25.87
rs6904029	12	30051046	NON_SYNONYMOUS_CODING	HCG9	0	36.36	25.75
rs2394255	2	30057800	DOWNSTREAM	HCG9	3625	59.09	48.62
rs3869070	6	30131847	INTRONIC	Q6ZU40_HUMAN	0	56.25	44.75
rs9366752	1	30132656	3PRIME_UTR	C6orf12	0	33.33	20
rs6909253	15	30163622	INTERGENIC	RNF39	12015	54.55	37.25
rs9261394	16	30172541	INTERGENIC	TRIM31	-6114	54.55	38
rs2523990	13	30185208	INTRONIC	TRIM31	0	60.61	47.38
rs1345229	18	30290374	UPSTREAM	TRIM26	1191	15.15	11.87
rs9357097	7	30393100	INTERGENIC	TRIM39	-9500	15.15	30.95

SNP	rank	physical position	type	Closest gene	Distance to gene	MAF	MAF
						Rejectors	Controls
rs1264583	19	30401462	UPSTREAM	TRIM39	-1138	12.12	5
rs1264581	5	30405484	SYNONYMOUS_CODING	TRIM39	0	14.06	5.29
rs3095150	17	31040511	INTERGENIC	DPCR1	10534	45.45	42.8
rs6457699	3	33089625	UPSTREAM	HLA-DOA	4258	53.12	46.75
rs9276994	8	33092233	INTERGENIC	HLA-DOA	6866	46.88	37.75
rs6933994	14	33095098	INTERGENIC	HLA-DOA	9731	56.25	45.99
rs9296068	9	33096673	INTERGENIC	HLA-DOA	11306	46.88	36.75

Parental Allele test and Gamete Competition (GC) test for the same snps as above

The 5'UTR flanking region of HLA-DOA is represented by 4 SNPs, of which rs6457699 lies roughly 4kb from the first HLA-DOA exon, and also shows differences in parental allele comparisons, which approach significance at $p=0.055$ (yellow cell).

rs9296068 is more frequent in parents of Rejectors, compared with parents of Non-Rejectors. However, differences are not statistically significant, due to reduced power in the Rejector group, and among Rejector parents, as a result of removing seven non-Caucasians, and adding 5 caucasian trios.

Appendix 5

SNP	rank	physical position	type	Closest gene	Distance to gene	Parental Allele	
						Chi-Sq p-value	LC
rs2975033	4	29930240	INTERGENIC	HLA-G	23348	0.08	-0.0
rs12206499	10	30045106	INTERGENIC	HCG9	-5765	0.22	-0.0
rs3823355	11	30050062	UPSTREAM	HCG9	-809	0.19	-0.0
rs6904029	12	30051046	NON_SYNONYMOUS_CODING	HCG9	0	0.22	-0.0
rs2394255	2	30057800	DOWNSTREAM	HCG9	3625	0.42	-0.0
rs3869070	6	30131847	INTRONIC	Q6ZU40_HUMAN	0	0.46	-0.0
rs9366752	1	30132656	3PRIME_UTR	C6orf12	0	0.42	-0.0
rs6909253	15	30163622	INTERGENIC	RNF39	12015	0.16	-0.0
rs9261394	16	30172541	INTERGENIC	TRIM31	-6114	0.19	-0.0
rs2523990	13	30185208	INTRONIC	TRIM31	0	0.10	-0.0
rs1345229	18	30290374	UPSTREAM	TRIM26	1191	0.01	0.0
rs9357097	7	30393100	INTERGENIC	TRIM39	-9500	0.00	-0.0
rs1264583	19	30401462	UPSTREAM	TRIM39	-1138	0.26	-0.0
rs1264581	5	30405484	SYNONYMOUS_CODING	TRIM39	0	0.25	-0.0
rs3095150	17	31040511	INTERGENIC	DPCR1	10534	0.35	-0.0
rs6457699	3	33089625	UPSTREAM	HLA-DOA	4258	0.06	0.0
rs9276994	8	33092233	INTERGENIC	HLA-DOA	6866	0.08	-0.0
rs6933994	14	33095098	INTERGENIC	HLA-DOA	9731	0.12	-0.0
rs9296068	9	33096673	INTERGENIC	HLA-DOA	11306	0.40	-0.0

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Figure 1.

Upper panel shows $-\log_{10}(p\text{-values})$ for comparison of minor allele frequencies of 1,776 SNPs in parents of Rejectors, and parents of Non-Rejectors. Fifty seven SNPs show large differences ($p < 0.05$, $-\log_{10}(p\text{-value}) > 1.3$) in allele frequencies in this comparison and are shown in the lower panel. Only one of these 57 SNPs (lower panel) also shows significant differences in parent-to-child transmission when Rejectors were compared with Non-Rejectors in the gamete competition statistic. This SNP rs9296068, marked by the red square is located in the HLA-DOA gene.

Figure 2.

Splicing index values, defined as the fold change between mean gene-level normalized exon intensities in the Rejector and Non-Rejector groups and 95% confidence intervals for the 10 exons that map to the HLA-DOA gene. The physical position ranges of each exon are represented on the x-axis and any exon point with a significant p-value ($p < .05$) is shaded red. Significant negative fold changes represent exon skipping or repression, while significant positive fold changes represent exon enrichment for the gene.

Figure 3.

a). Significant correlation exists between B-lymphocyte content in liver grafts with ACR, and numbers of risk allele G at rs9296068 ($r^2=0.194$, $p=0.021$). Accompanying micrographs show ACR, with portal areas containing brown, horseradish-peroxidase-stained CD79a+ B-lymphocyte/plasmacytoid cells. B-lymphocyte content is lowest, when the risk allele is absent (panel b), higher in heterozygotes (panel c), and highest when the risk allele is homozygous (panel d).

Figure 4.

The HLA-DOA gene is transcribed from the minus strand. The upstream 5' flanking UTR region defined by five discriminatory SNPs in simple association testing of Rejectors vs Non-Rejectors lies between rs9296068 to the right and rs6457699 to the left, and shows marked linkage disequilibrium. Rs6457699 lies ≈ 4 kb from the first HLA-DOA exon, on the left. Rs9296068 is associated with allele-dependent decrease in HLA-DOA expression among CEU and YRI in public databases (Source: WGAViewer).

Table 1

Etiology of liver disease leading to LTx in 80 children.

Etiology of liver disease	Rejectors	Non- Rejectors
Allagilles	1	1
Autoimmune Hepatitis	3	0
Biliary Atresia	8	13
BRIC: Benign Recurrent Intrahepatic Cholestasis	0	1
Budd-Chiari	0	1
Bylers Disease PFIC type I	1	0
Carolis Disease	1	0
Congenital Hepatic Fibrosis	1	2
Crigler-Najjar	1	5
Cystic Fibrosis	1	1
Fulminant Hepatic Failure	2	2
Giant Cell Hepatitis	0	1
Glycogen Storage Disease	1	0
Hepatic Fibrosis	1	0
Hepatoblastoma	1	1
Hepatocellular Carcinoma	0	1
Methylmalonic Acidemia	1	0
Maple Syrup Urine Disease	7	8
Ornithin Transcarbamylase Deficiency	1	2
Primary Sclerosing Cholangitis	2	1
Secondary Biliary Cirrhosis	0	1
Paucity of Bile Ducts	1	0
Tyrosenemia	1	1
Cirrhosis-unknown	2	1
Total	37	43

Table 2

Only one SNP, rs9296068 near the HLA-DOA gene, met criteria for selection and hypothesis testing in our two-tier approach. This SNP demonstrated a large difference ($p=0.041$) in MAF when parents of Rejectors were compared with parents of Non-Rejectors, as well as significant differences in parent-to-child transmission between Rejectors and Non-Rejectors, on the GC test ($p=0.0183$). Transmission data show more parents ($n=19$) transmitting the minor allele of rs9296068 to their Rejector offspring than parents who did not ($n=12$). The reverse was true among Non-Rejectors, whose parents were less likely to transmit this risk allele (7 transmissions vs 17 non-transmissions).

HLA-DOA/ SNP=rs9296068 (physical position=33,096,673)	
Rejector vs. Non-Rejector MAF p value	0.041
Rejector vs. Non-Rejector GC p value	0.018
GC transmission parameter (τ_2) [†]	1.9
Allele	2
Rejector parents	
MAF [‡]	46.3%
Number transmitted	19
Number non-transmitted	12
Non-Rejector parents	
MAF [‡]	33.8%
Number transmitted	7
Number non-transmitted	17

[‡]MAF=minor allele frequency

[†]For the GC statistic, under the null hypothesis of Mendelian segregation, $\tau_k=1$ for all k alleles (11)

Table 3

Confirmatory association testing shows that MAF for rs9296068 are greater among Rejectors, and less among Non-Rejectors, compared with a large cohort of 400 normal Caucasian children. Together, these results confirm parent-to-child over-transmission among Rejectors, and under-transmission among Non-Rejectors, observed in the GC test.

Rejector vs. Controls	MAF [‡] in Rejector children	51.4%
	MAF [‡] in Controls	36.8%
	χ^2	5.90
	P value	0.015
	Odds ratio	1.82
Non-Rejector vs. Controls	MAF [‡] in Non-Rejector children	26.8%
	MAF [‡] in Controls	36.8%
	χ^2	3.18
	p value	0.074
	Odds ratio	0.63

[‡]MAF=minor allele frequency

* MAF differences are based on the samples used. For the unrelated case/control association shown in the table above, children are used to calculate allele frequencies. For the GC test in Table 2, trio information is used, resulting in minor differences in allele frequency.

Table 4

Results of confirmatory association testing for SNPs, ordered by physical position, which showed the most significant differences in MAF ($p \leq 0.010$) between Rejectors and Non-Rejectors (last column). Of 14 such SNPs, five are near HLA-DOA. Among them, rs9296068 and rs9276994 show MAF, which are greater among Rejectors (R), and less among Non-rejectors (NR), compared with 400 normal control Caucasian children (NC). Only rs9296068 shows differences in PAF between outcome groups, satisfying selection criteria for candidacy, while rs9276994 fails the PAF test.

			Allele	Frequency		Chi-square test p-values		
SNP	Physical location	Closest Gene	R	NR	NC	R vs NC	NR vs NC	R vs NR
rs2517904	29,976,963	HCG2P7	2.9%	15.9%	7.5%	0.147	0.009	0.007
rs3134879	30,123,884	ZNRD1	26.6%	48.7%	41.1%	0.023	0.192	0.007
rs9366752	30,132,656	ZNRD1	34.3%	15.9%	20.0%	0.005	0.368	0.008
rs9261441	30,199,737	TRIM31	15.7%	3.7%	13.6%	0.623	0.01	0.01
rs1345229	30,290,374	TRIM26	14.3%	2.4%	11.9%	0.553	0.009	0.007
rs1264583	30,401,462	TRIM39	14.3%	2.4%	5.0%	0.001	0.3	0.007
rs1264581	30,405,484	TRIM39	16.2%	3.7%	5.3%	0.000	0.524	0.009
rs3094097	30,741,854	DHX16	14.3%	2.4%	9.8%	0.228	0.028	0.007
rs602875	32,681,607	HLA-DRB1	42.9%	22.0%	23.8%	0.000	0.707	0.006
rs6457699	33,089,625	HLA-DOA	54.3%	31.7%	46.8%	0.226	0.009	0.005
rs9276994	33092233	HLA-DOA	48.6%	28.1%	37.8%	0.075	0.083	0.009
rs6933994	33095098	HLA-DOA	57.1%	36.3%	46.0%	0.614	0.002	0.01
rs9296068	33,096,673	HLA-DOA	51.4%	26.8%	36.8%	0.015	0.074	0.002
rs9277015	33,101,244	HLA-DOA	44.3%	24.4%	37.0%	0.228	0.023	0.01