The Journal of Immunology

# Allele *1 of HS1.2 Enhancer Associates with Selective IgA Deficiency and IgM Concentration



Vincenzo Giambra, Rossella Cianci, Serena Lolli, Claudia Mattioli, Giacomo Tampella, Marco Cattalini, Sebnem S. Kilic, Franco Pandolfi, Alessandro Plebani and Domenico Frezza

*J Immunol* 2009; 183:8280-8285; ;
doi: 10.4049/jimmunol.0902426
http://www.jimmunol.org/content/183/12/8280



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Allele *1 of HS1.2 Enhancer Associates with Selective IgA Deficiency and IgM Concentration[1]

**Vincenzo Giambra,[2]* Rossella Cianci,[2†] Serena Lolli,[†] Claudia Mattioli,[‡] Giacomo Tampella,[§] Marco Cattalini,[§] Sebnem S. Kilic,[¶] Franco Pandolfi,[†] Alessandro Plebani,[§] and Domenico Frezza[3‡]**

**Selective IgA deficiency (IGAD) is the most common primary immunodeficiency, yet its pathogenesis is elusive. The IG (heavy) H chain human 3′ Regulatory Region harbors three enhancers and has an important role in Ig synthesis. HS1.2 is the only polymorphic enhancer of the 3′RRs. We therefore evaluated HS1.2 allelic frequencies in 88 IGAD patients and 101 controls. Our data show that IGAD patients have a highly significant increase of homozygousity of the allele *1 (39% in the IGAD patients and 15% in controls), with an increase of 2.6-fold. Allele *4 has a similar trend of allele *2, both showing a significant decrease of frequency in IGAD. No relationship was observed between allele *1 frequencies and serum levels of IgG. However, allele *1 was associated in IGAD patients with relatively low IgM levels (within the 30th lowest percentile of patients). The HS1.2 polymorphism influences Ig seric production, but not IgG switch, in fact 30th lowest or highest percentile of IgG in patients did not associate to different frequencies of HS1.2 alleles. The control on normal healthy subjects did not correlate high or low levels of IgM or IgG with HS1.2 allelic frequence variation. Overall our candidate gene approach confirms that the study of polymorphisms in human diseases is a valid tool to investigate the function of these Regulatory Regions that confers multiple immune features.** *The Journal of Immunology,* **2009, 183: 8280–8285.**

The humoral and cell-mediated immuno-response is the product of complex interactions in a fine coordinated manner modulated by multiple regulatory elements leading to the specificity of the immune-response. Alterations of this equilibrium may induce immonological diseases. Selective Ig A deficiency (IGAD)[4] (OMIM 137100) is the most common primary immunodeficiency, with a prevalence of ~1/600 in Caucasians. The affected individuals have low or absent IgA levels in serum (usually below 5 mg/dl) and altered mucosal secretions and may suffer from frequent respiratory and gastrointestinal infections. The genetic basis of IGAD is poorly understood and susceptibility loci have been described, such as *IGAD 1* located at 6p21 (1). A linkage to chromosome 16 has also been reported (2). Moreover, the class switch recombination is under the control of several factors including regulatory genes, such as TACI (TNFR13B) (3, 4). TACI mutants do not respond to its ligands. At least two ligands have been identified and are referred to as 1) the proliferation-inducing ligand (APRIL) (5) and 2) the B cell activating factor (BAFF). Mutations in the receptor ligand system are associated with IgA defect and other Ig defects (3, 6, 7). Bruton tyrosine kinase (BTK) is pivotal for normal B cell differentiation, BTK also has a consensus for NF-κB in the promoter and is probably co-regulated with BAFF and TACI (8). It can be relevant the presence of NF-κB binding site in allele *2 absent in allele *1 of HS1.2. The human Ig (heavy) H chain 3′ regulatory region, harbors three enhancers and has an important role in maturation and expression of immunoglobulins (9). This statement is based on available studies in both mice and humans (10, 11). In contrast to the single murine 3′RR, in humans there are two 3′RRs, each one located downstream of the two Cα regions (11, 12). The 3′RR enhancers are structured as a regulatory complex and play a role in the germline transcription (13). This is a prerequisite for class switch recombination and create an architectural scaffolding by physical interactions with H chain variable region (14, 15). In addition to its activity on Ig production, the HS1.2 is thought to be responsible for a wider range of regulatory actions as B cell control (16, 17). The network of interactions on B cell development involves also the function of these regulatory regions activated in a time-dependent manner (18). In humans, in respect to mice, the different organization of the 3′RRs could also have acquired diversified functions.

The human Ig H chain 3′RR is derived by the large ancestral duplication of four regions codifying for the constant Ig portion and harbor three enhancers, shown in Fig. 1*A*. The duplication of the four H chain regions probably occurred in apes with a hinge region represented by the pseudo constant γ region provoking a deletion of one of the two HS3 enhancers present in the large palindrome found in rodents (19, 20). The sequence, structure, and position of the enhancers is highly retained in the Ig H chain 3′ Regulatory Region from rodents to humans. The presence of this region is critical for class switch recombination (9, 21). Activation of 3′RR is regulated during B cell maturation by progressive demethylation that activates chromatine districts (18). More recent studies on mouse targeted for HS4 show that Ig switch can be alternatively regulated by still unknown regulatory factors (17). One unexpected consequence is the down-regulation of both Ig levels and B circulating cell number. The function of enhancers acts through the formation of complexes with transcription factors

*Terry Fox Laboratory, British Columbia Cancer Research Centre, Vancouver, BC, Canada; †Institute of Internal Medicine, Catholic University, Rome, Italy; ‡Department of Biology "Enrico Calef", University of Roma Tor Vergata, Italy; §Pediatric Clinic, University of Brescia, Italy; and ¶Department of Pediatrics, University School of Medicine, Bursa, Turkey

Received for publication July 27, 2009. Accepted for publication October 13, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Part of this work was done with the University of Tor Vergata funding of Domenico Frezza MIUR PRIN N. 20073RH73W003.

[2] V.G. and R.C. contributed equally to this work.

[3] Address correspondence and reprint requests to Domenico Frezza, Dept. of Biology "Enrico Calef", University of Roma Tor Vergata, Viale della Ricerca Scientifica, Rome, Italy. E-mail address: frezza@uniroma2.it

[4] Abbreviations used in this paper: IGAD, Ig A deficiency; BAFF, B cell activating factor; BTK, Bruton tyrosine kinase.



www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902426

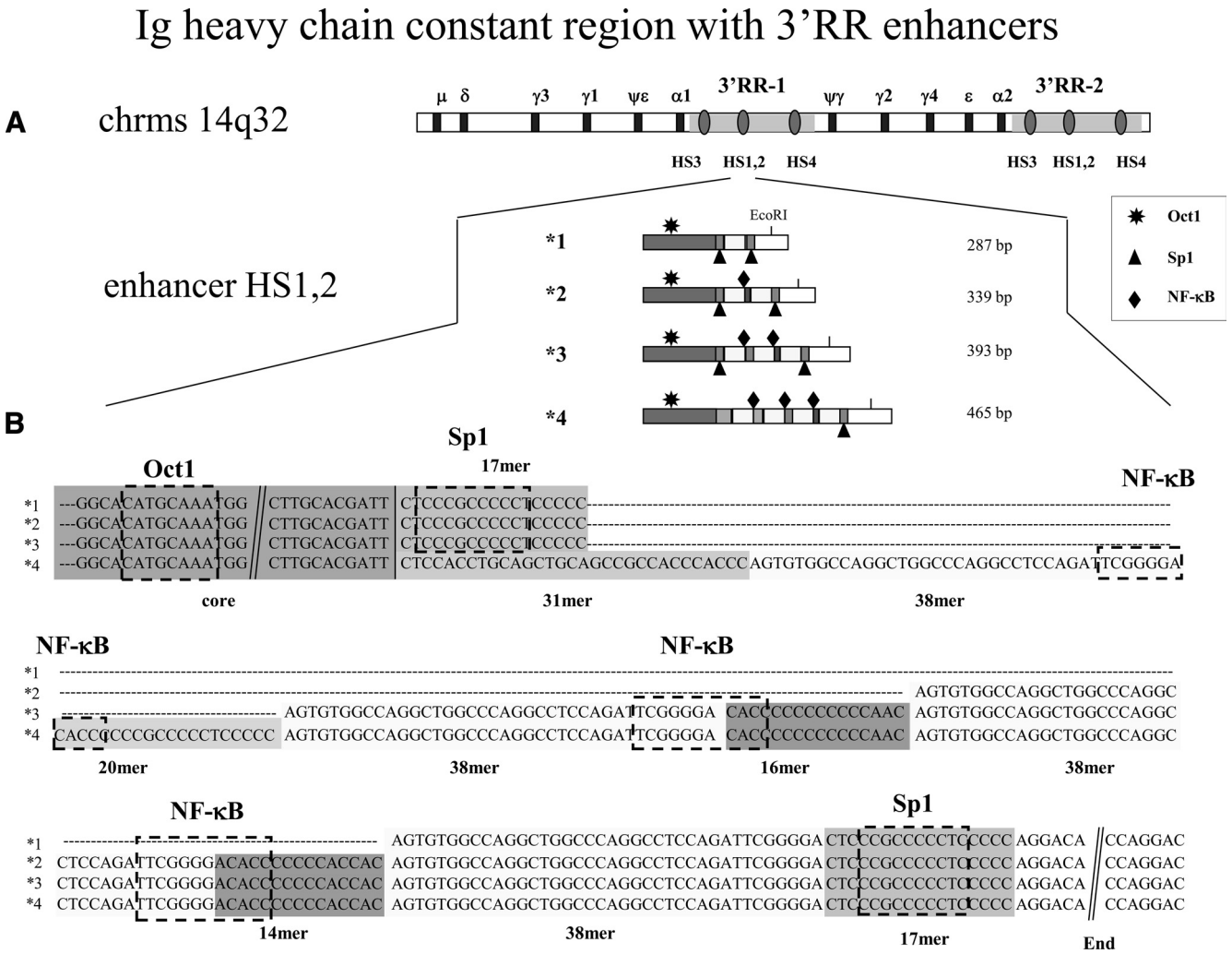


**FIGURE 1.** The human IgH chain cluster with the constant regions. *A*, The map of the constant regions on the 14q32 chromosome, the orientation corresponds to the transcription direction from left to right. The telomeric side is on the left. The two 3′RRs are evidenced with the position of the three enhancers. In the bottom part is shown the enlargment of the HS1.2 alleles *1, *2, *3, and *4. *B*, The alignment of the allele *1, *2, *3, and *4 sequences show the polymorphic part of the enhancer. Starting from the 5′: in dark gray the conserved structure with the Oct1 binding site; in gray the 17 bp spacer in two copies at the 5′end of the polymorphic part and at the 3′ of all alleles of the enhancer, all alleles bear the SP1 consensus site; the 38 bp element that harbors part of the NF-κB consensus in the allele *2 together with the 14 bp spacer (absent in allele *1) is present in two, three, and four copies in the *2, *3, and *4 alleles. The 38 bp element of allele *1 does not bind NF-κB. Other NF-κB binding sites are virtually present also in allele *3 and *4, but were determined only by "in Silicio" analysis and not with EMSA as for allele *2.

(22, 23). Because 3′RR in humans is duplicated it can be hypothesized there is a coordinated activity for the two 3′ RRs during Ig maturation. In addition, they are considered relevant for germline transcription of constant regions during B cell maturation (24). When the switch involves the duplicated constant regions (γ-2, γ-4, ε, α-2) the 3′RR-1 will be deleted and the entire region could be regulated in the next steps of maturation or in memory cell and plasma cell by the presence of 3′RR-2 alone (22). In humans, only HS1.2 was found polymorphic, while HS3 and HS4 was not (25). The polymorphism consists of a 38 bp satellite repeated from one to four copies separated by different spacers of 17, 20, 16, 1 or 4 bp rich in GC. Giambra et al. (26) observed the change of the consensus for several transcription factors in the four alleles. The specific binding of SP1 for HS1.2 allele *1 and *2 and the exclusive binding of NF-κB for allele *2 which is absent in allele *1 was confirmed by competition EMSA. A different mechanism of activation can be hypothesized for the two alleles (see Fig. 1*B*) (27). These structural differences can give rise to a change in the function of the enhancer and consequently on the entire regulatory region, and therefore can influence the maturation and regulation of B cells. In vitro studies with B cell transfected with the different human alleles of HS1.2 occurring from one to four repeats showed an increasing effect on the reporter gene (28). The biological relevance and specific functions in humans of the 3′RRs in vivo can be studied only with correlative studies of polymorphisms with immuno-alterations or pathologies.

Several case control studies show correlation of 3′RR1 HS1.2 alleles with immune diseases. These evidences indicate a specific role of this enhancer in B cell. Allelic frequency of 3′RR1 HS1.2 is highly variable and it is linked to different immunological diseases. In contrast, the frequency of 3′RR2 HS1.2 alleles is conserved among different populations (29). Taken together these data support that the two 3′RRs harbor different functions in the development of immune responses (30, 31).

The polymorphisms of the enhancer HS1.2 (see Fig. 1*B*) was found significantly associated to several immune-mediated diseases (32, 33). Compared with the healthy population, IGAD subjects have a highly significant increase of allele *1 frequency. To

Table I. *HS1.2 allelic frequencies of the IGAD patients and control population*

A

| | Controls | | | IgA defect | | | |
|---|---|---|---|---|---|---|---|
| Genotypes | Observed | % | Expected | Observed | % | Expected | *p* value |
| 1/1 | 15 | 14.85 | 12 | 34 | 38.64 | 22.5 | 0.0004 |
| 2/2 | 25 | 24.75 | 26 | 24 | 27.27 | 14.7 | NS[a] |
| 3/3 | - | - | - | - | - | 0.02 | NS |
| 4/4 | - | - | 2 | - | - | 0.4 | NS |
| 1/2 | 33 | 32.67 | 35 | 15 | 17.05 | 36 | 0.0218 |
| 1/3 | 1 | 0.99 | 0.7 | 2 | 2.27 | 1.5 | NS |
| 1/4 | 6 | 5.95 | 9 | 4 | 4.54 | 6 | NS |
| 2/3 | - | - | 1 | 1 | 1.14 | 1.2 | NS |
| 2/4 | 20 | 19.80 | 14 | 8 | 9.09 | 4.8 | NS |
| 3/4 | 1 | 0.99 | 0.3 | - | - | - | NS |

B

| Alleles | Frequencies | Observed | Frequencies | Observed | $\chi^2$ TEST |
|---|---|---|---|---|---|
| *1 | 0.346 ± 0.033 | 70 | 0.506 ± 0.037 | 89 | $\chi^2 = 18.541$ |
| *2 | 0.510 ± 0.035 | 103 | 0.409 ± 0.037 | 72 | d.f. 4 |
| *3 | 0.010 ± 0.006 | 2 | 0.017 ± 0.009 | 3 | $p$ value = 0.001 |
| *4 | 0.134 ± 0.023 | 27 | 0.068 ± 0.018 | 12 | |

[a] NS, not significant.

further expand our understanding of the range of immunological effects linked to a given phenotype of HS1.2 alleles, we also investigated whether Ig serum concentrations (IgG and IgM) were related to HS1.2 allelic frequencies.

## Materials and Methods

### *Subjects with IGAD and control of local population*

Eighty-eight patients with a median age of 11.75 years (ranges: from 23.3 to 3.1 years), 49 males and 39 females with a diagnosis of selective IgA deficiency based on IgA serum levels below 5 mg/dl, without impairment of IgG and IgM, were included in the present study. None of them had defect of T cell compartment as evaluated by T cell subsets evaluation. A group of 101 subjects, age and sex matched from the same geographical region, was included as control. The Ig levels of a normal group was analyzed in 114 subjects of same geographical area and HS1.2 allelic frequencies determined.

### *Lymphocyte cells and immunoglobulin serum determination*

IgA, IgM, and IgG serum levels were evaluated with standard techniques by the service of clinical analysis of the Pediatric Clinic of the University of Brescia. The IGAD subjects and Control were stratified for two groups of 30% each of the total with relatively low and high IgM or IgG levels.

### *PCR assay*

To estimate the frequencies of the four alleles of HS1.2-A respectively (Gene Bank acc. num. AJ544218, AJ544219, AJ544220, AJ544221), we conducted a selective PCR, which amplified the HS1.2-A region, but not the identical inverted HS1.2-B region (26). Genomic DNA was extracted from peripheral blood nucleated cells or from buccal mucosal swabs and amplified with the primers described previously (26). The cycle conditions were 94°C 2′ for a first step, followed by 94°C 30′′, 61°C 30′′, 68°C 5′ for 10 cycles and 94°C 30′′, 59°C 30′′, 68°C 5′ for 20 cycles, ending with 72°C 10′. PCRs were conducted in 50 microliters of reaction volume containing: 2 microliters of extracted DNA (10 ng), 1.5 U *Platinum Taq*DNA Polymerase High Fidelity (Invitrogen), 15 pmols of each primer, 1.5 mM $MgSO_4$, 50 microM each dNTP, and 1× buffer *High Fidelity* (600 mM Tris-$SO_4$ (pH 8.9), 180 mM ($(NH_4)2SO_4$) (Invitrogen), by using GeneAmp PCR System 9700 (Applied Biosystems). To prevent carryover contamination, pre-PCR procedures were performed with dedicated equipment in a laminar flow hood, using aerosol-resistant plugged pipette tips (ART, Molecular Bio-Product). Permanent devices were sterilized by UV irradiation between uses. Negative and positive controls, without DNA template or with a control DNA of a heterozygote, were always included. The nested second PCR to amplify the polymorphic core of the enhancer HS1.2-A was performed with 1/50 of the volume of the first PCR, avoiding the carryover of the genomic DNA of the first reaction. Control reactions were performed with 1 and 5 ng of total genomic DNA and resulted in no visible amplification in those conditions on gel agarose electrophoresis. The primers for the PCRs are reported in Giambra et al. (26). The second PCR was conducted with the same volumes and concentrations used in the first PCR, except for the use of 1 U of *Platinum Taq*DNA polymerase (Invitrogen). PCR products were analyzed on a 3.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen).

### *Statistical analysis*

The frequencies of the variables considered are described in percentages. Comparisons between frequencies of categorical variables in different groups were performed by chi-square test. The $p$ value was set at 0.05. Smith's Statistical Package, version 2.80 (Pomona College), GraphPad Instat, and Stata 6.0 TM software were used for statistical analysis.

## Results

### *The frequency of the HS1.2 3′RR1 alleles and IGAD patients*

The analysis of 3′RR-1 HS1.2 allelic frequencies of 88 IGAD patients and 101 healthy subjects of the same geographical area is reported in Table I. Comparison of the results shows a significant difference: homozygosity of the allele *1 that has a frequency of 39% in the IGAD patients and 15% in the control, with an increase of 2.6-fold; the heterozygous subjects with genotype *1/*2 also have a marked shift from 17% in IGAD to 33% in the control subjects; frequency of *2/*4 genotype changes from 9% in the IgAD patients to 19% in the control population. The two cohorts are in the conditions expected for the Hardy Weinberg equilibrium. The differences of the allelic frequencies among the two groups were investigated with the chi square test and the results are reported in Table I*B*. Chi-square analysis of the allelic frequencies among the two groups resulted highly significant ($p = 0.001$). The histogram in Fig. 2 shows the inversion of the frequencies among allele *1 and *2 in the two groups, where the relative ratio shifted from 1.24 in the patients with IGAD to 0.68 in the control. Allele *4 has a similar trend of allele *2 with a ratio of control vs patients with a significant decrease. The frequency of allele *3 is too low to determine a significant variation. Differences among the IGAD group of patients and the control for allele *1 and *2 shows a $\chi^2$ value of 7.34 with a $p$ value of 0.0067 (O.R. = 1.819).

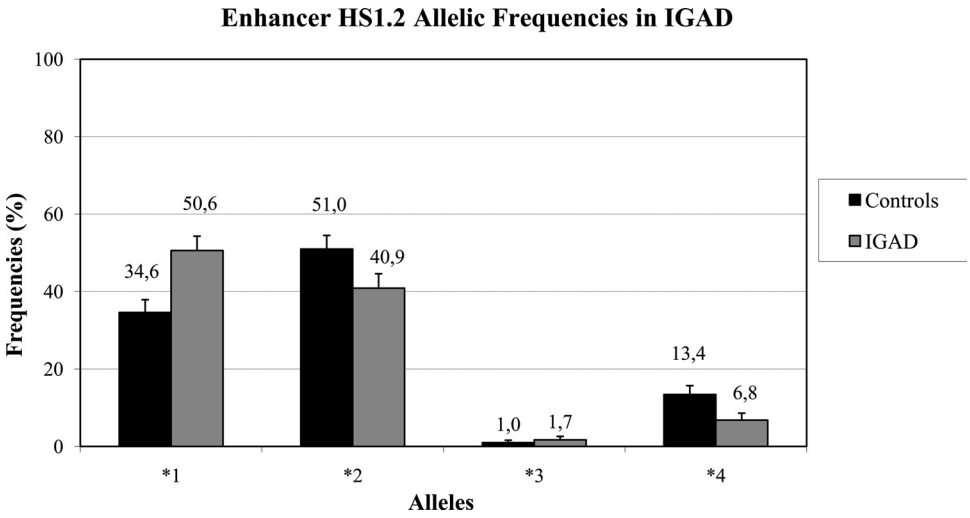


**FIGURE 2.** The histogram represents the comparison of HS1.2 allelic frequencies in IGAD patients and a control population of same geographical area. Inversion of frequency is observed for allele *1 in respect to alleles *2 and *4. The frequency of allele *3 is statistically not relevant. The $\chi^2$ analysis on the allelic frequencies shows a difference with a $p$ value of 0.001 highly significant. Values are indicated with the SEM. Total number of subjects is 88 for IGAD and 101 for the control. The single genotypes are reported in Table I.

*HS1.2 allelic frequency in IGAD patients and control related to IgG and IgM seric concentration*

Because HS1.2 is involved in a coordinated manner in Ig maturation, indirectly it could be involved in B cell maturation and survival (13, 17). Therefore, we investigated whether in our patients beside the IgA defect 3′RR-1 HS1.2 could be involved in differences in plasma IgM concentration or IgG. We evaluated serum levels of IgM and IgG to determine a possible correlation in the production of circulating Ig with the polymorphisms before and after isotype switch. The subjects with the values of IgM or IgG corresponding to the 30th percentile with low serum levels vs 30th percentile with high serum levels were compared for the frequencies of HS1.2 alleles in IGAD patients and Control. The results reported in Table II indicate that there is a remarkable difference of HS1.2 allelic frequencies as far as concerns the IgM values in IGAD subjects with relatively low or high levels in the serum. No differences were observed for IgG concentrations and HS1.2 frequencies. The frequency of the 1/1 homozygous individuals with low or high levels of IgM from 46% changes to 25% with a decrease of 1.84-fold and with an increase of 1.85-fold, respectively, for the 1/2 genomes, respectively. However for the IgG high and low values a change of frequency is observed only for the 1/2 genotypes, but on the whole does not influence the frequency of the four alleles among the two groups that show almost identical values (Table II). The variation of the four allele frequency is statistically significant for the comparison of patients with low or high IgM values with a $p$ value of 0.0391 (O.R. = 2.023) (Table II*B*). The analysis in the Control group stratified for the 30% of subjects with normal high or low levels of the IgM and IgG in the serum showed no significant difference for the genotype frequencies of the HS1.2 alleles as reported in Table III.

## Discussion

In this study, we first report a significant increase of HS1.2 allele *1 in the cohort of patients with IGAD, suggesting a possible role of this allele in the pathogenesis of the disease. The precise role of HS1.2 in the pathogenesis of IGAD is not known, but its strong association

Table II. *HS1.2 frequencies in IGAD patients of the 30th percentile with low or high values of IgM and IgG in the serum*

| | IgM IGAD[a] | | | | | | IgG IGAD[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Low values (30%) | | | High values (30%) | | | Low values (30%) | | | High values (30%) | | |
| Genotypes | Obs | % | Exp | Obs | % | Exp | Obs | % | Exp | Obs | % | Exp |
| 1/1 | 27 | 45.7 | 18.4 | 7 | 25 | 4 | 13 | 45 | 7.7 | 12 | 41.3 | 9.3 |
| 2/2 | 15 | 25.4 | 7.8 | 9 | 32.1 | 7 | 8 | 27.6 | 4.5 | 7 | 24.1 | 4.2 |
| 3/3 | - | - | - | - | - | - | - | - | - | - | - | - |
| 4/4 | - | - | - | - | - | - | - | - | - | - | - | - |
| 1/2 | 8 | 13.5 | 24 | 7 | 25 | 10.6 | 3 | 10.3 | 12 | 7 | 24.1 | 12.5 |
| 1/3 | 2 | 3.5 | 1.7 | - | - | - | - | - | - | 1 | 3.5 | 0.6 |
| 1/4 | 2 | 3.5 | 3.3 | 1 | 3.6 | 1.5 | 1 | 3.4 | 2 | 1 | 3.5 | 1.2 |
| 2/3 | 1 | 1.7 | 1 | - | - | - | 1 | 3.4 | 0.4 | - | - | - |
| 2/4 | 4 | 6.7 | 2 | 4 | 14.3 | 2 | 3 | 10.3 | 1.5 | 1 | 3.5 | 0.7 |
| 3/4 | - | - | - | - | - | - | - | - | - | - | - | - |

| Alleles | Frequencies | Obs | Frequencies | Obs | Frequencies | Obs | Frequencies | Obs |
|---|---|---|---|---|---|---|---|---|
| *1 | 0.559 ± 0.045 | 66 | 0.392 ± 0.063 | 22 | 0.517 ± 0.065 | 30 | 0.569 ± 0.065 | 33 |
| *2 | 0.364 ± 0.044 | 43 | 0.518 ± 0.065 | 29 | 0.397 ± 0.064 | 23 | 0.379 ± 0.063 | 22 |
| *3 | 0.026 ± 0.014 | 3 | 0.001 ± 0.001 | 0 | 0.017 ± 0.016 | 1 | 0.017 ± 0.016 | 1 |
| *4 | 0.051 ± 0.020 | 6 | 0.089 ± 0.033 | 5 | 0.069 ± 0.033 | 4 | 0.035 ± 0.023 | 2 |

[a] IgM: $\chi^2$ = 4.257; $p$ value (two sided) = 0.0391; OR (95% CI) = 2.023 (1.031–3.971).
[b] IgG: $\chi^2$ test is not significant.

Table III. *HS1.2 frequencies in control population of the 30th percentile with low or high values of IgM and IgG in the serum*

A

| | IgM Controls[a] | | | | | | IgG Controls[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Low values (30%) | | | High values (30%) | | | Low values (30%) | | | High values (30%) | | |
| Genotypes | Obs | % | Exp | Obs | % | Exp | Obs | % | Exp | Obs | % | Exp |
| 1/1 | 6 | 18.2 | 4 | 9 | 27.3 | 6 | 6 | 17.6 | 6 | 7 | 20.6 | 5 |
| 2/2 | 7 | 21.2 | 8 | 7 | 21.2 | 5 | 7 | 20.6 | 7 | 7 | 20.6 | 7 |
| 3/3 | - | - | - | - | - | - | - | - | - | - | - | - |
| 4/4 | - | - | - | - | - | - | - | - | - | - | - | - |
| 1/2 | 10 | 30.2 | 56 | 6 | 18.2 | 11 | 9 | 26.5 | 12 | 9 | 26.5 | 12 |
| 1/3 | - | - | - | - | - | - | - | - | - | 1 | 2.9 | 1 |
| 1/4 | 2 | 6.1 | 3 | 4 | 12.1 | 4 | 5 | 14.7 | 4 | 2 | 5.9 | 3 |
| 2/3 | 2 | 6.1 | 1 | 1 | 3 | 0.5 | 2 | 5.9 | 1 | 1 | 2.9 | 1 |
| 2/4 | 6 | 18.2 | 4 | 6 | 18.2 | 4 | 5 | 14.7 | 5 | 7 | 20.6 | 4 |
| 3/4 | - | - | - | - | - | - | - | - | - | - | - | - |

B

| Alleles | Frequencies | Obs | Frequencies | Obs | Frequencies | Obs | Frequencies | Obs |
|---|---|---|---|---|---|---|---|---|
| *1 | 0.364 ± 0.059 | 24 | 0.424 ± 0.060 | 28 | 0.382 ± 0.058 | 26 | 0.382 ± 0.058 | 26 |
| *2 | 0.485 ± 0.061 | 32 | 0.409 ± 0.060 | 27 | 0.441 ± 0.060 | 30 | 0.456 ± 0.060 | 31 |
| *3 | 0.030 ± 0.020 | 2 | 0.015 ± 0.014 | 1 | 0.030 ± 0.020 | 2 | 0.030 ± 0.020 | 2 |
| *4 | 0.121 ± 0.040 | 8 | 0.152 ± 0.044 | 10 | 0.147 ± 0.042 | 10 | 0.132 ± 0.041 | 9 |

[a] IgM: $\chi^2$ test is not significant.
[b] IgG: $\chi^2$ test is not significant.

with the disease and the complex effects of this enhancer on the immune systems prompted us to make some speculations. HS1.2 might be directly associated to IGAD pathogenesis. Alternatively, and more likely, the presence of a high frequency of allele *1 and low frequency of allele *2, may be associated to a given pattern of immune response characterized by several features: both IGA and IGM production. It has been shown that specular (high allele *2, low allele *1) pattern of HS1.2 allelic distribution is present in diseases with increased or abnormal IgA production such as IgA nephropathy and Celiac disease (30, 34). These results expand our knowledge of the possible role of HS1.2 alleles in the regulation of Ig production and in the pathogenesis of human immunological diseases. Overall, our data suggest that allele *1 may be linked to defective IgA production. In contrast, allele *2 may be associated to diseases with abnormal or hyperproduction of Ig. Our results on IGAD patients support previous evidence that high frequency of allele *1 is associated with reduced (albeit normal) serum level of at least one Ig class, while at high frequency of allele *2 corresponds significantly serum increase of at least one class of Ig (27).

The analysis on IGAD subjects shows that relatively low level of IgM correlates with high frequency of allele *1, in contrast high frequency of allele *2 is associated in patients with relatively high levels of IgM. The same pattern is not observed for IgG concentrations. Because IgM levels but not IgG are influenced by 3′RR1 HS1.2 allele frequencies, this suggests that the alleles cooperate differently before and after the isotype switch at least for the haplotypes associated in IGAD patients, though HS1.2 alleles interfere positively and negatively with Ig and also affect IgM production. It is intriguing why it does not affect IgG production in IGAD patients. This effect can be ascribed to the presence of two HS1.2 enhancers in both 3′RRs. It can be hypothesized that HS1.2 enhancer of 3′RR1 influences IgM production but differently than the isotype switch. This effect is not present in a control cohort. In a sample of 52 IGAD patients where CD19 data were available, we observed decreased percentages of $CD19^+$ cells associated with reduced allele *1 frequency as compared with subjects with higher levels of $CD19^+$ cells (data not shown). Possibly these data indicate a coregulation or coordinated way to support survival and/or activation of B cells by HS1.2 allele interactions. So far, HS enhancers of IgH cluster have been almost exclusively linked to Ig production; in light with this data in the present study, we report in IGAD patients HS1.2 enhancer alleles association with IgM seric levels. These findings are elusive, but a clue for their interpretation can be obtained by recent results by Vincent-Fabers et al. (17) who described a mouse KO for HS4. This mouse has a reduced number of spleen B cells. The two findings, KO for mouse HS4 and polymorphism of Human HS1.2, may appear contradictory, however the enhancers of 3′RR act synergistically and the KO of HS4 in mouse or polymorphisms of HS1.2 in IGAD patients both can alter the 3′RR activity and consequently B cell circulating number and Ig production.

The association between allelic variability in 3′RR-1 and immune diseases is still unclear, but some speculation can be made. HS1.2 allele *2 has a binding site for NF-κB, while allele *1 does not. It has been shown that BAFF, proliferation-inducing ligand (APRIL), and BTK, all regulate B cell survival via NF-κB (3, 8), and therefore in subjects with allele *1 homozygosis, the lacked binding sites for NF-κB may interfere with this pivotal transcription factor leading to Ig maturation and production thus affecting also survival of B cells. The penetrance of the allele *1 for the absence of circulating IgA is partial but can coregulate also IgM production. We do not exclude that the presence of specific haplotypes associated to IGAD and determined by polymorphisms distributed on the entire 3′RRs can cause conformational changes with different structural variation of the Regulatory Regions relevant for methylation changes and other epigenetic events. Interestingly, in mouse where polymorphisms are not studied was observed the relevance of the 3′RR for the epigenetic regulation (35). However further studies are needed to clarify the role, if any, of HS1.2 allele *1 in the pathogenesis of IGAD and to confirm a pattern of allelic frequencies associated to increased (allele *2 high; allele *1 low) or decreased (allele *1 high; allele *2 low) IgA

production. It is also worth noting that IGAD increases susceptibility to celiac disease (34, 36).

One additional point that can be made based on the results presented herein and those already available of a correlation between allelic frequency and immune mediated diseases is related to the functions of 3′RR-1 and 3′RR-2. In humans it is present an allelic hypervariability in 3′RR1, while alleles in 3′RR-2 have constant frequency. The variability in 3′RR-1 is associated with different diseases as we have discussed above. These findings suggest independent roles of the two 3′RRs in the development of the immune function as well as in susceptibility to immune-mediated diseases. Thus we suggest that the two human 3′RRs can play different roles for Ig maturation and B cell control. They could act differently either for different polymorphisms that can bear, either for different time activation caused by the position that have in the cluster (Fig. 1).

Only further studies in different diseases will confirm whether that pattern of IgM is specific for IgAD or is related to the activity of a given set of alleles present not only in IgAD but synergic with other cofactors leading in each case to different pathologies. Only the whole pattern of polymorphisms of both 3′RRs will show whether the presence of specific haplotypes may condition the regulatory activity in linkage to polymorphic enhancer HS1.2.

## Acknowledgments

We thank Arteo Ciancarelli, Pasquale Mancini, and Marcello Brancato of the secretariat of the department of Biology "E.Calef" of Tor Vergata for the budget administration.

## Disclosures

The authors have no financial conflict of interest.

## References

1. Vorechovsk, I., A. D. B. Webster, A. Plebani, and L. Hammastrom. 1999. Genetic linkage of IgA deficiency to the major histocompatibility complex: evidence for allele segregation distortion, parent-of-origin penetrance differences, and the role of anti-IgA antibodies in disease predisposition. *Am. J. Hum. Genet.* 64: 1096–1109.
2. Schaffer, A. A., J. Pfannstiel, D. B. Webster, A. Plebani, L. Hammarstrom, and B. Grimbacher. 2006. Analysis of families with common variable immunodeficiency (CVID) and IgA deficiency suggests linkage of CVID to chromosome 16q. *Hum. Genet.* 118: 725.
3. Martin, F., and V. M. Dixit. 2005. Unraveling TACIt functions. *Nat. Genet.* 37: 793–794.
4. Salzer, U., H. M. Chapel, A. D. Webster, Q. Pan-Hammarström, A. Schmitt-Graeff, M. Schlesier, H. H. Peter, J. K. Rockstroh, P. Schneider, A. A. Schäffer, et al. 2005. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat. Genet.* 37: 820–828.
5. Castigli, E., S. Scott, F. Dedeoglu, P. Bryce, H. Jabara, A. K. Bhan, E. Mizoguchi, and R. S. Geha. 2004. Impaired IgA class switching in APRIL-deficient mice. *Proc. Natl. Acad. Sci. USA* 101: 3903–3908.
6. Salzer, U., and B. Grimbacher. 2005. TACItly changing tunes: farewell to a yin and yang of BAFF receptor and TACI in humoral immunity? new genetic defects in common variable immunodeficiency. *Curr. Opin. Allergy Clin. Immunol.* 5: 496–503.
7. Castigli, E., S. A. Wilson, S. Scott, F. Dedeoglu, S. Xu, K. P. Lam, R. J. Bram, H. Jabara, and R. S. Geha. 2005. TACI and BAFF-R mediate isotype switching in B cells. *J. Exp. Med.* 201: 35–39.
8. Shinners, N. P., G. Carlesso, I. Castro, K. L. Hoek, R. A. Corn, R. T. Woodland, M. L. Scott, D. Wang, and W. N. Khan. 2007. Bruton's tyrosine kinase mediates NF-κB activation and B cell survival by B cell-activating factor receptor of the TNF-R family. *J. Immunol.* 179: 3872–3880.
9. Saleque, S., M. Singh, and B. K. Birshtein. 1999. Ig heavy chain expression and class switching in vitro from an allele lacking the 3′ enhancers DNase I-hypersensitive hs3A and hs1,2. *J. Immunol.* 162: 2791–2803.
10. Pinaud, E., C. Aupetit, C. Chauveau, and M. Cogné. 1997. Identification of a homolog of the C α 3′/hs3 enhancer and of an allelic variant of the 3′IgH/hs1,2 enhancer downstream of the human immunoglobulin α 1 gene. *Eur. J. Immunol.* 27: 2981–2985.
11. Chen, C., and B. K. Birshtein. 1997. Virtually identical enhancers containing a segment of homology to murine 3′IgH-E(hs1,2) lie downstream of human Ig C α 1 and C α 2 genes. *J. Immunol.* 159: 1310–1318.
12. Mills, F. C., N. Harindranath, M. Mitchell, and E. E. Max. 1997. Enhancer complex located downstream of both human immunoglobulin Cα genes. *J. Exp. Med.* 186: 845–858.
13. Pinaud, E., A. A. Khamlichi, C. Le Morvan, M. Drouet, V. Nalesso, M. Le Bert, and M. Cognè. 2001. Localization of the 3′ IgH locus elements that effect long-distance regulation of class switch recombination. *Immunity* 15: 187–199.
14. Ju, Z., S. A. Volpi, R. Hassan, N. Martinez, S. L. Giannini, T. Gold, and B. K. Birshtein. 2007. Evidence for physical interaction between the immunoglobulin heavy chain variable region and the 3′ regulatory region. *J. Biol. Chem.* 282: 35169–35178.
15. Laurencikiene, J., V. Deveikaite, and E. Severinson. 2001. HS1,2 enhancer regulation of germline epsilon and γ2b promoters in murine B lymphocytes: evidence for specific promoter-enhancer interactions. *J. Immunol.* 167: 3257–3265.
16. Sepulveda, M. A., F. E. Garrett, A. Price-Whelan, and B. K. Birshtein. 2005. Comparative analysis of human and mouse 3′ Igh regulatory regions identifies distinctive structural features. *Mol. Immunol.* 42: 605–615.
17. Vincent-Fabert, C., V. Truffinet, R. Fiancette, N. Cogné, M. Cogné, and Y. Denizot. 2009. Ig synthesis and class switching do not require the presence of the hs4 enhancer in the 3′ IgH regulatory region. *J. Immunol.* 182: 6926–6932.
18. Giambra, V., S. Volpi, A. V. Emelyanov, D. Pflugh, A. L. Bothwell, P. Norio, Y. Fan, Z. Ju, A. I. Skoultchi, R. R. Hardy, et al. 2008. Pax5 and linker histone H1 coordinate DNA methylation and histone modifications in the 3′ regulatory region of the immunoglobulin heavy chain locus. *Mol. Cell Biol.* 28: 6123–6133.
19. Sadhu, A., M. L. Shen, M. Hackbarth, E. Hume, and T. W. McKeithan. 1997. CpG-rich sequences close to the site of duplication within the human IGH constant region. *Immunogenetics* 45: 365–370.
20. Saleque, S., M. Singh, R. D. Little, S. L. Giannini, J. S. Michaelson, and B. K. Birshtein. 1997. Dyad symmetry within the mouse 3′ IgH regulatory region includes two virtually identical enhancers (C alpha3′E and hs3). *J. Immunol.* 158: 4780–4787.
21. Manis, J. P., N. van der Stoep, M. Tian, R. Ferrini, L. Davidson, A. Bottaro, and F. W. Alt. 1998. Class switching in B cells lacking 3′ immunoglobulin heavy chain enhancers. *J. Exp. Med.* 188: 1421–1431.
22. Kim, E. C., C. R. Edmonston, X. Wu, A. Schaffer, and P. Casali. 2004. The HoxC4 homeodomain protein mediates activation of the immunoglobulin heavy chain 3′ hs1,2 enhancer in human B cells: relevance to class switch DNA recombination. *J. Biol. Chem.* 279: 42258–42269.
23. Zelazowski, P., Y. Shen, and C. M. Snapper. 2000. NF-κB/p50 and NFκB/c-*Rel* differentially regulate the activity of the 3′αE-hsl, 2 enhancer in normal murine B cells in an activation-dependent manner. *Int. Immunol.* 12: 1167–1172.
24. Komori, A., Z. Xu, X. Wu, H. Zan, and P. Casali. 2006. Biased dA/dT somatic hypermutation as regulated by the heavy chain intronic iEmu enhancer and 3′Eα enhancers in human lymphoblastoid B cells. *Mol. Immunol.* 43: 1817–1826.
25. Guglielmi, L., V. Truffinet, E. Magnoux, M. Cogné, and Y. Denizot. 2004. The polymorphism of the locus control region lying downstream the human IgH locus is restricted to hs1,2 but not to hs3 and hs4 enhancers. *Immunol. Lett.* 94: 77–81.
26. Giambra, V., A. Fruscalzo, M. Giufre', C. Martinez-Labarga, M. Favaro, M. Rocchi, and D. Frezza. 2005. Evolution of human IgH3′EC duplicated structures: both enhancers HS1,2 are polymorphic with variation of transcription factor's consensus sites. *Gene* 346: 105–114.
27. Frezza, D., V. Giambra, C. Mattioli, K. Piccoli, R. Massoud, A. Siracusano, M. Di Giannantonio, B. K. Birshtein, and I. A. Rubino. 2009. Allelic frequencies of 3′ Ig heavy chain locus enhancer HS1,2-A associated with Ig levels in patients with schizophrenia. *Int. J. Immunopathol. Pharmacol.* 22: 115–123.
28. Denizot, Y., E. Pinaud, C. Aupetit, C. Le Morvan, E. Magnoux, J. C. Aldigier, and M. Cogné. 2001. Polymorphism of the human α1 immunoglobulin gene 3′ enhancer hs1,2 and its relation to gene expression. *Immunology* 103: 35–40.
29. Giambra, V., C. Martínez-Labarga, M. Giufré, D. Modiano, J. Simporé, B. K. Gisladottir, R. Francavilla, G. Zhelezova, S. S. Kilic, M. Crawford, et al. 2006. Immunoglobulin enhancer HS1,2 polymorphism: a new powerful anthropogenetic marker. *Ann. Hum. Genet.* 70: 946–950.
30. Aupetit, C., M. Drouet, E. Pinaud, Y. Denizot, J. C. Aldigier, F. Bridoux, and M. Cognè. 2000. Alleles of the α-1 immunoglobulin gene 3 enhancer control evolution of IgA nephropathy toward renal failure. *Kidney Internat.* 58: 966–971.
31. Frezza, D., V. Giambra, B. Tolusso, M. De Santis, S. Bosello, S. Vettori, G. Triolo, G. Valentini, and G. Ferraccioli. 2007. Polymorphism of immunoglobulin (Ig) enhancer element HS1,2A: allele *2 associates with systemic sclerosis: comparison with HLA-DR and DQ alleles frequency. *Ann. Rheum. Dis.* 66: 1210–1215.
32. Cianci, R., V. Giambra, C. Mattioli, M. Esposito, G. Cammarota, G. Scibilia, G. Magazzù, A. Orlando, G. Sandri, L. Bianchi, et al. 2008. Increased frequency of Ig heavy-chain HS1,2-A enhancer *2 allele in dermatitis herpetiformis, plaque psoriasis, and psoriatic arthritis. *J. Invest. Dermatol.* 128: 1920–1924.
33. Tolusso, B., D. Frezza, C. Mattioli, A. L. Fedele, S. Bosello, F. Faustini, G. Peluso, V. Giambra, D. Pietrapertosa, A. Morelli, et al. 2009. Allele *2 of the HS1,2A enhancer of the Ig regulatory region associates with rheumatoid arthritis. *Ann. Rheum. Dis.* 68: 416–419.
34. Frezza, D., V. Giambra, R. Cianci, M. Giufre', C. Cammarota, C. Martínez-Labarga, A. Fruscalzo, O. Rickards, G. Scibilia, C. Sferlazzas, et al. 2004. Increased frequency of the immunoglobulin enhancer hs1,2 allele 2 in coeliac disease. *Scand. J. Gastroenterol.* 39: 1083–1087.
35. Garrett, F. E., A. V. Emelyanov, M. A. Sepulveda, P. Flanagan, S. Volpi, F. Li, D. Loukinov, L. A. Eckhardt, V. V. Lobanenkov, and B. K. Birshtein. 2005. Chromatin architecture near a potential 3′ end of the igh locus involves modular regulation of histone modifications during B-Cell development and in vivo occupancy at CTCF sites. *Mol. Cell Biol.* 25: 1511–1525.
36. Cianci, R., G. Cammarota, S. Lolli, G. B. Gasbarrini, and F. Pandolfi. 2008. Abnormal synthesis of IgA in coeliac disease and related disorders. *J. Biol. Regul. Homeost. Agents* 22: 99–104.