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ORIGINAL ARTICLE

Association between IgH enhancer hs1.2 and type 1 diabetes

Rossella Cianci[1] · Pietro D'Addabbo[2] · Giovanni Gambassi[1] · Serena Lolli[3] · Eliseo Serone[3] · Alessandro Rizzi[1] · Dario Pitocco[1] · Franco Pandolfi[1] · Domenico Frezza[3]

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Abstract

Aim To investigate the association of alleles of the 3′ immunoglobulin heavy-chain regulatory region 1 (3′RR-1) enhancer hs1.2 in patients with type 1 diabetes (T1D).

Methods Eighty-one patients with T1D [among which 12 had concomitant coeliac disease (CD) and 25 an autoimmune thyroid disease (AITD)] were compared to 248 healthy individuals. All subjects were recruited from the same geographical area. Blood samples were collected from all patients and a nested PCR was performed to amplify the core of the 3′RR-1 and detect the alleles of the hs1.2 enhancer.

Results Allele distribution in healthy individuals was significantly different when compared to that of patients with T1D ($p < 0.01$). Even greater differences were detected comparing allele distribution of patients with T1D alone versus those with concomitant CD, but not versus those with concomitant AITD. The frequency of *2 allele is increased by 23% in patients with T1D and CD.

Conclusions The present study establishes that the multiallelic hs1.2 enhancer of the 3′RR-1 is associated with T1D, with higher frequency when there is co-occurrence of CD. This evidence has been previously observed in other immune diseases.

Keywords Enhancer hs1.2 · Type 1 diabetes · Coeliac disease · Immunoglobulins · Non-HLA genes

Introduction

Development of B lymphocytes is a multistep process that includes the somatic rearrangement of Ig genomic region [1–3]. This regulatory action involves a long list of players and of functional genomic regions [4]. The Ig heavy-chain genomic region has a wide 3′ regulatory region (3′RR) composed by enhancers and other sequences that are essential to the functional folding of the 3′RR itself [5, 6].

The 3′RR of the Ig heavy chain is highly preserved in mammals. During evolutionary differentiation of primates, a large region including 4 Ig class-specific exons and 3′RR has been duplicated, originating a second copy of the 3′RR (i.e. 3′RR-1 and 3′RR-2, see Fig. 1) [7–9]. After class switching in B cells ending with the production of IgE, the only active control of the heavy chain is provided by the 3′RR-2 left. The other allele is believed to be inactive (see Fig. 1) [10, 11].

Recent evidence in mouse suggests a super-enhancer activity of the 3′RR influencing the maturation of B-lymphocytes for somatic hyper-mutation, class switch recombination, interallelic interactions such as transvection [12]. The structures of the 3′RR support the hypothesis that 3D conformations determine nuclear location in the centre or in periphery transcription factories with interallelic contacts as observed with 4C conformation-capture assay and Fish-3D [13, 14]. The evolutionarily constant presence of a palindrome surrounding the hs1.2 enhancer strongly indicates that a hairpin shape of the DNA influences the 3′RR functions [7]. Moreover, the conservation in many species of a putative tetraplex sequence in the hs1.2 enhancer confirms the relevance of 3D structures for the 3′RR functions [15]. It should be also considered that a four-allele polymorphism

Managed by Massimo Porta.

✉ Domenico Frezza
frezza@uniroma2.it

[1] Institute of Internal Medicine, Catholic University, Largo A. Gemelli, 8, 00168 Rome, Italy

[2] Department of Biology, University of Bari, Via Orabona, 4, 70125 Bari, Italy

[3] Department of Biology Enrico Calef, Tor Vergata University, Viale della Ricerca Scientifica, 1, 00133 Rome, Italy

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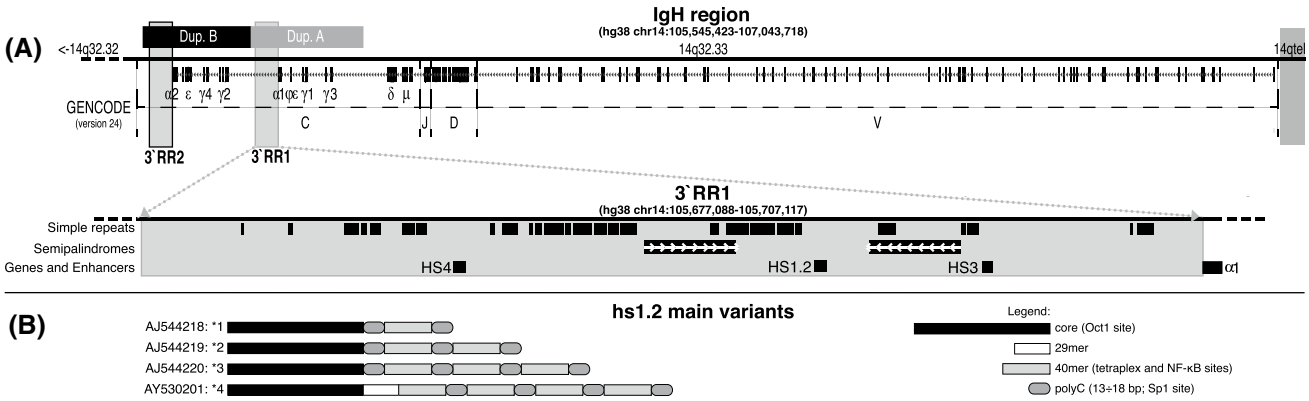

Fig. 1 Ig heavy-chain region as described in the Genome Reference Consortium Human Reference 38 (GRCh38/hg38), and hs1.2 alleles. **a** The upper part shows the whole IgH region on the human chr14. The Gencode (version 24) track represents the variable, diversity, joining and constant region exons. The two duplicated 3′ regulatory regions are labelled as 3′RR-1 and 3′RR-2. The lower part describes a more detailed view of the 3′RR-1, showing the position of internal features: simple repeats, inverted complementary repeats (semipalindromes) and enhancers. The direction of the two palindromic branches is indicated (arrows). The position of the three enhancers is also evidenced, with respect to the first portion of the alpha 1 constant gene. **b** Detailed description of the hs1.2 enhancer elements and tandem repeats that give rise to the four polymorphic alleles

of hs1.2 enhancer has been described (Fig. 1) [7], with the potential to influence these 3D structures. Indeed, hs1.2 is composed of two main regions: a constant sequence supporting an SP1-binding site and a variable-number tandem repeat with a monomer, repeated in humans from one to four times [9].

Correlation studies of allele frequency have concluded that in immune-related diseases the hs1.2 alleles restore control on circulating levels of Igs, as it is the norm in children under 5 years of age [16]. Indeed, an association between the *2 allele and high levels of circulating Igs has been confirmed in patients affected by systemic sclerosis, rheumatoid arthritis, lupus erythematosus, coeliac disease and psoriasis [17–20]. Likewise, in patients with an IgA defective disorder, the *1 allele is not only associated with the disease, but it also correlates with low levels of Igs [21]. In the initial studies, carried out in patients with IgA nephropathy, these polymorphisms correlate with the severity of the disease [22, 23]. Even in healthy subjects, frequency of the *2 allele is significantly higher when circulating Ig levels are above normal, whereas the *1 allele correlates with low Ig levels [21].

T1D is an autoimmune disease characterized by the activation of autoreactive T-cells that participate in pancreatic beta-cell destruction [24]. Genome wide association studies highlighted the strong link between *HLA* gene variants and T1D. HLA is located on chromosome 6p (6p21.3), but also other non-HLA genes are associated with T1D development, for example, the insulin gene (11p15.5), CTLA4 gene (2q33) and PTPN22 gene (1p13) [25]. All these genes regulate the complex interaction between B and T cells, during antigen presentation. This interaction results in the production of hyper-reactive T-cells that mediate the damage to the target organ. T1D could be considered a model for organ-specific autoimmune disorders such as coeliac disease (CD) and autoimmune thyroid diseases (AITD) [26] which frequently co-occur with T1D [27, 28].

This study has a background on two points deriving by previous studies on polymorphisms of hs1.2: association studies on levels of humoral Ig in several immune pathologies such as psoriasis [20], systemic sclerosis [18], rheumatoid arthritis [29], lupus erythematosus [19], IgA defect [21] and the effect of maturation of children of 5 years old [16]. Children under 5 years of age homozygous for the *2 allele have very high levels of circulating Ig that return to normal later in life. We hypothesize that juvenile T1D can be triggered by the presence of high levels of Ig in these children.

Thus, the aim of our study was to investigate the association of the enhancer hs1.2 of the Immunoglobulin heavy-chain 3′RR-1 polymorphisms in patients with T1D.

Materials and methods

Patients and healthy controls

Eighty-one patients (46 females and 35 males) with T1D were included in the study. Of these, 12 (15%) had a concomitant CD, 25 (31%) an AITD, 5 (6%) both CD and an AITD, while the remaining 39 patients had T1D alone. A total of 248 healthy subjects, sex-matched, from the same geographical area of Rome (Italy) were also studied.

Mean age was 67 years (SD ± 10.3) among T1D patients and 58 years (SD ± 10.0) among healthy controls. None of the individuals included in the study had defects of the T cell compartment as evaluated by a T cell subsets

cytofluorimetric count, using anti-CD45-peridinin chlorophyll protein (PerCP) mAb (Becton–Dickinson) for immunological gate, with anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE) and anti-CD8-PE mAb (Becton–Dickinson), performed on a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ, USA). Informed consent has been obtained from all patients. The institutional review board has given approval.

PCR assay

We run a selective PCR, which amplified the hs1.2-RR-1 region, but not the orthologous hs1.2-RR2 region, to estimate the frequency of the four alleles of hs1.2-RR-1 (Gene Bank acc. num. AJ544218, AJ544219, AJ544220, AJ544221). Genomic DNA was extracted from peripheral blood nucleated cells (PBMCs) or from buccal mucosal swabs. Genomic DNA was then amplified with primers and the nested PCR protocol as previously described [9].

Statistical analysis

The expected frequency of alleles/genotypes in patients was calculated using allele frequency in healthy control groups. Chi-square test was used for statistical comparisons.

Results

hs1.2 allele frequency

We have studied the frequency of the four alleles of hs1.2-RR-1 in 81 patients with T1D and 248 healthy subjects, sex-matched, from the same geographical area of Rome (Italy). Of T1D patients, 12 had a concomitant CD, 25 an AITD, 5 had both CD and an AITD, and the remaining 39 had T1D alone. hs1.2 genotype and allele frequencies in healthy controls and in patients with T1D alone or with concomitant CD or AITD are shown in Tables 1 and 2.

Table 1 hs1.2 genotype and allele frequencies in healthy controls and in patients with T1D. (A) Genotype frequency, (B) allele frequency and Chi-square tests

Genotypes	Control		T1D		
	Obs. *n*	Obs. *f* (%)	Exp. *n*	Obs. *n*	Obs. *f* (%)
(A)					
1/1	52	20.97	15.65	9	11.11
2/2	37	14.92	12.65	22	27.16
3/3	2	0.81	0.30	0	0
4/4	4	1.61	0.89	0	0
1/2	84	33.87	28.14	26	32.1
1/3	12	4.84	4.30	0	0
1/4	18	7.26	7.46	3	3.7
2/3	13	5.24	3.87	0	0
2/4	25	10.08	6.71	21	25.93
3/4	1	0.4	1.03	0	0
TOT	248	100	81.00	81	100.00
p				**2.47E−08**	

Alleles	Control		T1D			
	Obs. *n*	Obs. *f* (%)	Exp. *n*	Obs. *n*	Obs. *f* (%)	*p*
(B)						
*1	218	43.95	71.2	47	29.01	**1.28E−04**
*2	196	39.52	64.02	91	56.17	**1.45E−05**
*3	30	6.05	9.8	0	0	**1.24E−03**
*4	52	10.48	16.98	24	14.81	0.07
Total	496	100	162	162	100	
p				**4.53E−07**		

Expected genotype number in T1D patients was calculated using allele frequency in healthy control groups. Chi-square test between observed and expected T1D genotypes shows $p = 2.47E{-}08$, which means a highly significant difference in genotype distribution between cases and controls

Significant *p*-values are highlighted in bold ($p < 0.05$)

Table 2 hs1.2 genotype and allele frequency in patients with T1D alone, compared to patients with associated coeliac disease (CD) or AITD (genotypes absent in the cohorts, and the allele 3, absent as well, were omitted). (A) Genotype frequency, (B) allele frequency and Chi-square tests

Genotypes	T1D		T1D and CD			T1D and AITD		
	Obs. *n*	Obs. *f* (%)	Exp. *n*	Obs. *n*	Obs. *f* (%)	Exp. *n*	Obs. *n*	Obs. *f* (%)
(A)								
1/1	6	13.64	1.64	0	0.00	3.41	3	12.00
1/2	14	31.82	3.82	4	33.33	7.95	8	32.00
1/4	3	6.82	0.82	0	0.00	1.70	0	0.00
2/2	11	25.00	3.00	6	50.00	6.25	5	20.00
2/4	10	22.73	2.73	2	16.67	5.68	9	36.00
TOT	44	100.00	12.00	12	100.00	25.00	25	100.00
p				0.23			0.41	

Alleles	T1D		T1D and CD				T1D and AITD			
	Obs. *n*	Obs. *f* (%)	Exp. *n*	Obs. *n*	Obs. *f* (%)	*p*	Exp. *n*	Obs. *n*	Obs. *f* (%)	*p*
(B)										
1	29	32.95	7.91	4	16.67	0.09	16.48	14	28	0.46
2	46	52.27	12.55	18	75	**0.03**	26.14	27	54	0.81
4	13	14.77	3.55	2	8.33	0.37	7.39	9	18	0.52
Total	88	100	24	24	100		50	50	100	
p					**0.04**				0.69	

Significant *p*-values are highlighted in bold ($p < 0.05$)

In patients with T1D the most significant changes in frequency were found for the alleles that have a higher presence in the population, i.e. *1 and *2 alleles. The same change was previously observed in several association studies, reported above [18, 19, 29], describing the *2 allele as a possible factor of increased risk of autoimmune diseases, such as systemic sclerosis, rheumatoid arthritis and lupus erythematosus.

As observed in the analysis of the cohort of patients with T1D compared to a control group of healthy subjects, it appears evident that an increased frequency of *2 allele of hs1.2 3′RR-1 is significantly associated with T1D, as shown in Table 1, panel A and B.

In fact, Table 1 illustrates that there is a highly significant difference in genotype frequency comparing T1D and healthy controls ($p < 0.01$; panel A). This difference is due to an increased frequency of the *2 allele, relative to the *1 allele and *3 allele decreased frequency (panel B). The panel B shows a statistically significant alteration of the frequency of the single alleles in T1D and controls. In fact, the *1 allele has a frequency of 44% in controls and 29% in diabetic patients (p 1.28E−04), whereas the *2 allele frequency is 40% in controls and 56% in diabetic patients (p 1.45E−05). The *3 allele frequency is 6% in controls, and this allele is not present in enrolled T1D patients (p 1.24E−03).

The further analysis performed on two different subgroups of patients with T1D indicates that indeed the frequency of *2 allele is much higher in T1D patients with concomitant coeliac disease (CD), as shown in Table 2A and B. The frequency of patients that cumulate the presence of T1D and CD is quite low, but the number of these patients was sufficient to show a statistically significant high variation of *2 allele compared to patients with only T1D.

Table 2 shows genotype and allele frequency of the enhancer hs1.2 in two different subgroups of T1D patients. There is a significant difference of allele frequency between patients with T1D alone and those with concomitant CD ($p < 0.05$; panel B), but not when T1D co-occur with AITD ($p > 0.05$; panel B). The frequency of the *2 allele is further increased by 23% in patients with T1D and CD. The frequency of the *1 allele is reduced, but this reduction remains not statistically significant.

The results suggest a new case of association of *2 allele with another immune disease.

Discussion

The findings of the present study document that by comparing either genotype or allele frequencies, there is a highly significant difference between healthy individuals and patients with T1D. In T1D subjects with a concomitant CD diagnosis, the *2 allele frequency was significantly higher than in other T1D subjects (Table 2). This

result suggests that the co-occurrence of T1D and CD may be triggered by the activation of the same regulatory system under hs1.2 enhancer control, as part of modulation of the 3′RR-1 considered a super-enhancer region [31]. Instead, a different mechanism is probably responsible for the co-occurrence of T1D and AITD since these patients have unaltered allele frequencies.

The influence of the hs1.2 enhancer copy number variation polymorphisms is related to a possible 3D structure change that alters loci availability to the binding of transcription factors such as NF-kB or aryl hydrocarbon receptor (AhR)-binding site and 4G tetraplex structure [15, 17, 19, 30]. Other polymorphisms in the 3′RR-1 could be linked to different pathological conditions, may be generating different 3D spatial organization of the 3′RR with the essential palindromic interenhancer genomic area [31].

Heterogeneity of discrete pathophysiological mechanisms of T1D could explain distinct clinical features including response to treatment, as well as the association of complex autoimmune and inflammatory diseases. On the other hand, the discrepancy of CD autoimmunity between Swedish and Danish T1D cohorts strongly suggests that geographical variations are explained by differences in environmental factors [32]. Indeed, an important role of environmental variables was conclusively confirmed by the evidence that monozygotic twins have incomplete concordance of CD susceptibility [33, 34]. In fact, environmental factors play an important role in CD pathogenesis by inducing alterations of gene expression via histone modifications, DNA methylation and miRNA regulation [25].

The findings of the present genetic association study suggest further investigation on the immune regulation mechanisms under control of the 3′RR-1 in patients when T1D co-occur with CD. It is noteworthy that also in patients with dermatitis herpetiformis the co-occurrence of CD further increases the frequency of *2 allele [20]. This provides further support to the evidence that CD in association with other immune-related diseases further amplifies hs1.2 enhancer activity. This hypothesis is corroborated also by the fact that T1D is a juvenile disease and children under 5 years of age with *2 allele show a higher level of Ig [16]. A recent study on a cohort of CD patients in the USA (Dayton, OH) has failed to confirm an association between CD and the *2 allele [30]. However, association studies need an extremely valid cohort of control subjects as comparison group. Most specifically, healthy controls must have the same geographical origin and in the US population, even after few generations the descendants do not abolish the signatures from such genetic inhomogeneous origins.

Conclusions

The present study confirms that there is a genetic association between hs1.2 3′RR-1 and T1D. This new evidence together with previous association studies suggests a relevant role of the hs1.2 enhancer and possibly of the different haplotypes linked to the surrounding palindromic structure in immune diseases. Future studies will have to investigate this association in larger populations of the same geographical origins and to explore other polymorphisms of the 3′RR-1 as well as other specific mechanisms underlying the disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was performed according to the ethical principles of the Helsinki Declaration of 1964, revised by the World Medical Organization in Edinburgh in 2000 and 2008 and was approved by the ethics committee of our hospital.

Informed consent All participants provided written informed consent before the enrollment in the study.

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