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BALKAN ENDEMIC NEPHROPATHY RISK ASSOCIATES TO THE hs1.2 Ig ENHANCER POLYMORPHISM

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Balkan Endemic Nephropathy (BEN) is a kidney degenerative disease with a high incidence in the valleys of the Danube and tributary rivers. Many studies describe it as a multifactorial disease. Environmental as well immuno-inflammatory and genetic cofactors have been suggested to trigger the onset of the disease. Recently, high levels of C-reactive protein were demonstrated in BEN patients. We performed this study to evaluate the possible correlation of BEN with the polymorphism of the Ig heavy chain 3'Regulatory Region enhancer hs1.2 that is related to changes of consensus for trans activators binding within the DNA sequence and probably consequently autoimmune and inflammatory diseases. Therefore, we studied three cohorts: 1) 111 control subjects, 2) 95 BEN patients in dialysis therapy and 3) 133 components of a large family "J" in the same geographical area. The allelic frequencies of hs1.2 of BEN patients and family "J" components had similar decrease frequency of allele *1 and increase of allele *2 in respect to the controls. This trend suggests the association of allele *1 as a protective and allele *2 as a risk component for the disease. The presence of a consensus sequence for NF-κb in the allele *2 may link the polymorphism to the inflammatory activity of BEN. This study supports the presence of an inflammatory pathway in BEN through the involvement of polymorphic enhancer hs1.2 influencing differently binding complexes and consequently the 3D structure of 3' Regulatory Region of IgH. Our work is the first study that clearly links BEN to a gene involved in the regulation of immune response.

Balkan endemic nephropathy (BEN) is a chronic renal disease affecting tubulointerstitial tissue of kidney and associates with urothelial cancer. It is endemic for people living in the plains along tributaries of the River Danube. Broad investigations lead to the definition of BEN as a multifactorial disease (1-2). Epidemiological studies associated many risk factors (3). To date, it has not been possible in this complex pathogenesis to define the precise roles of the different factors involved (4). Consistent data have been accumulated in the past 50 years sustaining the possible role of genetic factors,

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environmental agents and immune mechanisms (5). Important analysis on the polymorphisms of detoxification enzymes, such as P450, has been suggested as a possible associated factor (6). Among the environmental agents, mycotoxins and polycyclic aromatic hydrocarbons, aristolochic acid and metabolic derivates were proposed as risk (7-9). Epidemiological studies considered the possible contamination of soil and drinking water (10). Bitter or sour receptor polymorphisms can influence the food uptake, possible natural toxins and indirectly also ingestion of aristolochic acid present in crops or farm animals (11-12). Association of chromosomal aberrations on chromosome 3 are observed during the evolution of the disease and on tumors of urothelial tract of BEN patients by FISH screening (13).

Multiple risk factors operate at the stage of initiation of kidney damage and in its progression. Until a significant decline in function is assessed the first symptoms are not specific (14). The degenerative action of inflammatory immune reaction depends on genetic factors and immune response leading to inflammation with increased levels of C-reactive protein reported as a relevant factor for the progression of BEN (15). Recent studies investigated the association of the polymorphism of the IgH 3' Regulatory Region (3'RR) to systemic inflammatory diseases such as systemic sclerosis, rheumatoid arthritis, plaque psoriasis, psoriatic arthritis and dermatitis herpetiformis (16-18).

We investigated a conformational polymorphism of the hs1.2 enhancer included by the Immunoglobulin's heavy chain 3'RR involved in the humoral immuno response (19-20). The relevance of this regulatory region for Ig and relative maturation of B cells was reviewed recently (21) and it was assessed in transgenic mice harboring the deletion of the entire region encompassing the four enhancers and the boundary region (22). Moreover, the incidence has been recently reported of polymorphism on the human hs1.2 DNA structure compared to mouse and the different response of the alleles to trans-activator factors activating differently the 3'RR (23-24). The polymorphism of the enhancer analyzed consists of a microsatellite of a 40bp element harboring an NF-κB consensus and repeated from 1 to 4 times in humans as shown in Fig. 1 (23, 25) leading to Ig serum control (17, 26, 27).

It has been reported that the polymorphism of this regulatory region of Ig was also associated with such immune-diseases as IgA nephropathy, celiac disease and IgA deficiency (26, 28, 29). The increased frequency of the enhancer hs1.2 allele *2 and low frequency of allele *1 was associated with high values of IgM and inflammatory diseases. On the contrary, higher frequency of allele *1 with decreased frequency of allele *2 correlates with low levels of circulating IgM (17, 25, 29). Therefore, we suggest that this polymorphism may be related to autoimmune-response and pro-inflammatory reaction. In the present study, we determined the allelic frequencies of enhancer hs1.2 in BEN.

Previous investigation reported an increased incidence of BEN in several families of the Nis area (Serbia). In fact, the incidence of BEN was reported as 1.7% in the general population and 3% in the family under investigation (5). We enrolled for this study a family of 133 members with endemic nephropathy, 95 patients in dialysis with BEN in a local hospital and matched to a healthy population control.

MATERIALS AND METHODS

Cohort enrolling

The data on the family were collected from the first epidemiological study performed after the description of the disease in subjects of the family recovered in the Hospital of Nis (2). The epidemiological description of the "J" family includes the subjects of 6 generations from the founder as reported on the family tree in Fig. 2.

For this study, buccal swabs were taken from 133 members of BEN family "J", 72 females mean of age 48±13; 61 males mean of age 42±12, originating from the village of Brestovac a well known endemic place for BEN located in southern Serbia. It was not possible to collect the DNA of the entire family since several emigrated in other countries in Europe or died for the disease. The first cases of BEN in family "J" were described in 1957. There were 79 males, and 72 females; the age was in a wide range because of the presence of different generations (mean 45±13). Diagnosis of BEN was formulated according to reported guide-lines (30). The cohort of patients in dialysis was composed of 48 female with the age mean 53±8; 53 male age mean 55±8. Along with them buccal swabs were taken from 43 control males, with the age mean 21±1, and 97 females age mean 21±1 who were born in the Nis province (Southern Serbia) and lived in

the same area. No subjects of dialysis cohort analyzed were from "J" family. The control cohort was chosen for the geographic origin in the same endemic area of the "J" family and dialysis group. The age was not considered to influence allelic frequencies in this genetic analysis. The genetic background of the reference population cohort was considered a valid control, since at that age it included subjects possibly later developing the disease.

DNA extractions and PCR

Oral swab DNA extractions were performed with proteinase K Tris EDTA phenol chloroform ysoamil alcohol, Millipore (Massachussetts, USA) tube Amicon ultra 0.5 following the manufacturer's instructions. PCRs were performed with primers as described in Giambra et al. 2005 with a genomic selective preamplification of the 3'RR-1 region including the hs1.2A enhancer and a nested PCR for the amplification of the hs1.2 region (Fig. 1). Genotyping was performed on 20-50 ng of genomic DNA and the nested PCR on 1/50 of the first PCR using High fidelity hot start Taq polymerase of Invitrogen Company (New York, USA).

The direct genomic PCR amplification and a selective with nested PCR is shown in the top and lower part of Fig. 3

Statistical analysis

Chi-square analysis were run according to Graphpad program and p value evaluated. Online p-value calculator based on GraphPad Software chi square value http://www.graphpad.com/quickcalcs/pvalue1.cfm.

RESULTS

Familiar incidence of BEN in a large family of South Serbia

Analysis on the frequency observed in the reconstruction of a Southern Serbian family with a high incidence of BEN showed the data reported in Table I and Fig. 2. This large family ("J") of Brestovac village near Nis was enrolled since several subjects of the family were admitted to the nephrology clinic and diagnosed for BEN. The "J" family tree was reconstructed and is shown in Fig. 2 with 151 members in six generations. The frequency of BEN incidence is evaluated on 79 subjects in four generations including the founder parents as shown in Table I. The last two generations after the parental founders were not included to avoid the bias represented by the fact that BEN develops in advanced adulthood. In the group of 15 patients afflicted by the disease, the ratio of female to male is 9 to 6 as shown in Table I. In the second generation the affected subjects are 3 in a total of 8 newborn. In the second generation two marriages were with a partner who later developed BEN. In one of the two cases the progeny (III generation) had the incidence of 100% with 3 sons and one daughter with BEN, in the other case only 1/4 of the progeny had BEN. In the fourth generation only 2 had BEN out of 43 newborns. From Table I the dilution effect is clear of the risk factors associated to the father alleles in the new generations. In fact, from the third to the fourth generation BEN frequency drops from 37% to ~ 5%, bringing the mean to 20% that is anyway almost the double of the prevalence reported in the local population (2). This can be interpreted with different models. Table II shows the separated analysis of the family, dividing the progeny (offspring) in respect to the three wives ("M, "D" and "S") of the founder. The frequencies change interestingly giving rise to new interpretations of the penetrance of the partnership. The separated analysis in Table II shows that the first wife M, had all descendants without the disease for a total of 16 descendants. The second wife D. had 2 sons and 1 daughter, two of whom presented symptoms of BEN. The healthy son married a wife who presented symptoms of BEN and one daughter had BEN. The

Table I. *Frequency of BEN in "J" family from Brestovac (Serbia).*

generations	n.	female	male	n. with disease	female	male
I	*p4	3	1	1	0	1
II	8	4	4	3 (37.5%)	2 (50%)	1 (25%)
III	27	10	17	10 (37.0%)	6 (60%)	4 (23%)
IV	43	22	21	2 (4.65%)	1 (4.5%)	1 (4.7%)
total	79	36	43	15 (18.9%)	9 (25%)	6 (14%)

**p the parental number is 4 with the founder (male affected by BEN) and 3 wives (healthy)*

Table II. *Progeny of the separate wives of the "J" family founder and descendants affected by BEN.*

generations	M.			*D. family			**S. family		
	f	m	Affected by BEN	f	m	Affected by BEN	f	m	Affected by BEN
II	1	0	0	1	2	2	2	2	1
III	1	4	0	5	4	3	4	9	7
IV	3	3	0	7	8	0	12	10	2
V	2	2	0	7	10	0	18	18	0
VI	0	0	0	0	0	0	7	8	0
total	7	9	0	20	24	5	43	47	10

** one marriage was with a wife affected by BEN of a healthy son of D.*
***one marriage was with a wife affected by BEN of one healthy son of S.*

Table III. *Genotypes and allelic frequencies of hs1.2 enhancer of the three cohorts of Nis area.*

genotype	controls		dialysis patients		J. family	
	observed	frequency %	observed	frequency %	observed	frequency %
1/1	32	0.29	8	0.08	14	0.11
2/2	16	0.14	15	0.16	39	0.29
4/4	2	0.02	2	0.02	2	0.01
1/2	38	0.34	24	0.25	49	0.37
1/3	1	0.01	-	-	-	-
1/4	3	0.03	11	0.12	9	0.07
2/3	-	-	-	-	1	0.01
2/4	19	0.17	34	0.36	18	0.14
3/4	-	-	1	0.01	-	-
total	111		95		132	
alleles						
***1**	106	0.478±0.033	51	0.269±0.032	86	0.326±0.028
***2**	89	0.401±0.032	88	0.463±0.036	146	0.553±0.030
***3**	1	0.004±0.004	1	0.005±0.005	1	0.004±0.003
***4**	26	0.117±0.021	50	0.263±0.032	31	0.117±0.018
total	222		190		264	

χ^2 was calculated for the allelic frequencies in the three cohorts, one of control, the second of BEN patients and the third of a family with high frequency of BEN, all from same geographical area of Nis (southern Serbia). p value Controls/Dialysis frequencies <0.0001; p value Controls/J.Family frequencies <0.0001.

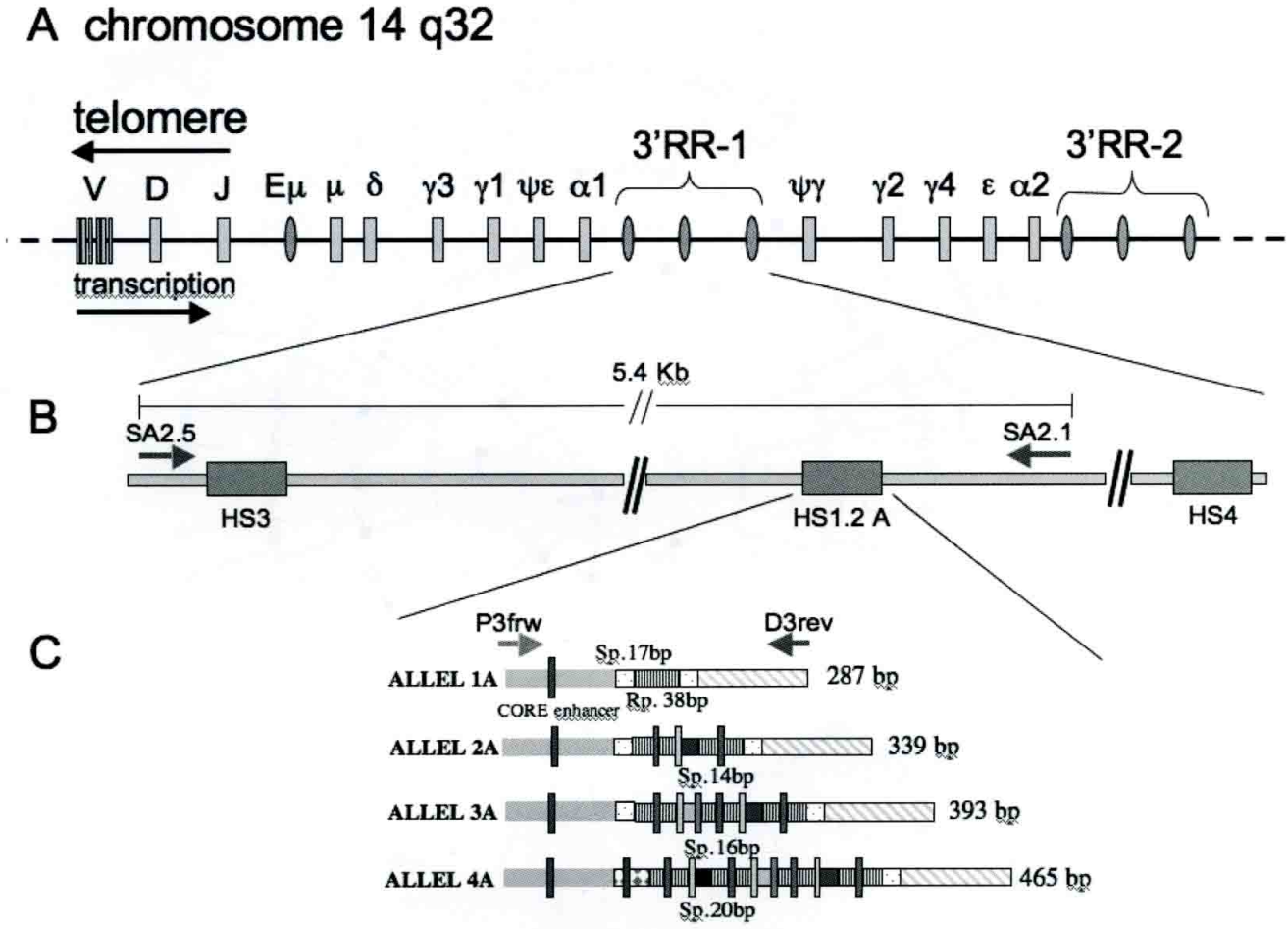

Fig. 1. *The Ig heavy chain locus. **A)** Schematic representation of the variable and constant exons of the human locus positioned on chromosome 14q32. The wide duplication of four constant genes harbors the 3'Regulatory Region at both 3' alfa genes. **B)** Enlargement of the 3'RR1 corresponding to the region amplified with selective PCR, gray boxed the enhancers hs3, hs1.2, hs4. The arrows show the position of the primers utilized for the selective PCR of the fragment encompassing the hs3 and hs1.2 enhancer. **C)** Schematic enlargement of the hs1.2 polymorphic enhancer. The four alleles are depicted for the different elements. The main difference consists in a 40 bp GC rich sequence that along with the spacer of 14 bp in alleles *2, *3 and *4 harbor a NF-κB consensus missing on allele *1. Rectangles show the position from left of Oct1, NF-κB and Sp1 transcription factors observed by EMSA experiments. Arrows indicate the position of the primers for the nested PCR.*

son and daughter with BEN had each 1 child with the disease from a total of 44 descendants. No cases of BEN were demonstrated in the last two generations. The third wife S. had 2 sons and 2 daughters and only one daughter had BEN. One healthy son married a wife with illness and 4/4 of the offspring had BEN (see pedigree in Fig. 2). In the next two generations no BEN was demonstrated in 19 descendants. Both daughters (1 had BEN and 1 healthy) had healthy descendants in a total of 24 newborns. The other healthy son, probably with the risk alleles, married a healthy wife and had 2 daughters (both of them affected by BEN) and two sons (one with BEN). In the next generation, two were affected by BEN and one was the son of a healthy father and the other from a sister with BEN. The total descendants were 38 with five subjects affected by BEN but only in the first two generations. Table II, shows the offspring of the six generation, but none had BEN in the last two.

A similar trend of BEN distribution with high frequency of BEN in the first two generations and a dilution effect was also shown in another large family (for a total of 36 members) of a village from the same area (data not shown).

Frequency analysis of hs1.2 alleles in the control population, the BEN dialysis patients and "J" family

The comparison of the genotypes was carried out

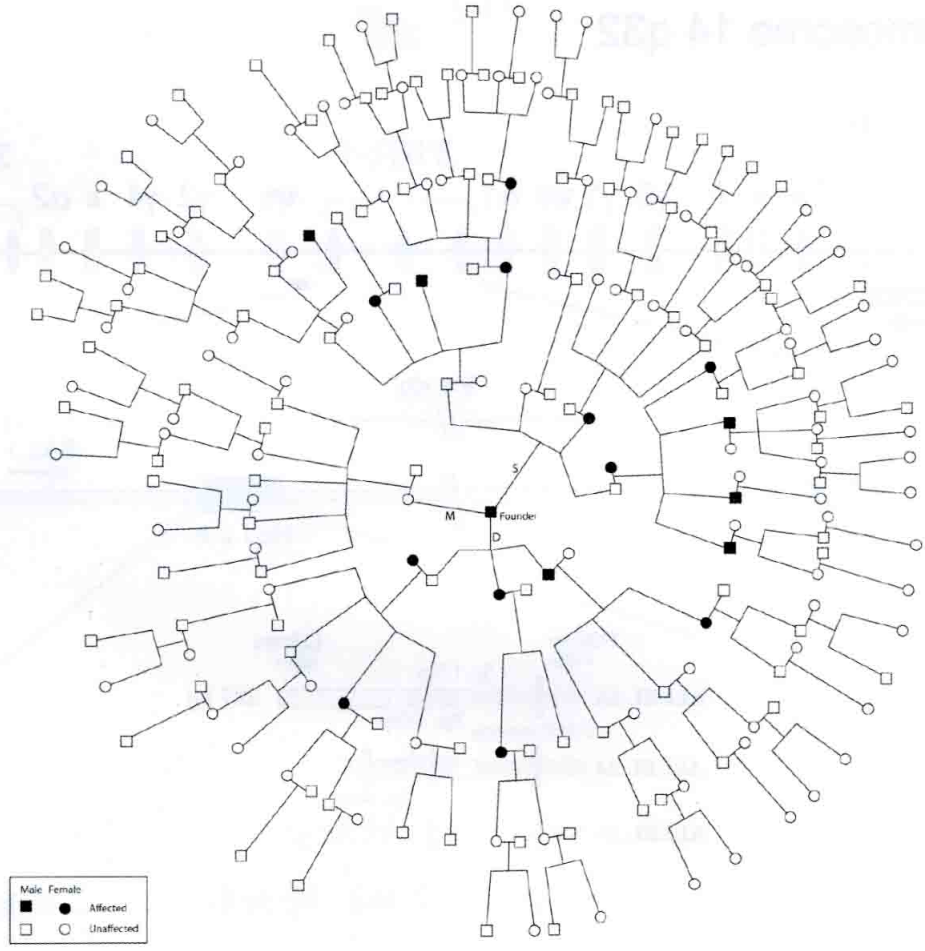

Fig. 2. *Schematic circular representation of the Family "J" pedigree. Square for males and circle for females represent a subject affected by BEN when darkened. The three marriages of the founder give rise to three descendants marked as M, D and S for a total of 151 members in six generations. Each circle corresponds to a generation considering as the first that one with the founder.*

in this study with: 1) controls (111subjects), 2) 95 BEN patients in dialysis therapy in the hospital of Nis and 3) 133 members of "J" family. It was not possible to collect the DNA from members affected by BEN of the "J" family in order to determine the haplotype carrying associated alleles, however from our data the significantly modified frequency is clear of the hs1.2 alleles in the entire family and its statistical significance. The dialysis cohort and the "J" family showed a variable pattern of the genotype frequencies shown in Table III. In the dialysis cohort subjects of the "J" family were not included. The number of homozygous and heterozygous subjects changes significantly in the three cohorts, suggesting allelic correlation in the dialyzed patients and in the "J" family compared to healthy controls. The *chi*-squared test and p value reported in Table III shows a significant reduction for homozygous frequency of allele *1. On the contrary, heterozygous genotypes 1/2 and 1/4 are increased in the dialysis group and "J" family. The changes for 2/2 and 2/4 genotypes have different trends in the two cohorts with BEN. Overall, in BEN patients and "J" family the allelic frequencies significantly change in respect to the controls, showing a decrease of allele *1 and an increase of the allele *2 frequencies. We show a correlation for the inversion of the frequency of allele *1 and *2 in the BEN patients and "J" family as compared to the general population. Moreover, the allele *4 frequency in dialysis patients increased as a result of heterozygous genotypes (1/4 and 2/4). The χ^2 test value for the difference of the allelic frequencies of BEN family and patients toward the general control population was highly significant ($p<0.001$). The significance of our observation is suggested by the same trend observed in both the

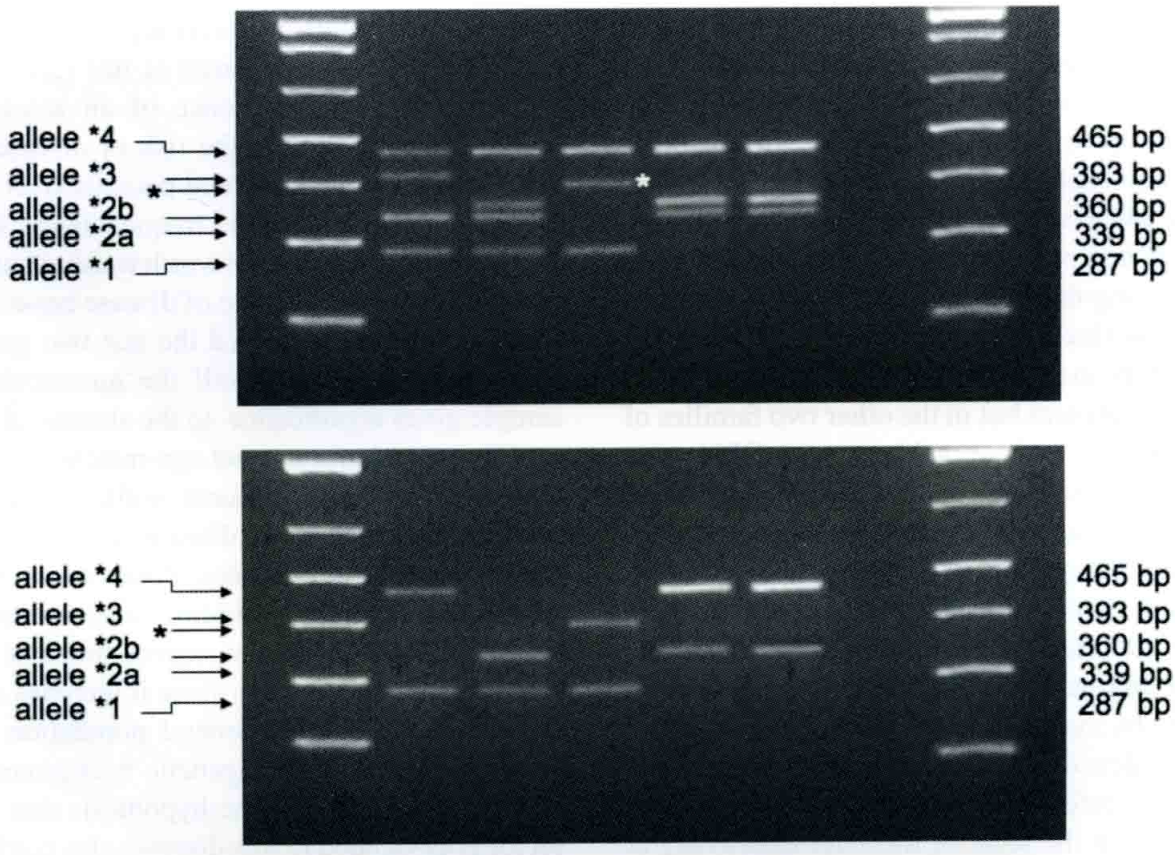

Fig. 3. *Gel electrophoresis of 5 DNA PCR amplifications from different heterozygous genomes: upper gel the genomic PCR amplification of both hs1.2 loci with four alleles, lower gel the nested selective PCR amplification where only the hs1.2 alleles from the 3'RR-1 are present. The DNA were selected from subjects double heterozygous for the hs1.2 enhancers of both 3'RR-1 and 3'RR-2 regions.*

dialysis patients and in the "J" family where the frequencies of the most frequent allele *1 and *2 change in a similar mode. On the other hand, allele *4 frequency increased only in the dialysis patients. Allele *3 frequency is too low to gain a significant relevance. The allelic frequencies in the controls with median age lower than BEN patients could include potential subjects who will later develop the disease, but this possibility eventually increases the validity of the results, increasing the level of the genetic background and though diminishing the allelic difference with patients and "J" family that resulted anyway very significative.

DISCUSSION

In this communication we show that HS1,2 allele *2 frequency is significantly increased and allele *1 frequency reduced in BEN patients and in a large family affected by BEN as compared to control population. This is the first time that a risk factor for BEN is associated with a polymorphism of the regulatory region of the humoral immune-response.

The frequency of BEN studied in four generations of descendance of an affected person strongly shows a typical dilution effect of the founder alleles. Consanguinity could be a confounding factor which is present by definition in a family, but as the frequency of allele *2 is similar in the group of BEN non familiar subjects, this suggests that there is no false correlation. Moreover, subsequent marriages with healthy non-family members dilute the alleles linked to disease. In fact BEN is present at a higher frequency in the first two generations, rarely in the third generation and it is absent in the fourth generation. The marriages were all with partners of

the same geographic area and same ethnical origin. The founder married three times and we analysed the family descendants of the three wives. The BEN frequency in these families and in the other analyzed previously (data not shown) have a higher prevalence when compared to the general population. The dilution and segregation effect can be observed also considering the frequencies of BEN in the three families of the three wives ("M" "D" and "S") of the founder. In fact, in the descendants of "M" no affected subjects were present but in the other two families of "D" and "S" five and ten subjects with BEN were present. Two marriages in the second generation were with two partners affected by BEN and only in these large progenies BEN patients were present. This suggests that the genetic risk factors for BEN present in "D" and "S" were diluted at the second generation, but new genetic risk was introduced to the progeny by marriage with 2 partners with BEN.

BEN incidence is reduced over the last decades. This could be referred to a lower inbreeding and/or modifications in the style of life with improvement of the environmental conditions (31). In the fourth generation the effect of age retardation in the offset of the disease and improved environmental condition of life contribute to a reduced rate of BEN cases as observed in recent years (31). The majority of the "J" family is still living in the same village and if an environmental contaminant factor contributing to the onset of BEN was still present this should have resulted in the same frequency of BEN in all generations, but this was not observed by us and no reports support this possibility. Therefore, we suggest that the genetic dilution effect may be a more solid theory to explain the reduced incidence of BEN in this family still living in the same environment. In any case, hs1.2 allele frequency is significantly different in the BEN patients and in the "J" family as compared to controls suggesting a correlation of this polymorphism with the risk of disease.

The age distribution of disease is a complicating factor in this analysis since the youngest generations that are disease-free may just reflect the age distribution in these individuals (i.e; too young to show the onset of the disease). If this is the case, there would be data missing because the younger subjects could develop BEN later. Therefore, we considered only the generations at the age of median onset of the disease, and the correlation is highly significative.

The age of disease onset is not relevant to the fact that we show evidence of an association of allelic frequencies with the risk of disease. In fact, the analysis on the unrelated patients with BEN has the same pattern of allelic frequencies as in the "J" family. Moreover, it is not worth waiting until the last generation reaches the age of disease onset. To be on the safe side, we excluded the last two generations from our analysis and still the numerosity of the sample gives significance to the statistic analysis.

The control group is not age-matched because we consider "that it is not influent on allelic frequencies". As the typical peak of the disease takes place between the third and fourth decades. Potential BEN patients who could develop the disease later are included in the control group. We considered this fact useful to reinforce our conclusions since it includes all alleles present in the control general population giving a complete representative genetic background. These considerations support the hypothesis that a genetic factor is associated to this disease, also confirmed by the evidence that the same alleles change frequency in a similar mode in two independent cohorts both affected by BEN when compared to the general population. Elevated frequencies of allele *2 are largely associated with inflammatory diseases or diseases with an abnormally high or abnormal (autoimmune) antibody production. Therefore, the association of increased allele *2 frequency with BEN is an indication that this allele may contribute to the inflammatory status present in BEN (15).

This is the first report of an immunogenetic status that links BEN pathogenesis to inflammation and Ig regulation. We suggest that this polymorphism can be one of the different factors involved in the onset of BEN where inflammation plays a role at the level of tubular interstitial tissue. The mechanism by which this occurs is not known, although the 3' regulatory region is known, in mouse, to be essential for class switch recombination to all isotypes and for the high levels of expression of Ig in plasma cells.

Since our data suggest a link between BEN and an abnormal immune response leading to elevated inflammation could be confirmed and expanded, it is conceivable that a modulation of the immune-response may eventually result in a new therapeutical approach to prevent, or at least to slow down, the

progression of kidney failure.

In summary, we propose that allele *2 is one of the factors leading to the development of the disease. On the contrary, allele *1 that has a lower frequency compared to control could be considered a protection factor against BEN mainly when in homozygosity. In fact, its frequency declines from 37% in the general population to 11% in the "J" family and to only 8% in the dialysis patients. A similar trend with allele *2 frequency increase and allele *1 decrease was described also in other immuno-mediated diseases characterized by inflammation and or abnormally high levels of antibody or autoantibody production (17, 18, 28).

The increase of allele *4 is intriguing and we can only formulate some hypotheses: 1) with a low frequency there could be a higher variability; 2) to produce a clear cut exclusion of an association risk a bigger cohort should be evaluated. However, the increase of allele *4 could fit with our data because allele *4 also has a Nf kappa B consensus.

Converging mechanisms linking hs1.2 allelic frequencies to the induction of the abnormal immunological features summarized above are not defined, but we want to emphasize that this polymorphism probably acts by remodeling the secondary structure of the 3'Regulatory Region influencing the three dimensional structure of the chromatine and the different binding to transcription factors such as NF-κB and others (25, 27, 32, 33). The activation of NF-kB is a well known pathway leading to inflammation. The resequencing analysis of the 3'RR could evidence more polymorphisms also influencing its palindromic region and 3D structure modulating the regulatory activity of IgH production (20).

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