Polymorphism in the Neuropeptide Y Y1/Y5 Receptor Gene Cluster is Associated with Proteinuria and Blood Pressure in Patients with IgA Nephropathy

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Accepted for publication on September 3, 1997

ABSTRACT. A *PstI* RFLP has been described within the first intron of the human Neuropeptide Y Y1 receptor (NPYY1R) gene, which is the receptor subtype most likely to mediate renal and systemic vasoconstriction and is associated with antinatriuretic effects. Therefore, this RFLP and its clinical features in patients with IgA nephropathy was evaluated.

A total of 105 patients with IgA nephropathy (49 males and 56 females) were genotyped, and the clinical findings in each genotype were compared. To evaluate the patients' long-termprognosis, only those who had a clinical course of over eight years duration were selected, and they were divided into a "poor-group" and a "fair-group", and compared their gene frequency in each group. The genotypes of the NPYY1R gene were determined by the PCR-RFLP method using the restriction enzyme *PstI*.

The results suggested that RFLP is associated with blood pressure, as systolic blood pressure was significantly higher in the Yy and yy genotypes, compared to YY genotype [YY : 123.1 ± 18.2, Yy : 131.8 ± 16.9, yy : 132.0 ± 14.9 mmHg, ($P<0.05$; YY to Yy, $p<0.05$; YY to yy)] . With regard to proteinuria, the yy genotype tended to be severer than the YY genotype, although there was no significant correlation between Yy and YY [YY : 0.82 ± 0.69, Yy : 1.29 ± 1.46, yy : 1.87 ± 2.08 mg/24hr, ($p<0.05$; yy to YY)] . Most interestingly, however, was that 11 patients with massive proteinuria (>3g/24hr), had at least one y allele (YY ; 0, Yy ; 5 and yy ; 6) suggesting a strong correlation between this y allele and proteinuria. These results suggest that this *PstI* RFLP of NPYY1R gene could be correlated with blood pressure and proteinuria, especially massive proteinuria in patients with IgA nephropathy.

Key words : neuropeptide Y Y1/Y5 receptor gene RFLP — IgA nephropathy — blood pressure — proteinuria

IgA nephropathy (IgAGN) which was first reported by Burger in 1969,[1] is the most common glomerular disease. Chronic haemodialysis patients suffering from the progression of IgAGN account for 30% in over 150,000 Japanese haemodialysis population,[2] however, the pathogenesis of IgAGN still remains unclear. The presence of heavy proteinuria and/or hypertension in the clinical findings of these patients seems to be indicative of a poor prognosis.[3,4] Vasoactive agents acting through the renin-angiotensin-axis on hypertension and renal haemodynamic could participate in the progression of IgAGN. Therefore, polymorphic markers of candidate genes encoding such proteins have been analyzed for possible linkage to this disease. Especially the involvement

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of an insertion/deletion polymorphism within the angiotensin converting enzyme gene (ACE) and the progression of IgAGN has been discussed.[5-7] Although our laboratory has also analyzed this ACE polymorphism as well as several polymorphism of other candidate genes, we have not found any significant association with IgAGN.[8,9]

In this study, however, a significant association between the *PstI* RFLP located withn the human neuropeptide Y-Y1/Y5 receptor gene cluster, proteinuria and blood pressure in Japanese IgAGN patients was described. Neuropeptide Y (NPY) receptors, members of the G protein coupled receptor superfamily, are activated by one of the most abundant peptides in the mammalian nervous system and subsequently influence a diverse range of important physiological parameters, including effects on psychomotor activity, central endocrine secretion, anxiety, reproduction, appetite and most importantly, potent effects on the cardiovascular system. NPY also regulates renal and systemic vasoconstriction and has antinatriuretic effects.[10-12] A *PstI* RFLP has been described within the first intron of the human NPY Y1 receptor gene,[13] which is the receptor subtype responsible to mediate this renal effects. Although no association between this RFLP and any diseases has been reported so far, it is very likely that this gene correlates with hypertension and its associated diseases.[14] Therefore the association between this RFLP and its clinical features was evaluated in patients with IgAGN.

MATERIALS AND METHODS

Patients and controls

A total of 105 patients (49 males and 56 females) were the subjects of this study. IgAGN was diagnosed from predominant granular IgA deposits which were mainly found in the glomerular mesangium during immunofluorescence studies, as well as from mesangial electron dense deposits found during ultrastructural examinations.[15] Patients who had diabetes mellitius, chronic liver diseases or collagen disease were excluded. To evaluate the patients' long-term prognosis, only those who had a clinical course of over eight years duration were selected, and divided into two groups. A "poor-group" consisting of 27 patients (16 males and 11 females) whose creatinine clearance had fallen to less than 50% of that during the initial period or who had developed end stage renal disease (ESRD) within 3 to 10 years after diagnosis, and a "fair-group" consisting of 35 patients (23 males and 12 females) whose creatinine clearance had been kept at over 80% of that during the initial period even 10 years after the diagnosis. As controls, 100 healthy Japanese subjects (50 males and 50 females) with normal urinalysis and renal function were analyzed. A detail mention of ethical approval was made in all patients and controls before entered into the study.

Clinical and biochemical data

Clinical and biochemical data were extracted from the case records. Blood pressure was evaluated as the average during one week of the patients' hospitalization for a first renal biopsy. Proteinuria was also evaluated as the average data obtained from 24 hour collected urine samples for week. Renal haemodynamic were measured as RBF, RPF, GFR and FF by the thiosulfate

and para-amino hippuric acid infusion method.

Genotyping of the Y1/Y5R gene cluster RFLP

Genomic DNA was extracted from peripheral leukocytes by conventional methods.[16] The *PstI* RFLP, which results from a point mutation located within the first intron of the Y1 gene, was detected by the PCR-RFLP method. The polymerase chain reaction (PCR) was carried out as previously described.[17,18] The PCR mixture consisted of 50ng of genomic DNA, 10mM of Tris-HCl, 50mM KCl, 1.5mM of $MgCl_2$, 0.2mM of dNTP, 12.5pmol of each primer and 0.2U of Taq polymerase (AmpliTaq™, Parkin-Elmer) at pH 8.3 in a total volume of 20μl with 5 min of denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C, 75 sec at 60°C, and 15 sec at 72°C, and a final extension of 6 min at 72°C. Ten microliters of the post-PCR mixture was digested for 2 hr at 37°C with 16U of *PstI*, and analyzed by 5% polyacrylamide gel electrophoresis visualized by silver-staining (Figure 1). The diallelic polymorphism (designated as Y and y) were detected as bands corresponding to fragment sizes of 412bp (*PstI* cutting site absent; YY), 156bp and 256bp (PstI cutting site present; yy) and 156, 256 and 412bp (Yy) (see Figure1).

Statistical analysis

All values are expressed as means ± SD. Significant differences in allele frequency and genotype between the groups were tested by χ^2 test. Biochemical data between the groups were tested by Student's t test.

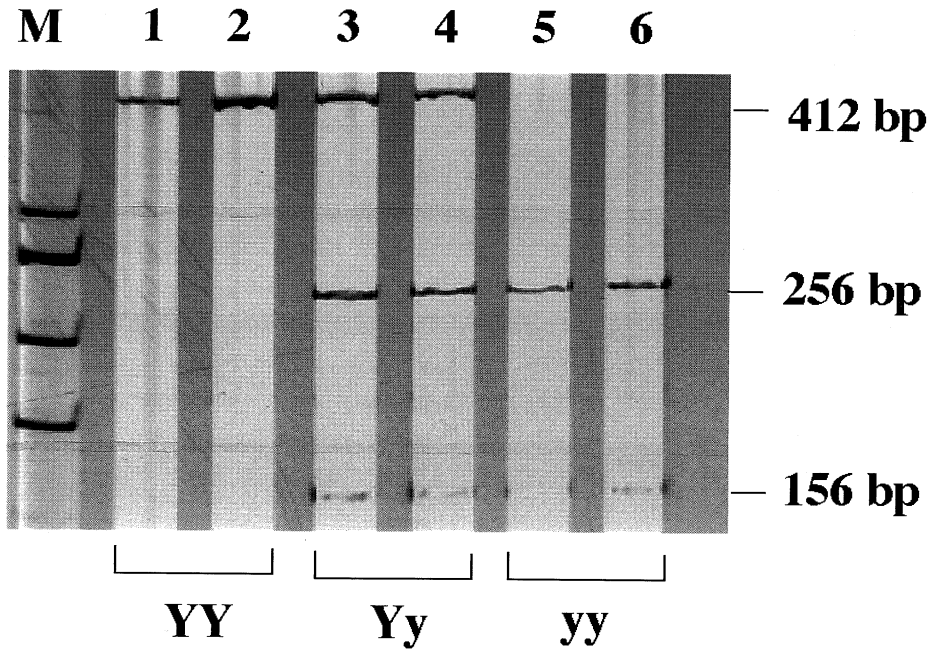

Fig 1. *PstI* RFLP of the NPYY1R gene. Shown is a silver stained 5% acrylamide gel after electrophoresis of *PstI* digested PCR-samples. Lanes 1 and 2 show the homozygote for the Y allele (YY). Lanes 3 and 4 show the heterozygote (Yy). Lanes 5 and 6 show the homozygote for the y allele (yy).

RESULTS

1. Genotype and allele frequencies of each group

Comparison of the frequency of each group revealed no statistically significant differences, but the y allele frequency in the poor group was slightly higher than that in the control group (Poor: y=63%. Control: y=48%. $p<0.1$). The genotype and allele frequencies for the RFLP in each group are summarized in Table 1.

TABLE 1. NPYY1R genotype and the frequency of alleles. $p<0.1$ compared with control group.

	IgAGN n=105	Fair n=35	Poor n=27	Control n=100
Y/Y	26 (0.25)	8 (0.23)	4 (0.15)	26 (0.26)
Y/y	49 (0.47)	16 (0.46)	12 (0.44)	50 (0.50)
y/y	30 (0.28)	11 (0.31)	11 (0.41)	24 (0.24)
Allele Frequency	Y=0.48 y=0.52	Y=0.46 y=0.54	Y=0.37 y=0.63*	Y=0.51 y=0.49

*$p<0.1$

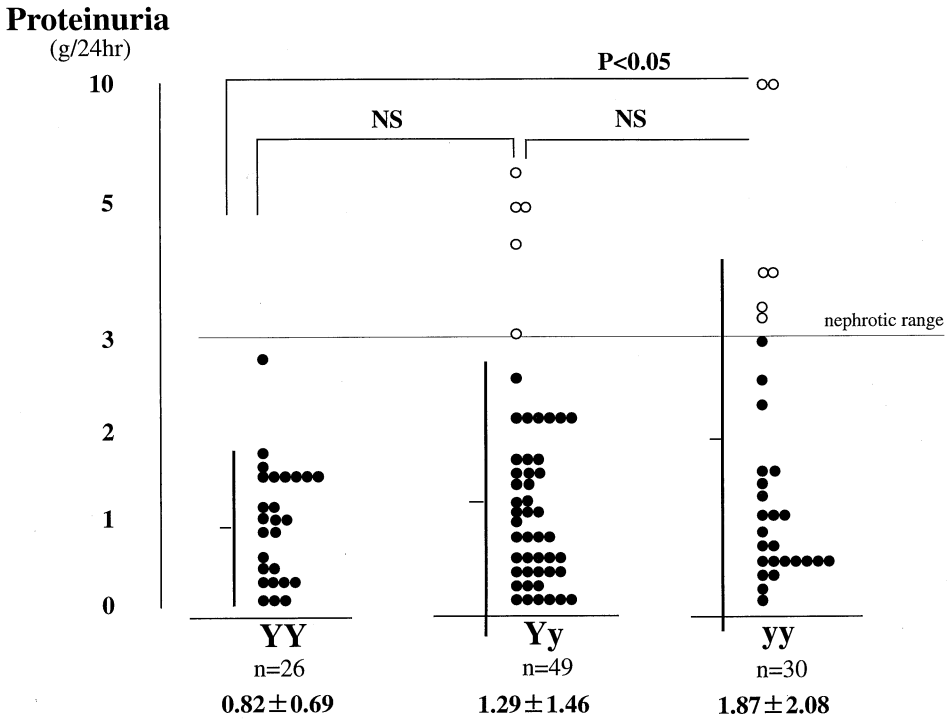

Fig 2. Proteinuria associated with the NPYY1R genotypes. Symbols are: nephrotic patients (○; N=11), non-nephrotic patients (●; N=94).

2. Genotype vs clinical and biochemical data

In this study, there were ten patients who had been administered some anti-hypertensive drugs during their first hospitalization. Their genotypes were YY ; 1, Yy ; 9 and yy ; 0, and they had been also analyzed in the study. Systolic blood pressure (SBP) was significantly higher in the Yy and yy genotypes, compared to YY genotype [YY : 123.1 ± 18.2, Yy : 131.8 ± 16.9, yy : 132.0 ± 14.9 mmHg, ($p<0.05$; YY to Yy, $p<0.05$; YY to yy)] . With regard to proteinuria, the yy genotype tended to be severer than the YY genotype [YY : 0,82 ± 0.69, Yy : 1.29 ± 1.46, yy : 1.87 ± 2.08 mg/24hr, ($p<0.05$; yy to YY)] , although there was no significant correlation between Yy and YY. Proteinuria data for the patients in each group are shown in Figure 2. Most interestingly, however, all 11 patients with massive proteinuria (>3g/24hr), had at least one y allele (YY ; 0, Yy ; 5 and yy ; 6) suggesting a strong correlation with that allele. The clinical and biochemical data for each genotype group are summarized in Table 2.

TABLE 2. Clinical and biological characteristics associated with NPYY1R genotypes. $p<0.01$ and $p<0.05$ compared with respect to YY genotype.

	YY n=26	Yy n=49	yy n=30	yy+Yy n=79
BP(Syst)(mmHg)	123.1±18.2	131.8±16.9**	132.0±14.9**	131.9±16.1**
BP(Dias)(mmHg)	74±13	79±13	77±11	78.2±11.9
Cho(mg/dl)	189±40	205±47.7	203±53.3	204±49.5
Proteinuria(g/24hr)	0.82±0.69	1.29±1.46	1.87±2.08**	1.45±1.71*
Ccr(ml/min)	85.3±29	81.2±28.7	85.1±26.1	82.72±27.6
Crn(mg/dl)	1.04±0.38	1.05±0.38	0.98±0.29	1.02±0.35
RBF(ml/min)	782.7±346.9	834.7±279.9	859.7±324.5	844.1±295.6
RPF(ml/min)	492.1±188.0	488.8±166.7	513.2±164.3	498.0±155.1
GFR(ml/min)	113.1±39.9	114.2±44.2	128.6±53.5	119.6±48.1
FF	0.24±0.06	0.24±0.08	0 .25±0.06	0.24±0.07

*$p<0.01$ **$p<0.05$

DISCUSSION

The results suggest that the *PstI* RFLP of the NPY Y1/Y5 receptor gene cluster could be correlated with proteinuria and blood pressure in Japanese IgAGN patients. NPY is a strong vasoconstrictor[10,11] and is present in renal sympathetic nerves, which innervate the intra-renal arteries, the juxtaglomerular, afferent and efferent arterioles of the glomeruli in rats and humans.[19,20] It is thought that NPY is one of the substance associated with hypertension as injection of NPY gives arise to renal vasoconstriction[10,11] and leads to the inhibition of natriuresis.[12] The NPY Y1/Y5 receptor gene cluster encodes, within 30 kb and on opposite strands, both the Y1 and Y5 receptor subtype.[21] The *PstI* RFLP originally identified within the first intron of the Y1 receptor subtype is also considered as a possible marker associated with the Y5 receptor subtype. However, the Y5 receptor subtype seems to be mainly expressed within the central nervous system and more likely to be associated with the regulation of food intake and so far has not been shown to be present in the

kidney. The Y1 receptor subtype, on the other hand, is widely expressed in the central and peripheral nervous system and has been shown to be mainly responsible for the effects of NPY on hypertension.[10,11]

In the present study, blood pressure and proteinuria were significantly higher in the group with the y allele compared to the group without the y allele. Moreover, all patients who had massive proteinuria over 3g/day shared at least one y allele. Therefore, it seems very likely that the y allele is associated with proteinuria, especially with massive proteinuria. This correlation might be based on the effects of NPY on renal haemodynamic, acting through its Y1 receptor subtype. However, further study would be necessary to confirm our findings. As noted in the introduction, IgAGN patients with heavy proteinuria and/or hypertension seem to have a poor prognosis.[3,4] Although there is no statistical significance in the allele frequency between the genotype and prognosis, the y allele frequencies in the poor group were slightly higher than those in the control group [Poor : $y=63\%$. Control : $y=48\%$. $p<0.1$] . Since it is well known that patients with IgAGN usually develop ESRD very slowly, for this study only patients who could be followed up over a period of eight years were evaluated. Among the patients, there tended to be a higher frequency of the y allele, especially in those in the poor group.

Despite of many investigations, the cause and progression of IgAGN still remains quite unclear. Recently, the correlation between the ACE gene polymorphism and the progression of IgAGN has been a matter of intense discussion within the field of nephrology.[5-8] However, it has also been proposed that the disease may be a polygenic disease,[22] and it has been suggested that many genetic traits could be associated with its progression. The Y1/Y5 receptor RFLP should be considered as one of these candidate genetic traits for the progression of this disease. Although further studies would be necessary to clarify the detailed mechanism and association between this receptor polymorphism and nephropathy, it is the first association shown to be linked on this disease.

ACKNOWLEDGMENT

The author would like to thank Dr. Herbert Herzog (Garvan Institute of Medical Research, Neurobiology Program, St. Vincent's Hospital, Sydney, Australia) for the information of PCR primer. The author would also like to thank Dr. Shinsuke Nomura and professor Gengo Osawa (Division of Nephrology, Department of Medicine, Kawasaki Medical School) to access the patients of this study.

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