*Rapid communication***Anomalous behaviour of the 5' insulin gene polymorphism allele 814: lack of association with Type I diabetes in Basques****I. Urrutia, B. Calvo, J. R. Bilbao, the GEPV-N Group*, L. Castaño***

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Summary A susceptibility locus (*IDDM2*) for Type I (insulin-dependent) diabetes mellitus has been identified as allelic variation at a variable number of tandem repeats polymorphic region upstream of the human insulin gene. In Caucasian populations, individuals homozygous for the short length alleles (26 to 63 repeats; class I) have a two- to fivefold increased risk of developing the disease, while the long alleles (more than 140 repeats; class III) are dominantly protective. Recent evidence has shown that class I alleles are not equally predisposing, and in particular, the 42-repeat allele (*allele 814*) can be protective when paternally inherited. We have assessed the contribution of *IDDM2* to disease in a group of Basque families with Type I diabetes. As in other Caucasoid popula-

tions, we found that class I alleles, as a whole, are associated with an increased risk of developing the disease. Using a polymerase chain reaction-based assay to more accurately resolve the different sizes of individual class I alleles, we identified 14 different variants and observed that *allele 814* has an anomalous behaviour in Basques, being the only class I allele that does not have an increased frequency in the diabetic alleles group. These findings provide additional support for the recently published allele-specific effects of *IDDM2* in Type I diabetes pathogenesis. [Diabetologia (1998) 41: 1121–1123]

Keywords Type I diabetes, insulin gene VNTR, *IDDM2*, Basques, association analysis.

The greatest genetical contribution to the risk of developing Type I (insulin-dependent) diabetes mellitus has been located in the HLA class II genomic region on chromosome 6p21.31, (termed *IDDM1*). Several other loci with a smaller contribution have been identified. The *IDDM2* susceptibility locus has been mapped to and identified as variation at the variable number of tandem repeats (VNTR) polymorphic region upstream of the human insulin gene (*INS*) on chromosome 11p15.5 [1]. In Caucasians, the shortest VNTR alleles (class I) are more frequent among pa-

tients with Type I diabetes, and long alleles (class III) are associated negatively with the disease. Recent reports have indicated allele specific effects on the risk of the disease [2, 3]. Basques are a population living in the western Pyrenees (northern Spain and south-western France) which maintain distinctive genetic characteristics due to the relatively low admixture with other Caucasian populations [4]. HLA association with Type I diabetes in this ethnic group shows a number of characteristic features, including low frequency of subtype HLA-DR4 and its associated DQA1*0301 and DQB1*0302 alleles, and a high frequency of haplotype DR3-F1C30, which is the most frequent among Basque diabetic patients, and is uncommon in other Caucasian populations [5, 6]; little is known about the role of non-HLA genes in the susceptibility to Type I diabetes in this population. We analysed a group of families of Basque origin with Type I diabetes, to help characterise the contribution of *IDDM2* to Type I diabetes suscepti-

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Abbreviations: VNTR, Variable number of tandem repeats; INS, human insulin gene; CI, confidence interval.

bility and to investigate the possibility of differential association of particular class I alleles with the disease.

Subjects and methods

Patients and control subjects. We analysed 71 (64 simplex and 7 multiplex) Basque families ($n = 326$) originating from the Basque and Navarre regions in Spain, comprising a total of 78 patients with Type I diabetes (42 males, 36 females; mean age 23.2 ± 13.9 years) and 248 first-degree relatives (120 males, 128 females; mean age 36.5 ± 19.3 years). All Type I diabetic patients were diagnosed according to the World Health Organization (WHO) criteria [7]. The mean age of Type I diabetes onset was 14.5 ± 9.9 years. We also studied 45 unrelated healthy subjects who did not have a family history of diabetes (18 males, 27 females; mean age 34.3 ± 7.4 years). All people included in the study had at least four generations of family names of undoubted Basque origin. All studies were performed after informed consent had been given by the subjects or their parents. The I/I, I/III and III/III VNTR genotype frequencies in parents (70, 45 and 12 respectively; $\chi^2 = 0.75$, 1df) satisfied Hardy-Weinberg equilibrium.

Insulin VNTR typing

Southern blot. Genomic DNA was digested with *Pvu* II, separated on a 1% agarose gel, blotted onto nylon membranes and hybridised overnight at 65°C with ^{32}P -labelled *phins310* probe (ATCC 57400, Rockville, Md., USA). Bands were detected by autoradiography and VNTR allele classes were assigned according to Bell et al. [8].

Class I allele size analysis. To resolve the differences in size between alleles falling into class I, a fluorescence based DNA-sizing technique was used. Polymerase chain reaction amplification of class I VNTR alleles was performed basically as described by Bennett et al. [1], except that amplification primer *VNTR6* was fluorescently labelled with Cy5 (Pharmacia-Biotech, Uppsala, Sweden) at its 5' end. Electrophoresis was carried out on a 25 cm long, 6% sequencing gel in an ALFexpress DNA Sequencer (Pharmacia-Biotech) for 10 h at a limiting power of 25 W at 55°C . Each sample was mixed with an equal amount of an internal size standard and polymerase chain reaction fragments were sized in mobility units which approximate to fragment length in base pairs using the Fragment Manager software (Pharmacia-Biotech). In order to standardise the lengths of our alleles to sizes previously reported, DNA samples with known alleles were genotyped at the Wellcome Trust Centre for Human Genetics, University of Oxford, using published methods [1].

Statistical analysis. Association of the VNTR class I alleles with Type I diabetes was tested using affected family-based control subjects. Out of the four parental VNTR alleles in each family, those never transmitted to a diabetic member were categorised as "non-diabetic alleles", and those harboured by diabetic patients were classified as "diabetic alleles". Relative risk values of the genotypes were calculated using a case-control study design, comparing genotype frequencies of diabetic patients with those of unrelated healthy control subjects. Chi-squared and Fisher's exact tests were used for statistical comparison. Only two-tailed p values are reported.

Table 1. Allelic frequencies of the VNTR polymorphism in the family-based study

VNTR alleles	Diabetic alleles ($n = 147$)	Non-diabetic alleles ($n = 125$)
CLASS I	0.857	0.600
CLASS III	0.143	0.400

$$p = 1.5 \cdot 10^{-6}$$

Results and discussion

Our family-based study (Table 1) showed an increased frequency of class I alleles in the diabetic alleles group (86%) compared with the non-diabetic alleles group (60%), confirming an association of class I alleles with Type I diabetes among Basques ($\chi^2 = 23.16$, 1df; $p = 1.5 \cdot 10^{-6}$). When genotype frequencies were compared between diabetic patients and unrelated control subjects, class I homozygous (I/I) were present in 74.4% of diabetic patients and only in 31% of healthy control subjects, and both I/III and III/III were significantly more frequent in the control group (51 and 18%, respectively) than among diabetic patients (24.4 and 1.2%, respectively). Based on this distribution, relative risk values were 6.42 for I/I [95% confidence interval (CI) 2.66–15.76; $p = 2.7 \cdot 10^{-6}$], 0.31 (95% CI 0.13–0.72; $p = 2.5 \cdot 10^{-3}$) for I/III and 0.06 (95% CI 0.00–0.49; $p = 1.3 \cdot 10^{-3}$) for III/III. Thus, in the Basque population the class I/I homozygous genotype is associated with a sixfold increased risk of developing Type I diabetes. These results support the recessive mechanism for VNTR class I-mediated mediated susceptibility described for other Caucasoid populations [1].

Using a fluorescence-based polymerase chain reaction method, we identified 14 different class I alleles in our Basque data set (Fig. 1), ranging from 30 to 44 repeats (641 to 843 mobility units). Among the class I alleles that are frequent in Basques, the 42-repeat allele, which corresponds to *allele 814*, was the only one that was not increased in the diabetic category and its distribution was deviant from the rest of class I alleles ($\chi^2 = 4.14$, 1df; $p = 0.04$), but did not differ significantly from class III alleles, thus suggesting it does not contribute to the risk of development of Type I diabetes. A recent report has also found evidence of the singularity of *allele 814* among a large group of Caucasian families of diverse origin with Type I diabetes [3]. Ours is the first report presenting differences in contribution to risk of individual class I VNTR alleles in a sample from a relatively homogeneous Caucasian population such as Basques. Although this strict design limits the size of the available population and reduces the probability of detecting differences, our results identify *allele 814* as non-predisposing to Type I diabetes and provide additional support for the recently published *INS* VNTR *allele 814* anomalous effect in the disease pathogenesis [3].

Fig. 1. Distribution of class I VNTR subtypes among diabetic and non-diabetic alleles in the Basque population. The 42-repeat, allele 814 reported by Bennett et al. [3], was more increased in the non-diabetic group when compared with the remaining class I alleles (2×2 contingency test; $\chi^2 = 4.14$, $p = 0.04$) * $p < 0.05$

The importance of this finding in other populations and its possible role in the mechanism linking *IDDM2* and disease are still to be determined, but recent reports have put forward an interesting hypothesis that insulin VNTR allele-mediated control of the level of insulin expression could influence the induction of immune tolerance towards insulin in the thymus [9, 10]. The analysis of genetically homogeneous populations and large data sets, together with functional studies of the different VNTR alleles will help clarify the contribution of individual class I alleles to Type I diabetes.

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