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Alleles of the $\alpha 1$ immunoglobulin gene 3′ enhancer control evolution of IgA nephropathy toward renal failure

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Alleles of the $\alpha 1$ immunoglobulin gene 3′ enhancer control evolution of IgA nephropathy toward renal failure.

Background. IgA nephropathy is the most common glomerular disease. Mechanisms leading to its occurrence and controlling the evolution of the disease remain largely unknown. Various genetic factors have been found, mostly implicating immunologically relevant genes (IgH, TCR, human lymphocyte antigen, and complement loci). A regulatory region recently identified downstream, the *α1* gene of the IgH locus, was a likely candidate for the control of IgA1 production in patients. Alleles of this region, differing by size, sequence, and orientation of the *α1 hs1,2* transcriptional enhancer, were first identified through Southern blot hybridization.

Methods. We established a polymerase chain reaction (PCR) method suitable for routine testing that amplifies minisatellites within the *α1 hs1,2* enhancer, with variable numbers of tandem repeats (VNTR) defining the two alleles. This assay allowed the typing of 104 patients with IgAN and 83 healthy volunteers. Results from typing of *α1 hs1,2* alleles were compared with long-term clinical outcome in patients. Enhancer alleles were compared in a luciferase reporter gene assay.

Results. The *α1 hs1,2* alleles do not constitute a predictive factor for IgA nephropathy, since similar allelic frequencies were observed in healthy individuals and in unrelated European patients. In contrast, among patients, homozygosity for the weakest enhancer allele (AA genotype) was significantly correlated with a milder form of the disease, whereas the allele B was associated with severe evolution. The minisatellite region within the *α1 hs1,2* enhancer carried potential transcription factor-binding sites, and its duplication increased the transcriptional strength of the *α1 hs1,2* allele B over that of allele A.

Conclusion. Altogether, these alleles may constitute a risk factor for the prognosis of IgA nephropathy.

IgA nephropathy (IgAN) is defined by IgA deposits in the kidney mesangium. Although secondary IgA deposits occur in association with various systemic pathologies, IgAN, the so-called Berger's disease, stands as an idiopathic nephropathy. It is the most common nephropathy worldwide, and especially affects white people and mostly males [1]. The initial features are often mild, with hematuria and proteinuria, usually after an infectious episode. Diagnosis can also be suggested later on, at the time of hypertension, edemas, or renal failure. Although initially considered as benign, it is now clear that the disease frequently leads to renal failure. Factors for a poor prognosis are hypertension, microhematuria, renal failure at the stage of diagnosis, elevated proteinuria (>3 g/day), and pathologic alterations of the mesangium [2]. Whatever the stage, diagnosis relies on a kidney biopsy that shows IgA deposits associated with complement (mainly C3 or C4) or other immunoglobulins (IgG or IgM).

The pathophysiology of mesangial deposits involves immunologic mechanisms that are not elucidated; they may rely on both qualitative and quantitative changes in IgA production. Negatively charged and poorly glycosylated IgA may become able to bind positively charged collagen, fibronectin, or the membrane of mesangial cells [2–4]. Specific antigens have been suggested to elicit an abnormal immune response skewed toward the synthesis of IgA1. Such a skewed response may yield circulating immune complexes comprised of antigens, IgA, occasionally IgG, and complement, ultimately forming pathogenic deposits in the kidney.

Familial predisposition has been documented [5, 6], but the inheritance of the disease has not been firmly associated with specific genes. Many factors seem to influence the outcome of IgAN, such as human lymphocyte antigen (HLA) haplotypes [7–9], the level of HLA expression on natural killer (NK) lymphocytes [10], the C4 null complement factor phenotype, allelic forms of the angiotensin-converting enzyme [11, 12], or the genes for an IgA-specific glycosyl transferase [13–15].

Since serum IgA, specifically IgA1 in a dimeric form, is increased in nearly 50% of the patients, a genetic pro-

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pensity to a high expression of the IgH α gene or of cytokines that promote switching toward IgA has been suggested [16, 17]. Sμ and Sα switch regions of the IgH locus, and transcription of the Iα region is a key element of class switching to IgA. Their role in the pathophysiology of IgAN is controversial: Specific allelic forms have been found to be associated with a more severe evolution of IgAN in two studies [18, 19], but not in a third one [20]. Meanwhile, I$\alpha 1$ germline transcripts have been reported to be absent in IgAN patients by one group [21], whereas they were increased and their promoter frequently carried specific mutations in a study of Japanese patients [17].

In an attempt to find new genetic markers and to understand better the molecular basis of IgAN, we focused on the polymorphism of a region lying downstream the $\alpha 1$ gene. This region contains three enhancers, *hs*3, *hs1,2*, and *hs4*, and is homologous to the murine 3′ IgH palindromic locus control region [22–24]. By Southern blot we previously identified a restriction fragment length polymorphism of that region, with at least two different alleles ($\alpha 1^a$ and $\alpha 1^b$) [22]. These alleles differ by the orientation of the *hs1,2* element and by inclusion of variable numbers of tandem repeats (VNTR): The $\alpha 1^a$ *hs1,2* allele has one 53 bp repeat, and the $\alpha 1^b$ allele carries two; meanwhile, the $\alpha 2$ gene has four repeats [22, 23]. Since Southern blotting is tedious work and only indicates the orientation of *hs1,2*, we have designed a polymerase chain reaction (PCR) assay that specifically amplifies the VNTR within *hs1,2*. We named the alleles defined by PCR $\alpha 1^A$ and $\alpha 1^B$, by analogy to the nomenclature of Southern blot alleles ($\alpha 1^a$ and $\alpha 1^b$) [22]. The tandemly repeated minisatellite motif features the presence of several potential binding sites for transcription factors, including μE1 and nuclear factor-κB (NF-κB) [22]. We wished to address the hypothesis that the duplicated motif in the $\alpha 1^B$ allele may boost the transcription of the $\alpha 1$ gene and play a role in the pathophysiology of IgAN. The distribution of PCR alleles was thus determined in healthy individuals as well as in patients suffering mild or severe forms of idiopathic IgAN.

METHODS

Patients

All members of our study group gave their informed consent, and the study design was in agreement with the local ethical committee. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vials from 104 patients suffering from kidney biopsy-confirmed IgAN. These Caucasoid patients included 81 men and 23 women, originating from the Limoges, Poitiers, Brive, and Périgueux areas of France. Ages ranged from 13 to 69 years old (mean 41.24 ± 1.23 years old). Patients were ranked according to their clinical status:

Patients with renal failure. Sixty-four patients had creatininemia levels above 150 μmol/L or a deterioration of clearance over 3 mL/min/year as determined by the Cockroft formula [25, 26], and dialysis or kidney transplantation was necessary in 42 of them (65%). The mean duration of follow-up in this group before the occurrence of renal failure was 7.25 ± 0.87 years.

Patients without renal failure. This group of 40 patients had creatininemia values below 150 μmol/L at the beginning of the study (mean follow-up 8.3 ± 0.9 years).

Whole blood cell DNA was prepared from all 104 patients and from 83 healthy volunteers of the Limoges area, with no history of renal disease. Our control population included 27 men and 56 women ages 21 to 55 years old (mean 40.20 ± 0.89 years old).

Polymerase chain reaction

DNA was digested overnight with proteinase K (1 mg/mL), phenol and chloroform extracted, alcohol precipitated, and adjusted to a concentration of 1 μg/μL in Tris 10 mmol/L, EDTA 0.1 mol/L.

DNA fragments including the VNTR region of the *hs1,2* enhancer were amplified with consensus primers flanking the 53 bp repeats in both $\alpha 1^A$ and $\alpha 1^B$ (Fig. 1): primer 1, 5′-GGGTCCTGGTCCCAAAGATGGC-3′ (67°C); primer 2, 5′-TTCCCAGGGGTCCTGTGGGTCC-3′ (69°C).

The following sizes were expected for amplification products: $\alpha 1^B$ gene, $409 + (2 \times 53) = 515$ bp, and $\alpha 1^A$ gene, $409 + (1 \times 53) = 462$ bp.

Reactions were carried out in 30 μL of a mix made up of dNTP 8 mmol/L, 100 nmol/L of each primer, 1 × *Taq* buffer, and 0.2 U *Taq* Polymerase (Pharmacia, Uppsala, Sweden). PCR was performed on a Robocycler Gradient 96 (Stratagene, La Jolla, CA, USA). DNA was denaturated three minutes at 94°C and then submitted to 10 cycles that consisted of 94°C for 45 seconds, 66°C for 55 seconds, and 72°C for 45 seconds, followed with another 20 cycles at 64°C for 105 seconds for the annealing step. A final elongation step was realized at 72°C for 10 minutes. The product was then analyzed on a 2% agarose gel stained with ethidium bromide; 280 nm ultraviolet illumination was allowed to score the homozygotes (AA or BB) and heterozygotes patterns (AB; Fig. 1).

Luciferase assays

A lymphoid-specific vector for reporter gene assays was constructed by inserting an immunoglobulin heavy chain VH promoter upstream the luciferase gene (*Luc*) in the pGL3 vector (Promega Corporation, Madison, WI, USA). Fragments containing either one repeat (*hs1,2* $\alpha 1^A$ allele) or two repeats (*hs1,2* $\alpha 1^B$ allele) of the 53 bp minisatellite were first cloned in pCRII-topo cloning vector (Invitrogen, Groningen, The Netherlands) and inserted downstream the *Luc* gene in the pVH promoter-

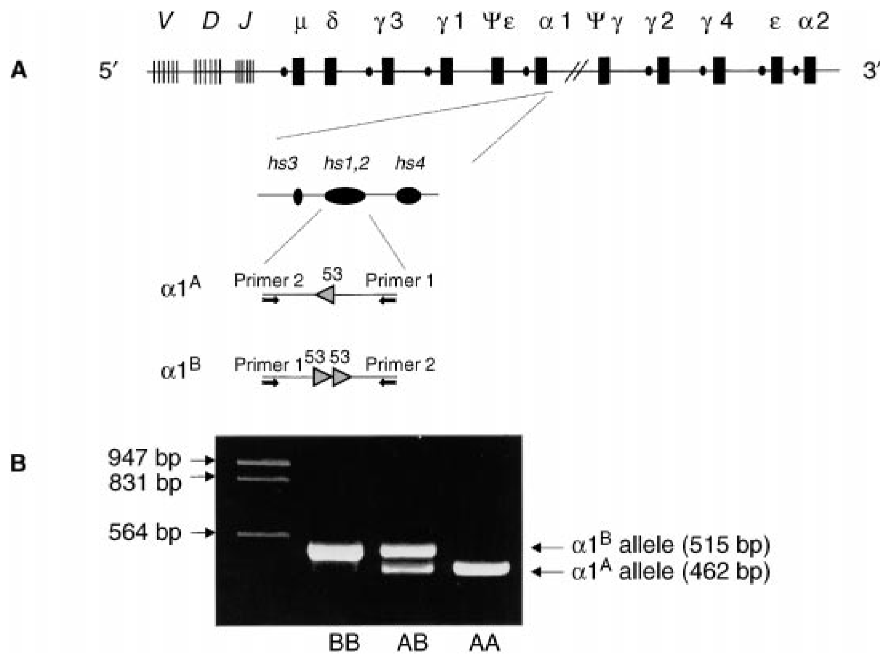

Fig. 1. (*A*) Map of the IgH locus showing the *hs1,2* enhancer downstream from the *α1* gene and also showing the polymerase chain reaction (PCR) amplification. Different orientation and numbers of tandem repeats in the *α1* gene can be seen in the $\alpha 1^A$ allele (one repeat) and $\alpha 1^B$ allele (two repeats). (*B*) Patterns of PCR amplification: AB genotype ($\alpha 1^A$ and $\alpha 1^B$ allele fragments), BB genotype ($\alpha 1^B$ allele fragment), and AA genotype ($\alpha 1^A$ allele fragment).

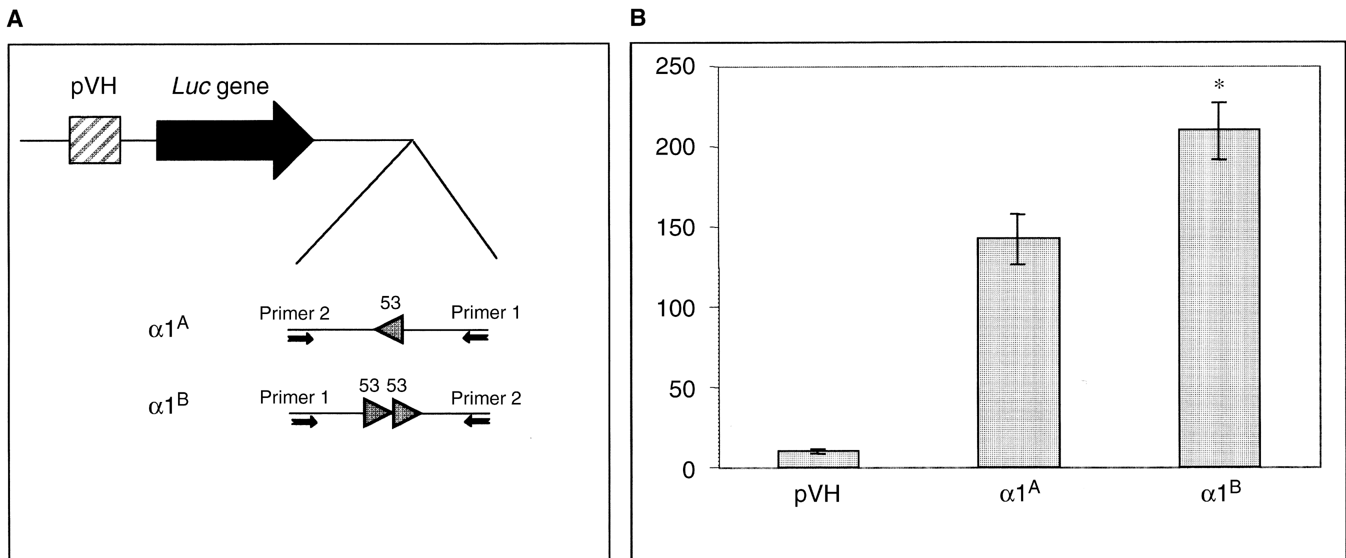

Fig. 2. (*A*) Construction: *hs1,2* elements were cloned as BamH1-Xho1 fragments in BamH1-Sal1 sites of the pGL3-pVH vector. (*B*) Estimation of the luciferase activity in the $\alpha 1^A$ and $\alpha 1^B$ fragments compared with the VH promoter basal activity (pVH). Results are reported as mean ± SEM of six samples. One representative experiment out of two is shown. *P = 0.01 (Mann-Whitney U-test) as compared with $\alpha 1^A$.

driven *Luc* reporter plasmid in their normal respective orientations (Fig. 2). An enhancerless construction, including only the pVH promoter-driven *Luc* gene, was transfected in the same conditions to evaluate basal transcription. Transfection efficiencies were controlled by simultaneous transfection of a control β galactosidase expression vector (pCMV β vector; Clontech, Palo Alto, CA, USA). Plasmid DNAs were prepared using plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany).

For transient transfection assay, 5×10^5 U266 human plasma cells were transfected with equal molar amounts (corresponding to 400 ng of pGL3-pVH construct) of each construct, using Superfect transfection reagent (Qiagen GmbH). For each assay, 400 ng of pCMV β vector (Clontech) were added as an internal control, and for each construct, six assays were performed in parallel. PGL3 Control vector plasmid (Promega Corp.) was used as a positive control. After 18 hours, cultures in RPMI 1640

Table 1. Distribution of the three *hs1,2* genotypes among controls and patients

	Patients			
	Patients with renal failure	Patients without renal failure	Total	Control
AA	4 (6.2%)	8 (20%)	12 (11.5%)	13 (15.6%)
BB	30 (46.9%)	10 (25%)	40 (38.5%)	36 (43.4%)
AB	30 (46.9%)	22 (55%)	52 (50%)	34 (41%)
Total	64	40	104	83
Follow-up	7.25 ± 0.86 years before kidney failure	8.3 ± 0.9 years		

Patients were divided according to the progression of the disease. Years of follow-up are indicated.

medium (Life Technologies, Tassin, France) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 μg/mL penicillin, and 10 μg/mL streptomycin, cells were centrifuged and lyzed five minutes at room temperature in 100 μL of lysis buffer (K_2HPO_4 100 mmol/L, KH_2PO_4 10 mmol/L, Triton O.2%, dithiothreitol 1 mmol/L). After centrifugation, the supernatant was used to assay luciferase activity in the Luclite Plus assay kit (Packard Bioscience BV, Groningen, The Netherlands) and β galactosidase activity with the luminescent β-gal detection kit (Clontech) to standardize the assay. The luminescent assays were all performed in 96-well Optiplates (Packard Bioscience BV) in a Lumicount microplate reader luminometer (Packard Bioscience BV).

Statistical analysis

Statistical analysis was performed with the Statviews software (Alsym, Meylan, France). Frequencies were compared by the χ^2 method, Mann-Whitney, or Fisher test when appropriate, and were considered significantly different when P values were less than 0.05. The odds ratios were calculated by the Haldane method [27].

A dynamic study of the outcome of the disease according to genotypes was carried out by considering time lapses between diagnosis and the occurrence of renal failure through a Kaplan–Meier statistical test, and differences in those rates were evaluated by means of log rank test.

RESULTS

Relationship between clinical status and *hs1,2* genotypes

First we compared the repartition of the different genotypes and alleles between the patients and the control population (Table 1). Allelic frequencies did not differ between the patients and control groups (A allele, 36.5 vs. 36.2%; B allele, 63.5 vs. 63.8%), and the distribution was in agreement with the Hardy–Weinberg equation.

In contrast, the patients separated into two groups of either mild or severe clinical evolution appeared to differ significantly, and the 64 patients with renal failure carried the B allele more often than the 40 patients without renal failure. The presence of allele A was significantly associated with stability of the disease and was present in 75% of the 40 patients without renal failure versus only 53% of the patients with renal failure ($\chi^2 = 4.1$, $P = 0.04$).

Relationship between rapid or slow evolution and *hs1,2* genotypes

We also wished to consider statistically how fast the patients progressed to renal failure. Rather than splitting patients in two groups at the time of insertion in the study, the Kaplan–Meier analysis allowed us to take into account the delay between diagnosis and occurrence or not of renal failure. This delay was 11.5 ± 4.1 years in B allele-negative patients versus 6.97 ± 1.24 years in all B allele-positive patients (Fig. 3). The mean time lapse after which 25% patients suffered renal failure was 14 ± 2.3 years in the B allele-negative group and only in 3 ± 2.2 years in the B allele-positive group.

Luciferase assays

Since the B allele of the *hs1,2 α1* enhancer apparently represented a risk factor for a disease involving increased IgA1 production, we compared the transcriptional strength of the various *hs1,2* VNTR regions in luciferase gene reporter assays. Fragments from the $hs1,2\ \alpha1^A$ allele manifested a weaker activity ($P = 0.01$, Mann-Whitney U-test) than the $hs1,2\ \alpha1^B$ allele (209.9 ± 18.0 vs. 142.3 ± 15.9 for the $\alpha1^B$ and $\alpha1^A$ alleles, respectively; Fig. 2).

DISCUSSION

Although genetic factors obviously play a role in the occurrence and evolution of idiopathic IgAN, most correlations reported between the disease and alleles at specific loci have proven to be weak and are subject to geographic or ethnical variations. Beside a strong prevalence in males, with a sex ratio varying from 2:1 to 6:1, depending on the geographic area (with a 3.5:1 ratio in the present study), several reports have documented the influence of alleles mapping within the IgH locus [14, 17, 19, 20].

We amplified the different allelic forms of the IgH *α1*

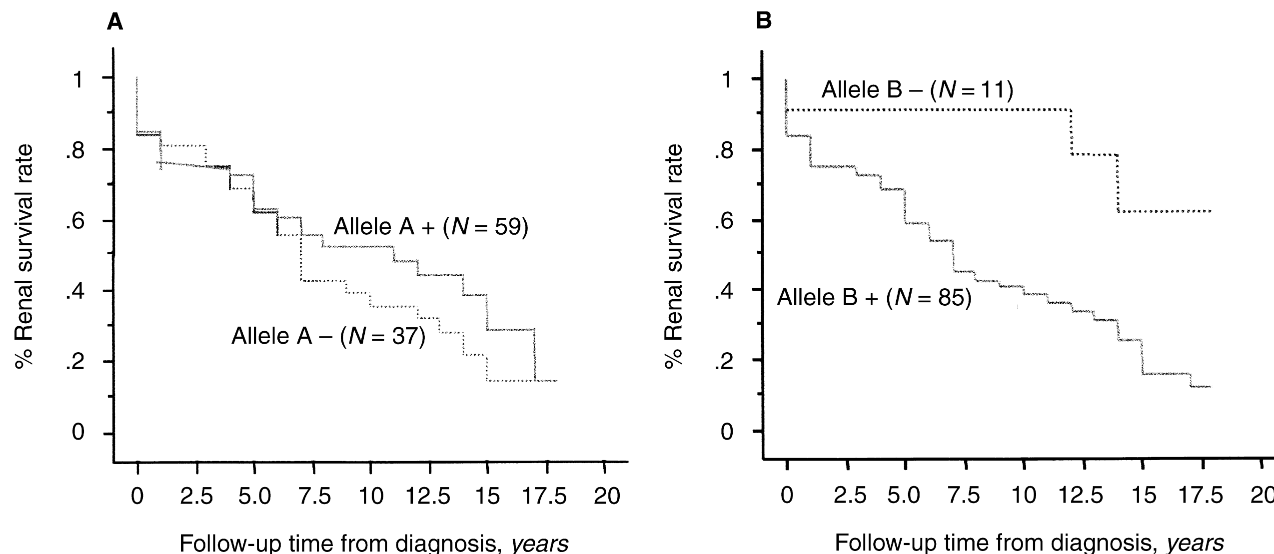

Fig. 3. Progression curves toward renal failure estimated by the Kaplan–Meier analysis. The AA genotype confers a protection in IgA nephropathy patients ($P = 0.04$). Symbols in A are: (solid line) allele A+ ($N = 59$); (dotted line) allele A– ($N = 37$). Symbols in B are: (solid line) allele B+ ($N = 85$); (dotted line) allele B– ($N = 11$). P values were not significant in A, but $P = 0.01$ in B.

gene *hs1,2* enhancer by PCR. This PCR identifies two alleles for the $\alpha 1$ gene: allele A characterized by a band of 462 bp, and allele B characterized by a band of 515 bp. We found that the allele frequencies in IgAN patients did not differ significantly from those defined in the control population. By contrast, allele frequencies significantly differed between patients with either mild or severe evolution of the disease. The B allele was significantly more represented in those patients with renal failure, who often required dialysis or kidney transplantation. Another remarkable feature appeared when the delay between diagnosis and occurrence of renal failure was taken into account for the statistical analysis: The B allele was associated with a fast slope on the progression curve ($P = 0.01$; Fig. 3).

The $\alpha 1$ gene *hs1,2* enhancer contains variable numbers of a 53 bp minisatellite sequence carrying consensus binding sites for transcription factors such as NF-κB. In transcriptional reporter gene assays, the minisatellite region of the B allele, which contains two repeats, carries a stronger transcriptional enhancer activity than the single-repeat allele A. Since the *hs1,2* enhancer has been postulated to play an important role in class switching [28], a tempting conclusion is that allele B of *hs1,2* increases switching toward the $\alpha 1$ gene. Allele B could then directly play a role in the evolution of IgAN by a long-term promotion of a higher rate of IgA1 production through increased switching and transcription of the $\alpha 1$ gene, thus finally yielding to heavier IgA1 kidney deposits.

It is also worth noting that the determination of polymorphism at the $\alpha 1$ *hs1,2* enhancer bears the advantage of being quickly carried out through PCR of the minisatellite region and to be much more easily done in routine testing than Southern blots, which previously have been used for the identification of switch region alleles.

Altogether, our results encourage us to think that polymorphism at the IgH 3′ regulatory region could play an important role in the outcome of idiopathic IgAN. The presence of the B allele described herein confers a more important calculated odds ratio [3.93 (1.13; 13.74)] for malign progression than the one conferred by the "reference" HLA-DQB1*0301 allele [1.64 (0.65; 4.18)] [9] when it is measured in our population of patients with renal failure and patients without renal failure who were followed for at least four years (data not shown). The B allele finally confers a significant risk of severe outcome and rapid evolution. The frequencies of *hs1,2* alleles and genotypes in normal individuals are such that their determination has no diagnostic value by itself; however, they could be useful markers for the evaluation of a prognosis, especially in association with other known genetic markers such as HLAs, complement factors, or switch region alleles. Finally, the role of such enhancer alleles in the transcriptional control of the $\alpha 1$ gene provides a new clue toward unraveling the complex pathophysiology of IgAN.

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