Brief Genetics Report

Haplotype Tag Single Nucleotide Polymorphism Analysis of the Human Orthologues of the Rat Type 1 Diabetes Genes *Ian4* (*Lyp/Iddm1*) and *Cblb*

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The diabetes-prone BioBreeding (BB) and Komeda diabetes-prone (KDP) rats are both spontaneous animal models of human autoimmune, T-cell–associated type 1 diabetes. Both resemble the human disease, and consequently, susceptibility genes for diabetes found in these two strains can be considered as potential candidate genes in humans. Recently, a frameshift deletion in *Ian4*, a member of the immune-associated nucleotide (*Ian*)-related gene family, has been shown to map to BB rat *Iddm1*. In the KDP rat, a nonsense mutation in the T-cell regulatory gene, *Cblb*, has been described as a major susceptibility locus. Following a strategy of examining the human orthologues of susceptibility genes identified in animal models for association with type 1 diabetes, we identified single nucleotide polymorphisms (SNPs) from each gene by resequencing PCR product from at least 32 type 1 diabetic patients. Haplotype tag SNPs (htSNPs) were selected and genotyped in 754 affected sib-pair families from the U.K. and U.S. Evaluation of disease association by a multilocus transmission/disequilibrium test (TDT) gave a *P* value of 0.484 for *IAN4L1* and 0.692 for *CBLB*, suggesting that neither gene influences susceptibility to common alleles of human type 1 diabetes in these populations. *Diabetes* 53: 505–509, 2004

Development of diabetes in the BB rat involves at least three genes: *Iddm1/lyp* on chromosome 4, *RT1*^{u} (at *Iddm2*) in the major histocompatibility complex (MHC) on chromosome 20, and a third unmapped gene (1,2). One unusual feature of this animal model is the severe lymphopenia that is essential for the development of the diabetic phenotype and that is inherited as a Mendelian trait (3). Life-long and profound T-cell lymphopenia is characterized by a reduction in peripheral CD4+ T-cells, an even greater reduction of CD8+ T-cells (4), and an almost total absence of RT6+ T-cells (5). The lymphopenia gene is involved in the regulation of apoptosis in the T-cell lineage and is, therefore, responsible for loss of critical T-cells, resulting in autoimmunity (6). Recently, two groups have independently shown, by positional cloning of *Iddm1/lyp*, that lymphopenia is due to a frameshift deletion in *Ian4* (also called *Ian5*) of the immune-associated nucleotide (*Ian*)-related gene family (6,7), resulting in a truncated protein product. This deletion was only found in strains that have lymphopenia and diabetes (6). The human orthologue of *Ian4* (*IAN4L1*) belongs to a family of at least 10 genes that encode GTP-binding proteins and are located in a 300-kb interval of human chromosome 7q36.

The KDP rat was derived as a substrain of the Long-Evans Tokushima lean (LETL) rat and shows 100% development of moderate to severe insulitis within 220 days of age (8,9). The LETL rat is characterized by sudden onset of polyuria, polyphagia, hyperglycemia, weight loss, and autoimmune destruction of pancreatic B-cells, while showing no significant T-cell lymphopenia and no sex-specific differences in rate of onset or severity (8). As with the BB rat, the KDP rat possesses the diabetogenic *RT1*^{u} haplotype, adding to its relevance as a model of type 1 diabetes. In addition to the MHC, another unlinked locus, *Iddm/kdp1*, is essential in the development of moderate to severe insulitis and the onset of diabetes (10). *Iddm/kdp1* has been mapped to a nonsense mutation in *CBLB* (Casitas B-lineage lymphoma b, or Cas-Br-M murine ecotropic retroviral transforming sequence b), a gene shown to have a role in the regulation of tyrosine kinase signaling pathways (11–14). This mutation results in the removal of 484 amino acids, including the proline-rich and leucine zipper domains of the protein, and is specific to the KDP rat and the original LETL strain. It is not found in the nondiabetic KND (Komeda nondiabetic) or LETO (Long-Evans Tokushima Otsuka) strains (15). Homozygous mice generated to be deficient in *Cblb* develop spontaneous autoimmunity, characterized by T- and B-cell infiltration of

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htSNP, haplotype tag single nucleotide polymorphism; LD, linkage disequilibrium; MHC, major histocompatibility complex; SNP, single nucleotide polymorphism.

TABLE 1
SNPs identified in *IAN4L1* and single-locus test results

Variant name	Map position	dbSNP	Minor allele frequency	Allelic R^2	Transmitted	Not transmitted	*P* value	Location
DIL4283	149746862	rs3757411	0.355	99.35	1612	1602	0.78	5′
DIL4284	149747929	**ss14452109**	0.012	—	—	—	—	5′
DIL4285	149748019	**ss13452110**	0.078	85.47	255	248	0.72	5′
DIL4287*	149748426	**ss13452112**	0.094	htSNP	343	333	0.67	5′
DIL4286*	149748431	**ss13452111**	0.281	htSNP	810	811	0.97	5′
DIL4344	149749043	rs3807383	0.281	100.00	1986	1985	0.97	5′
DIL4343*	149749122	rs917805	0.047	htSNP	49	66	0.07	5′
DIL4348	149749357	**ss13452128**	0.161	100.00	419	424	0.79	5′
DIL4347	149749574	**ss13452127**	0.016	—	—	—	—	Exon 1
DIL4345*	149750328	**ss13452125**	0.266	htSNP	838	859	0.54	Intron 1
DIL4346	149750358	**ss13452126**	0.375	100.00	1182	1192	0.78	Intron 1
DIL4288	149750835	**ss13452113**	0.016	—	—	—	—	Intron 1
DIL4289	149751082	**ss13452114**	0.172	100.00	2338	2323	0.60	Intron 1
DIL4349	149752812	**ss13452129**	0.266	100.00	838	859	0.54	Intron 1
DIL4350	149752813	**ss13452130**	0.016	—	—	—	—	Intron 1
DIL4351	149752974	rs4725936	0.391	100.00	1615	1611	0.92	Intron 2
DIL4352*	149753023	rs4725359	0.125	htSNP	2453	2470	0.49	Intron 2
DIL4353*	149753046	**ss13452133**	0.438	htSNP	838	861	0.50	Intron 2
DIL4354	149753102	**ss13452134**	0.016	—	—	—	—	Intron 2
DIL4338	149753443	**ss13452121**	0.300	97.40	845	866	0.54	Intron 2
DIL4355	149753684	**ss13452135**	0.276	100.00	838	859	0.54	Intron 2
DIL4342	149754385	rs759011	0.321	86.19	846	867	0.54	Exon 3
DIL4290	149754928	rs1046355	0.224	91.85	757	775	0.55	Exon 3
DIL4291*	149755077	**ss13452116**	0.172	htSNP	2681	2655	0.11	Exon 3
DIL4293	149755128	rs10361	0.269	88.14	775	796	0.51	Exon 3
DIL4292	149755534	rs6598	0.261	80.68	699	717	0.53	Exon 3
DIL4294*	149755599	rs2286899	0.097	htSNP	376	378	0.93	Exon 3
DIL4295	149755861	rs2286898	0.274	97.32	838	857	0.58	3′UTR
DIL4357*	149756048	**ss13452136**	0.160	htSNP	343	327	0.53	3′UTR
DIL4358	149757510	**ss13452137**	0.267	98.39	2001	1981	0.54	3′

Variant name based on local naming scheme for polymorphisms. Map positions on human chromosome 7 from NCBI build 33. Minor allele frequencies shown are based on the sequencing panel of 32 type 1 diabetic subjects. Transmitted and not transmitted were estimated for the ungenotyped SNPs with allele frequencies >0.03 from the regression equations computed in the preliminary sequencing study. *Denotes htSNP. dbSNP numbers for all novel polymorphisms in boldface. Single-locus tests at SNPs other than htSNPs were calculated from imputed data based on htSNP genotypes and LD information obtained from the sequencing panel. Single-locus *P* values were obtained using paired *t* tests. Data were not imputed and tests not performed for SNPs with allele frequencies <0.03. UTR, untranslated region.

multiple organs (16). Taken together, this evidence suggests that *Cblb* is probably the disease susceptibility gene at *Iddm/Kdp1* and, consequently, a major susceptibility gene for diabetes in the rat.

We, therefore, resequenced both *IAN4L1* and *CBLB* as candidates for human type 1 diabetes susceptibility. For *IAN4L1*, we resequenced the entire gene, covering 12.2 kb, comprising three exons and introns and 3 kb 3′ and 5′ of the gene in 32 type 1 diabetic subjects, identifying 30 single nucleotide polymorphisms (SNPs), 19 of which were novel (Table 1). Of the 30 SNPs, 7 were exonic: 1 in exon 1, which contains the 5′ untranslated region, and 6 in exon 3. At *CBLB*, which extends over 230 kb (including three alternative, untranslated exon 1s), we resequenced 12.6 kb in 96 type 1 diabetic subjects, encompassing exons, intron/exon boundaries, and 2.5 kb 3′ and 5′ of the gene. From the *CBLB* sequence data, we identified 37 polymorphisms, of which 26 were novel (Table 2). These comprised 32 SNPs and five insertion/deletions. Of the 37 polymorphisms, 7 were exonic: 1 in each of exons 6, 9, 11, and 12 and 3 in exon 10. However, no nonsynonymous variants were observed in either gene, nor were there any other obvious candidates for variants that might change function or expression (Tables 1 and 2). For *CBLB*, we were unable to sequence exons 18, 1A, or 1B (although we covered 135 of 195 bp of exon 1C), and consequently, it was not possible to fully represent them directly with our haplotype tag SNP (htSNP) selection.

From the 21 polymorphisms in *CBLB* and 25 in *IAN4L1* with allele frequencies >3%, we selected nine htSNPs for each, capturing the allelic variation within the genes with a minimum R^2 of 0.8 (Tables 1 and 2), using the htSNP selection method described by Chapman et al. (17). To further reduce genotyping costs, we adopted a two-stage strategy, in which we only proceed to the second stage of genotyping if the results from the first stage offered some possibility of an overall significant result. In stage 1, a collection of 754 affected sib-pairs, comprising 472 U.K. and 282 U.S. multiplex type 1 diabetic families (equivalent to ~1,400 trios; set 1), are genotyped and tested for association using the multilocus TDT, which tests for association between disease and htSNPs due to linkage disequilibrium (LD) with one or more causal variants (17). Transmissions of SNP alleles not genotyped in stage 1 can also be predicted using multiple regression equations computed in the course of htSNP selection from the initial sequencing data (17). Stage 2, genotyping in 1,708 additional families (set 2) only proceeds if the stage-1 multilo-

TABLE 2
Polymorphisms identified in *CBLB* and single-locus test results

Variant name	Map position	dbSNP	Minor allele frequency	Allelic R^2	Transmitted	Not transmitted	*P* value	Location
DIL4620	106873087	rs1503921	0.244	97.07	2220	2225	0.88	5′
DIL4621	106872769	**ss13452139**	0.023	—	—	—	—	5′
DIL4622	106872519	**ss13452140**	0.005	—	—	—	—	5′
DIL4623	106871969	rs1503922	0.005	—	—	—	—	5′
DIL4624*	106867943	**ss13452142**	0.229	htSNP	599	594	0.89	5′
DIL4625	106776408	**ss13452143**	0.028	—	—	—	—	Intron 4
DIL4649	106776404-5	**ss13452167**	0.128	87.18	323	321	0.94	Intron 4
DIL4650	106776371-2	**ss13452168**	0.021	—	—	—	—	Intron 4
DIL4651	106776332-6	**ss13452169**	0.261	88.48	663	655	0.79	Intron 4
DIL4626*	106776303	rs3772512	0.109	htSNP	261	259	0.92	Intron 4
DIL4627	106745995	**ss13452145**	0.016	—	—	—	—	Exon 6
DIL4628*	106740946	rs3213928	0.027	htSNP	2727	2731	0.74	Intron 6
DIL4629	106740829	**ss13452147**	0.005	—	—	—	—	Intron 6
DIL4652	106740755	**ss13452170**	0.006	—	—	—	—	Intron 6
DIL4630*	106737184	rs2289746	0.413	htSNP	1861	1879	0.62	Intron 8
DIL5960	106734298	**ss13452173**	0.026	—	—	—	—	Intron 8
DIL5961	106734124	**ss13452174**	0.005	—	—	—	—	Exon 9
DIL4634	106720318	**ss13452152**	0.005	—	—	—	—	Exon 10
DIL4633	106720255	rs2305035	0.258	93.40	624	609	0.61	Exon 10
DIL4632	106720186	rs2305036	0.258	93.40	624	609	0.61	Exon 10
DIL4631	106720058	**ss13452149**	0.012	—	—	—	—	Intron 10
DIL4636*	106704073	rs2305037	0.286	htSNP	781	771	0.76	Exon 11
DIL4635	106703765	**ss13452153**	0.286	100.00	781	771	0.76	Intron 11
DIL4637	106702263	rs3772534	0.043	99.58	2761	2745	0.19	Exon 12
DIL4663	106681919	**ss13452172**	0.005	—	—	—	—	Intron 14
DIL4638	106658744	rs1042852	0.290	97.59	2059	2070	0.75	3′UTR
DIL4639*	106658224	**ss13452157**	0.096	htSNP	199	199	0.99	3′
DIL4640	106657549	**ss13452158**	0.016	—	—	—	—	3′
DIL4641*	106656495	**ss13452159**	0.042	htSNP	2762	2745	0.18	3′
DIL4642	106656481	**ss13452160**	0.307	93.01	2012	2024	0.70	3′
DIL4653	106656362-3	**ss13452171**	0.135	81.28	378	373	0.83	3′
DIL4643	106656361	**ss13452161**	0.297	94.25	2050	2056	0.89	3′
DIL4644	106656043	**ss13452162**	0.286	100.00	2051	2061	0.76	3′
DIL4645*	106656038	**ss13452163**	0.130	htSNP	2547	2583	0.09	3′
DIL4646	106656037	**ss13452164**	0.005	—	—	—	—	3′
DIL4647*	106655931	rs2293148	0.443	htSNP	1626	1640	0.70	3′
DIL4648	106655861	**ss13452166**	0.005	—	—	—	—	3′

Variant name based on local naming scheme for polymorphisms. Map positions on human chromosome 3 from NCBI build 33. Minor allele frequencies shown are based on the sequencing panel of 96 type 1 diabetic subjects. Transmitted and not transmitted were estimated for the ungenotyped SNPs with allele frequencies >0.03 from the regression equations computed in the preliminary sequencing study. *Denotes htSNP. dbSNP numbers for all novel polymorphisms in boldface. Single-locus tests at SNPs other than htSNPs were calculated from imputed data based on htSNP genotypes and LD information obtained from the sequencing panel. The single-locus *P* values were obtained using paired *t* tests. Data were not imputed and tests not performed for SNPs with allele frequencies <0.03. UTR, untranslated region.

cus TDT *P* value is <0.1. By setting a threshold *P* value relatively high at the first stage, in order to avoid rejecting true positives, little power is lost when compared with a single-stage approach. After genotyping of set 2, statistical analysis is performed on the entire dataset (2,462 families). Given the currently available sample collection and the two-stage strategy adopted, we have over 90% power to detect an association with $P = 1 \times 10^{-4}$, assuming a relative risk of 1.5 conferred by each copy of the causal allele and a population frequency of the causal allele of 0.1, regardless of whether genotyping proceeds to stage 2.

Approaches to the statistical analysis of htSNPs have been described by Chapman et al. (17). It was demonstrated that in regions of strong LD, simple models considering only the main effects of htSNP genotypes were optimal or near optimal for detecting disease association. Consequently, the multilocus TDT is considered the most appropriate test. In stage 1, the multilocus TDT *P* value for association between type 1 diabetes and *IAN4L1* was 0.484 and for *CBLB* was 0.692. Therefore, we did not proceed to genotype the additional set 2 families in either gene. To illustrate the predictions of ungenotyped markers that are possible using this new approach, Tables 1 and 2 include single-locus tests for all the common polymorphisms in set 1 families.

These results suggest that common alleles of *IAN4L1* and *CBLB* do not contribute significantly to the familial clustering of human type 1 diabetes in the two populations analyzed. We cannot exclude the possibility that a common variant exists in either gene with an effect that is too

small to be detected in a study of this size or that there is an unidentified polymorphism that is in much weaker LD with the htSNPs we analyzed. Had we genotyped all identified markers, our probability of detecting disease association would not have been substantially increased. Large introns and more extensive flanking DNA regions can be analyzed for association in the future by using the genome-wide SNP map that is under construction (18). By adopting an htSNP and a two-stage strategy, these candidate genes were quickly and economically evaluated for association with type 1 diabetes. This approach has allowed us to significantly reduce the genotyping burden (by ~84% for *CBLB* and ~87% for *IAN4L1*) and decrease turnaround time *1*) by avoiding redundant genotyping of markers that can be imputed easily from the genotyping data of other markers and the patterns of LD across the gene and *2*) by refraining from genotyping additional families in which there is limited possibility of obtaining an overall significant result. Although, in these data, common allelic variation in neither the *IAN4L1* nor *CBLB* coding regions is associated with type 1 diabetes, genetic susceptibility data obtained from animal models can be directly applicable to humans, as has been found with the MHC (19) and *CTLA4* (20). In addition, in our study, we have not excluded the possibility that alleles with frequencies <3% affect susceptibility to type 1 diabetes, and this remains a possibility. Whether or not exactly the same disease susceptibility genes in animal models are contributors to the familial clustering of disease in humans depends on the frequencies of causal alleles of the gene orthologues in human populations, a parameter that is subject to wide random variation. Nevertheless, even if a direct genetic susceptibility concordance is not found, the pathways that emerge from genetic studies of representative models and humans improve our understanding of disease mechanisms and how these might be modulated to reduce the risk of disease.

RESEARCH DESIGN AND METHODS

The 754 type 1 diabetic families were white European or of Caucasian European descent, with two parents and at least one affected child (472 Diabetes U.K. Warren 1 multiplex [21] and 282 multiplex ascertained in the U.S., obtained from the Human Biological Data Interchange [22]).

SNP identification and genotyping. Direct sequencing of nested PCR products from 96 type 1 diabetic individuals for *CBLB* and 32 for *IAN4L1* was performed using an Applied Biosystems (ABI) 3700 capillary sequencer (Foster City, CA). Polymorphisms were identified using the Staden Package (http://www.mrc-lmb.cam.ac.uk/pubseq/) and mapped to the golden path sequence (NCBI build 33). htSNPs were selected from the polymorphisms with >3% minor allele frequency in our sequencing panel using Stata (http://www.stata.com) and the htSNP package available from http://www-gene.cimr.cam.ac.uk/clayton/software/stata/.

Genotyping was performed using either Taqman MGB chemistry (Applied Biosystems) (23) or the Invader biplex assay (Third Wave Technologies, Madison, WI) (24). All genotyping data were double scored to minimize error. All SNP sequences are in dbSNP; sequencing and genotyping data can be obtained upon request (http://www-gene.cimr.cam.ac.uk/todd/human_data.shtml).

Annotation. *CBLB* (European Molecular Biology Laboratory [EMBL] accession nos. U26710, full-length human *CBLB* mRNA; U26711, truncated form 1, human *CBLB*, lacking leucine zipper mRNA; amd U26712, truncated form 2, human *CBLB*, lacking leucine zipper mRNA) and *IAN4L1* (EMBL accession no. AK002158) were annotated locally, importing Ensembl information into a temporary ACeDB database. Here, the gene structure was verified following a more thorough Blast analysis and then reextracted from ACeDB in GFF format and submitted to a local Gbrowse database (National Center for Biotechnology Information build 33) (DIL annotations viewable at http://dil-gbrowse.cimr.cam.ac.uk).

Statistical analysis. All statistical analyses were performed within Stata making specific use of the *Genassoc* package (http://www-gene.cimr.cam.ac.uk/clayton/software/stata). All genotyping data were assessed for, and found to be in, Hardy-Weinberg equilibrium ($P > 0.05$).

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