Commonality in the genetic control of Type 1 diabetes in humans and NOD mice: variants of genes in the IL-2 pathway are associated with autoimmune diabetes in both species

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Abstract

Variants within the IL-2 (interleukin 2) and CD25 genes are associated with T1DM (Type 1 diabetes mellitus) in mice and humans respectively. Both gene products are essential for optimal immune tolerance and a partial failure to tolerize is linked to the autoimmune responses to insulin and other β-cell proteins that precede T1DM onset. Gene variants that contribute to common disease susceptibility often alter gene expression only modestly. Small expression changes can be technically challenging to measure robustly, especially since biological variation usually contributes negatively to this goal. The present review focuses on allele-specific expression assays that can be used to quantify genotype-determined expression differences such as those observed for IL-2, where the susceptibility allele is transcribed 2-fold less than the resistance allele.

Some T1DM (Type 1 diabetes mellitus) genes are shared by humans and NOD (non-obese diabetic) mice

The MHC region remains the major genetic determinant causing T1DM in humans and NOD mice [1,2] and the identification of non-MHC genes contributing to T1DM susceptibility has made significant progress. Of particular note, in 2007, it was demonstrated unequivocally that genome-wide association studies in humans could be used to discover novel genes causing autoimmune disease [3,4], including T1DM [5]. A conservative estimate of the number of genes influencing T1DM susceptibility is at least 50 in both humans and NOD mice. Of those discovered already or those that are strong candidate genes, most have one or more known functions in immune cells. Importantly, overlap with T2DM (Type 2 diabetes mellitus) causal regions has not been observed [6], whereas overlapping associations are present with autoimmune diseases such as rheumatoid arthritis, coeliac disease, Graves’ disease and systemic lupus erythematosus [5,7,8]. Humans and NOD mice have variations in some of the same T1DM genes or gene pathways and there are results in both species indicating that an early autoimmune response to insulin and other β-cell proteins that is caused by a partial failure in central and peripheral tolerance mechanisms precedes β-cell destruction [9,10]. In addition to sharing structural aspects of the MHC class II molecules that confer T1DM susceptibility or resistance [11], variation of the CTLA-4 (cytotoxic T-lymphocyte antigen 4) gene, which negatively regulates T-cells is also associated with T1DM in both species. Gene variants encoding the interacting ligand–receptor molecules IL-2 (interleukin 2) [12] and CD25 [13], which are essential for peripheral tolerance, are present in mice and humans respectively. Supporting the hypothesis that gene variants altering T1DM susceptibility can influence immune tolerance more generally, the success of a tolerance-induction protocol for islet transplantation is altered by T1DM alleles at IL-2, even when tested in the context of the non-autoimmune B6 genome [14].

Potential functions of T1DM genes in the IL-2 pathway

Signalling through the IL-2 receptor is critical for the function of the FOXP3+CD25+CD4+ (where FOXP3 is forkhead box P3) regulatory T-cells that dampen immune responses [15], and it appears that their function is reduced in T1DM patients and NOD mice [16–18]. In analysing the immune responses of IL-2 congenic mice, we observed a 2-fold increase in IL-2 production with the presence of a T1DM-resistance allele at Il2 [12]. Augmented FOXP3+CD25+CD4+ regulatory T-cell function and decreased islet-specific effector CD8+ T-cell proliferation in the pancreatic lymph nodes with subsequent migration to the islets also positively correlated with T1DM-resistance alleles. Our findings probably explain the observations that the IL-2 gene region also controls susceptibility to thymectomy-induced ovarian
dysgenesis [19], a disease that is initiated by the reduction of FOXP3+ CD25+ CD4+ regulatory T-cells caused by neonatal thymectomy [20]. These data are also consistent with the observation that neutralizing anti-IL-2 antibodies delivered in vivo can induce autoantibodies as well as cellular infiltrates in a variety of tissues [21]. We hypothesize that there has been selective pressure to maintain optimal levels of IL-2 production, since excessive IL-2 could reduce immune responsiveness via the induction of overly potent regulatory T-cells, whereas too little IL-2 increases the likelihood of autoimmune responses to reproductive or other organs because effector mechanisms are not held in check by regulatory T-cells. Only small differences in IL-2 production are needed for significant biological effects and we showed that haplodeficiency at the IL-2 gene increased T1DM susceptibility significantly [12].

The SNP (single nucleotide polymorphism), or SNPs, in the IL-2 gene that cause different levels of IL-2 production in mice having a susceptibility or resistance allele have not been defined [12]. However, from a sequence comparison of different T1DM-resistant and -susceptible IL-2 haplotypes, the causative variant does not alter the coding region and is not within the well-defined promoter region. Disease-associated candidate SNPs are within introns and the extended 5′ and 3′ regions of the IL-2 gene and levels of IL-2 pre-mRNA correlate with genotype. Together, these data support the hypothesis that T1DM-associated variants affect promoter accessibility and therefore transcription by altering the efficiency of chromatin remodelling in response to differentiation [12].

Quantifying phenotypic changes caused by T1DM genes
It remains a technical challenge to measure genotype-determined differences in protein or mRNA expression that are less than 2-fold. Biological variation that occurs in inbred mouse strains, assay variation and confounding influences in outbred populations from other variant loci that affect the pathway under examination can all blur a genotype-phenotype relationship. One strategy to compare the effects of variant alleles on the expression of disease-associated genes is to study them in a heterozygous individual or animal using allele-specific assays. Such assays utilize properties conferred either by the known causative SNP or most-associated SNP (or an SNP in linkage disequilibrium with this SNP) to determine which allele is producing the RNA product being measured. This experimental design ensures that the allelic products have arisen from cells subjected to similar environmental and activation conditions.

In studies of human CTLA-4 isoforms, we made use of an allele-specific assay known as hot-stop PCR [22] to assess the relative amounts of the full-length (four exons) and the Δexon 3 isoforms present in individuals heterozygous for a susceptible and resistant CTLA-4 allele [23]. This experimental approach confirmed results from a QPCR (quantitative PCR) analysis demonstrating a less than 2-fold difference in the expression of the Δexon 3 isoforms levels in the two groups of homozygous donors. The hot-stop PCR analysis also demonstrated that no difference was observed in the allele-specific expression of the full-length isoform.

Using QPCR, we observed that the B6 IL-2 allele makes approx. 2-fold more IL-2 pre-mRNA and mRNA than the NOD allele [12]. To confirm this difference, allele-specific pyrosequencing [24] was performed using cDNA derived from RNA isolated from activated (NOD × NOD.B6 Idd3)F1 T-cells, which are heterozygous for the IL-2 gene [12]. We observed that the B6 allele was transcriptionally more active than the NOD allele, with approx. 60–70% of the IL-2 mRNA derived from the B6 allele. When extrapolated to the homozygous genotypes, this equates to the approx. 2-fold difference observed by QPCR. However, the reliability of the pyrosequencing technique was dependent on having samples with a relatively high level of mRNA for the gene being measured. We therefore sought additional methods for analysing allele-specific expression differences in heterozygous samples.

Development of a novel allele-specific expression assay
We developed an allele-specific expression assay capable of analysing even rare pre-mRNAs and mRNA+ pre-mRNAs based on the technique of Frommer et al. [25] to calculate the percentage methylation at CpG sites in genomic DNA by cloning, sequencing and counting the number of clones that had either a C or G at the analysed site. The steps of this assay are depicted in Figure 1. The most critical aspect of this approach is the necessity that the causal variant, or a variant associated with the causal variant, is located within the transcribed region of the gene of interest. Since variants causing expression differences can occur outside of the pre-mRNA (i.e. those associated with certain categories of epigenetic changes), additional sequencing may be needed.

Figure 1 | Workflow of a clone-based allele-specific expression assay

![Workflow of a clone-based allele-specific expression assay](image-url)
Figure 2 | Allele specific expression analysis of the IL-2 gene

Three (NOD × NOD.B6 Idd3)F1 mice were stimulated in vivo with 5 μg of anti-CD3 (clone 2C11) for 1 h, and cDNA was made from DNase-treated total RNA extracted from whole spleen. The PCR product generated using primers flanking an NOD/B6 SNP in intron 2 of IL-2 (forward primer, 5′-AAAGAATGGCCCAACTTTCA-3′; reverse primer, 5′-TTTCATTGGGACAAATAGATTTA-3′) was cloned into a Topo vector (Invitrogen), and colony PCR was performed on the transformed cells. Restriction digestion with RsaI (New England Biolabs), which cuts the cloned PCR product of the NOD allele but not that of the B6, was used to score each clone.

<table>
<thead>
<tr>
<th>Heterozygous sample</th>
<th>IL-2 allele</th>
<th>Number of clones genotyped (% B6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD × NOD.B6 Idd3 heterozygous genomic DNA</td>
<td>NOD = 94, B6 = 93 (B6 = 49.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD × NOD.B6 Idd3 heterozygous Sample 1</td>
<td>NOD = 39, B6 = 76 (B6 = 66.1%)</td>
<td>P = 5.4 × 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>NOD × NOD.B6 Idd3 heterozygous Sample 2</td>
<td>NOD = 25, B6 = 83 (B6 = 76.8%)</td>
<td>P = 4.8 × 10^{-8}</td>
<td></td>
</tr>
<tr>
<td>NOD × NOD.B6 Idd3 heterozygous Sample 3</td>
<td>NOD = 26, B6 = 69 (B6 = 73.4%)</td>
<td>P = 2.4 × 10^{-4}</td>
<td></td>
</tr>
</tbody>
</table>

= NOD  = B6

to identify ‘read-out’ SNPs. Once the target sequence is selected, PCR primers flanking the SNP are used to amplify cDNA and genomic DNA (as a control for equivalent expression). The products are cloned into a vector, and transformed cells are analysed by colony PCR. The variant present in each PCR product is determined by sequencing or conventional genotyping assays.

In Figure 2, the cloning-based allele-specific expression assay was examined for its ability to produce results similar to those obtained with QPCR and allele-specific pyrosequencing in the case of IL-2 mRNA expression from T1DM-susceptible and -resistant alleles. Counting the number of clones that contained either the NOD or the B6 allele revealed that the B6 allele was more transcriptionally active, with an average 70% of the IL-2 transcripts derived from the B6 allele. Since these results confirmed those obtained for IL-2 gene expression when assessing allele-specific expression with the pyrosequencing-based technique [12], this adaptation of the Frommer et al. [25] technique represents another method that can be used to assess relatively small expression differences caused by disease-associated variants. An additional advantage of this adapted protocol is that the statistical power to detect small expression differences can be increased by genotyping larger numbers of clones obtained from each heterozygous sample examined.

Assessing variation in the IL-2 pathway

It is likely that many of the gene variants that contribute to the susceptibility of common diseases will only modestly alter gene function or expression. On the basis of the fact that small differences in the level of IL-2 cause large changes in T1DM frequency in NOD mice, we expect that the CD25 gene variants associated with human T1DM [13] will also produce relatively subtle expression changes. As is the case for mouse IL-2, T1DM-associated SNPs within CD25 are outside of the coding regions of the gene and are therefore likely to control the levels of mRNA expression.

We are currently using allele-specific approaches to study the expression of the human gene encoding CD25 as well as other genes associated with T1DM in humans and in NOD mice.

The importance of variation in the IL-2 pathway for human autoimmune disease is reinforced by the report that coeliac disease is associated with IL-2 SNPs [8] and there is strong evidence that T1DM has an IL-2 gene association; however, this latter finding requires replication [3,5]. Although the results from the NOD mouse [12] would predict that the human susceptibility allele at I2Z produces less IL-2, the pleiotropic aspects of the IL-2 pathway could yield surprises when the alleles are studied in detail. Although studies of the functional consequences of common alleles associated with autoimmune diseases are in their infancy, they will surely increase our understanding of disease pathogenesis and provide insights for the development of disease biomarkers as well as therapeutic and preventative approaches.

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