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Lack of Association between the S20G Missense Mutation of Amylin Gene and Essential Hypertension in Korean Population

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***ABSTRACT*: Essential hypertension is a heterogeneously multifactorial disease in which blood pressure is harmfully high without overt cause. Both genetic and environmental factors have been implicated in its etiology. In view of the regulatory role of this peptide in the carbohydrate metabolism and renin-angiotensin system, amylin gene has been proposed to a candidate gene for essential hypertension. Therefore, we scanned the amylin gene for mutations in 133 Korean normotensives and 61 essential hypertensives by single-strand conformational polymorphism, and found a single heterozygous S20G missense mutation. However, no significant difference was observed between normotensives and essential hypertensives in the distribution of allele and genotype frequencies of this mutation at the amylin gene ($P > 0.05$). This finding suggests that S20G missense mutation of the amylin gene are unlikely to contribute to the etiology of essential hypertension in the Korean population.**

Keywords : Amylin, Essential Hypertension and Genotype.

Introduction

Essential hypertension and non-insulin-dependent diabetes mellitus (NIDDM) are both characterized by insulin resistance (DeFronzo and Ferrannini, 1991; Ferrannini *et al.*, 1987; Haffner *et al.*, 1992; Reaven, 1988). Both conditions have a strong genetic component, and defects in carbohydrate metabolism can be demonstrated already in first-degree relatives of subjects with essential hypertension or NIDDM (Alleman et al., 1993; Eriksson *et al.*, 1989; Ohno *et al.*, 1993; Schalin-Jäntti *et al.*, 1992; Vagg *et al.*, 1992).

Amylin is a peptide composed of 37 amino acids which is co-secreted with insulin in the pancreatic β-cell (Cooper *et al.*, 1987; Westermark *et al.*, 1987), and a main component of pancreatic islet amyloid found in most patients with NIDDM (Nishi *et al.*, 1990). It has been hypothesized that amylin may be an important link between insulin resistance and essential hypertension. Because this peptide affects the carbohydrate metabolism such as inhibition of glucose incorporation into glycogen in skeletal muscle (Leighton and Cooper, 1988) and inhibition of insulin secretion by the pancreatic β-cell (Degano *et al.*, 1993), and regulates plasma renin and aldosterone concentration in human (Cooper *et al.*, 1995), it suggests an important role of amylin in cardiovascular and renal physiology. For this reason, amylin gene is considered to be a prominent candidate gene in ethiology of essential hypertension.

The gene of human amylin has been cloned and assigned to chromosome 12p (Christmanson *et al.*, 1990). Sakagashira *et al.* (1996) have reported the S20G missense mutation of the amylin gene in Japanese NIDDM patients. They found the mutation in 12 out of 294 patients with NIDDM (4.1%), whereas none of the group of non-diabetic subjects and insulin-dependent diabetic patients had the mutation. However, Yamada *et al.* (1998) found this mutation in 3 out of 184 non-diabetic subjects (1.6%) as well as in 4 out of 86 NIDDM patients (4.7%), indicating that the mutation is not necessary for development of diabetes.

Nevertheless, few data have been available regarding the role of the amylin gene in essential hypertension. For this reason, we scanned the amylin gene by single strand conformational polymorphism (SSCP) methods, and investigated the relationship between its genetic polymorphism and essential hypertension in Korean population.

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Materials and Methods

Subjects

Subjects were from the outpatients of Seoul Hygiene Hospital, Seoul, Korea, and we obtained 191 blood samples from subjects. Among the subjects, 61 essential hypertensives were defined as having a blood pressure above 140/90 mmHg. Subjects with secondary forms of hypertension were excluded from the study.

PCR reaction

Genomic DNA was prepared from buffy coat of 5ml blood after lysis of red blood cell (Sambrook *et al.*, 1989). Polymerase chain reaction (PCR) was performed according to the protocols by Saiki *et al.* (1988) with slight modification. The 5'-flanking region and exon 1~3 regions of amylin gene were amplified with each set of primers (Table 1). PCR reactions were performed in 50 ul of a mixture which included 100 ng of genomic DNA, 20 pmol of each primer, 200 uM each of the four dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.4 and 2.5 unit of *Taq* DNA polymerase. The reaction conditions included denaturation at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 51~56°C and 1 min at 72°C with final extension time of 10 min at 72°C. Amplified PCR products were used for SSCP analysis.

SSCP analysis

To detect the sequence variation at the amylin gene, SSCP analysis was performed according to the protocols by Orita *et al.* (1989) with slight modification. Five ul of PCR products were mixed with equal volume of loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol), and boiled for 5 min. These samples were loaded on a 0.5 x MDE (Mutation Detection Enhancement) gel (FMC Co. Ltd., U.S.A.) in 0.6x TBE buffer. Ten percentage polyacrylamide gel electrophoresis (PAGE) was carried out with adjustable vertical slab gel apparatus (Oncor Co. Ltd., U.S.A.) for about 18 hrs at room temperature. After electrophoresis, each band was stained by silver nitrate (Allen *et al.*, 1989).

RFLP analysis

For restriction fragment length polymorphism (RFLP) analysis, 4 ul of PCR products of exon 3 were precipitated by ethanol and digested by restriction enzyme *Msp* I at 37°C for 16 hrs. The digested samples were run on 10% PAGE gel and stained by silver nitrate (Allen *et al.*, 1989).

Statistical analyses

Allele frequencies were calculated from genotype frequencies, and the significant deviation from Hardy-Weinberg equilibrium (HWE) was analyzed by a χ^2-fitness test. The heterozygosity and polymorphism information content(PIC) was estimated by the methods of Bostein *et al.* (1980). The significance of the difference between allele and genotype distributions for the normotensives and essential hypertensives was tested by Fishers exact test. The odds ratio of essential hypertension associated with allelic variation or genotype was calculated by univariate logistic regression analysis. A $P<0.05$ was considered as statistically significant. All statistical analyses were performed with the SPSSWIN (version 8.0) computer program.

Results

All exons as well as 5-flanking region at the amylin gene were amplified by PCR (Fig. 1). We performed the mutation screening by using these amplification products. As results, any mutation was not found in 5'-flanking, exon 1 and exon 2 region of amylin gene with SSCP analysis. However, only one heterozygous patterns of abnormal band was observed in the amplification products corresponding to exon 3 in 2 out of 61 essential hypertensives (3.3%) and 1 out of 133 normotensives (0.8%) (Fig. 2). DNA sequencing of this exon revealed a single heterozygous missense

Table 1. Primer sequences used for PCR-SSCP analysis of amylin gene

Regions	Oligonucleotide	PCR product size[1]	Reference
5'-flanking region	5'-ACTGCACAAGGACACTGTGT-3'	230	Sakagashira *et al.*, 1996
	5'-TCCAAGCTTGTATCCACTGG-3'		
Exon 1	5'-TGCCTGATGTCAGAGCTGAG-3'	217	
	5'-ACACCAAGTGTGCATTTCTCT-3'		
Exon 2	5'-CTCTTGATTTCAGTGCTGGA-3'	202	
	5'-GGCTGTAGTTATTTGACAGT-3'		
Exon 3	5'-TCACATTTGTTCCATGTTAC-3'	239	
	5'-CAATAACTATAGAGTTACATTG-3'		

[1]base pairs.

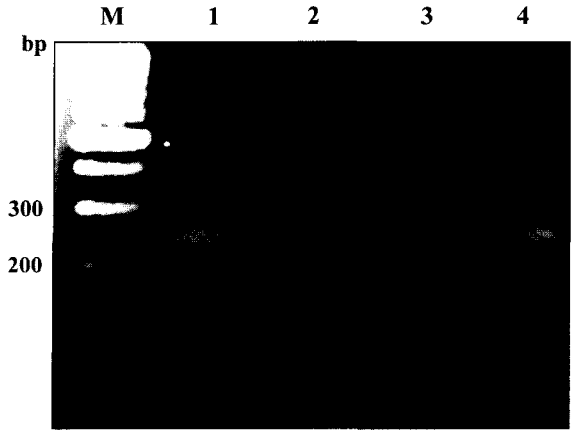

Fig. 1. PCR product of the four fragments containing the 5'-flanking region and all exons of amylin gene by PCR. Lane M, size marker; lane 1, 230 bp PCR product of 5'-flanking region; lane 2, 217 bp PCR product of exon 1; lane 3, 202 bp PCR product of exon 2; lane 4, 239 bp PCR product of exon 3.

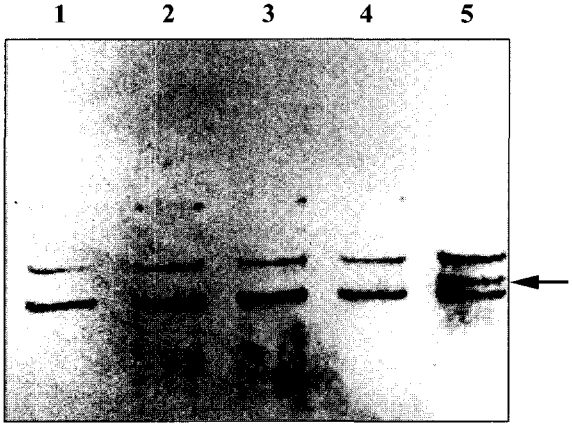

Fig. 2. SSCP patterns of exon 3 at the amylin gene. Lane 1~4, SS homozygotes; lane 5, SG heterozygote. Arrow indicates the S20G missense mutation site of the amylin gene.

mutation in codon 20 of the mature amylin molecule (AGC^{Ser} to GGC ^{Gly} : S20G missense mutation, data were not presented) (Sakagashira *et al.*, 1996). This nucleotide change (G allele) creates a *Msp* I restriction site (Sakagashira *et al.*, 1996). Wild type S allele shows the band pattern of the 239 bp, while mutant G allele results in the band patterns of 140 bp and 99 bp by using *Msp* I restriction digestion. In order to precisely demonstrate the existence of the S20G missense mutation, we performed the *Msp* I restriction digestion analysis for all subjects with abnormally migrating bands. As expected, all subjects showing abnormally migrating band pattern were identified to have band pattern digested by restriction enzyme *Msp* I (Fig. 3) and confirmed these variants as S20G missense mutation.

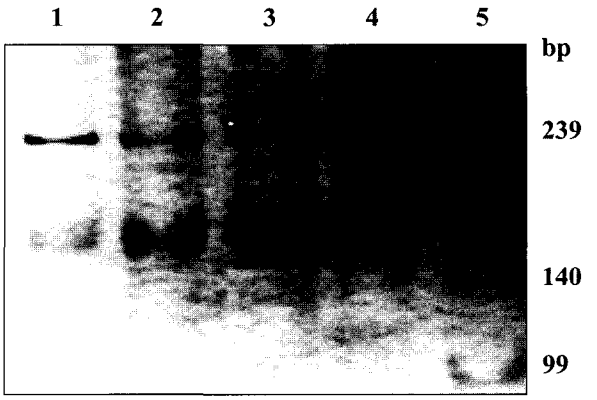

Figure 3. *Msp* I digestion of PCR product at the exon 3 of the amylin gene. Lane 1~4, SS homozygotes; lane 5, SG heterozygote.

Table 2. Allele and genotype distribution of the amylin gene in Korean

	Subjects	
	Normotensives (%)	Hypertensives (%)
Genotype Frequencies		
SS	132 (99.2)	59 (96.7)
SG	1 (0.8)	2 (3.3)
GG	0 (0.0)	0 (0.0)
Total subject number	133 (100)	61 (100)
Allele Frequencies		
S	265 (99.6)	120 (98.4)
G	1 (0.4)	2 (1.6)
Total chromosome number	266 (100)	122 (100)

There were no significant differences in allele and genotype frequencies between two groups (For genotype distribution, Fishers exact test, P = 0.2332; for allele distribution, Fisher's exact test, P = 0.2361).

However, any novel mutation, except for S20G missense mutation was not observed in the exon 3 region of amylin gene in our study.

The genotype and allele frequencies of S20G missense mutation at the amylin gene were displayed in Table 2. The observed genotype distributions of the amylin gene were not significantly different from those expected for Hardy-Weinberg equilibrium. The frequencies of SS and SG genotypes were 99.2 and 0.8% in normotensives, and 96.7 and 3.3% in essential hypertensives, respectively. GG homozygote was not detected in the both groups. Although the frequencies of SG genotype and G allele in essential hypertensives were slightly higher than that in normotensives (For SG genotype, odds ratio (95% confidence interval (CI)) = 4.47 (0.40-50.32); for G allele, odds ratio (95% CI) = 4.38 (0.39-48.77)), there were no statistically significant differences in allele and genotype frequencies between

normotensives and essential hypertensives. The heterozygosity and PIC values of S20G missense mutation represented the values of 0.0075 and 0.0075, respectively for normotensives, and 0.0320 and 0.0315 for essential hypertensives, respectively. According to the heterozygosity and PIC values, S20G missense mutation showed the low degree of polymorphism in the both groups.

In this study, the S20G missense mutation of the amylin gene is a rare mutation in Korean, and may not be a critical genetic marker in the etiology of essential hypertension.

Discussion

Essential hypertension accounts for 95% of all cases of hypertension. Many factors contribute to the development of essential hypertension. Twin and adoption studies revealed that essential hypertension is a heterogeneous disease with strong genetic components (Havlik *et al.*, 1977; Levine *et al.*, 1982: Ward, 1990). Therefore, the important task in essential hypertension research is to clarify the causative genes of this disease.

It has been reported that amylin exerts the pleiotrophic effects on carbohydrate metabolism (Leighton *et al.*, 1988; Degano *et al.*, 1993) and renin-angiotensin system (Cooper *et al.*, 1995). It suggests the probable role of amylin in cardiovascular diseases such as essential hypertension. Thus, these reports prompted us to examine the relationship between genetic variation of amylin gene and essential hypertension in Korean population.

After screening the mutation, we found only S20G missense mutation in the exon 3 of the amylin gene in our Korean subjects, however, there were no significant differences in the allele and genotype frequencies of S20G missense mutation at the amylin gene between normotensives and essential hypertensives in the Koreans. It suggests that amylin gene may not be applicable to genetic marker for the risk assessment or disease diagnostics of essential hypertension.

The G allele of the amylin gene has not been reported in Caucasianon (Birch *et al.*, 1997), however, detected as rare frequency in only Korean and Japanese populations (Sakagashira *et al.*, 1996; Yamada *et al.*, 1998). Similar genetic background or close geographic relationship between Korean and Japanese populations may affect the common existence of the G allele in gene pool. Our study is the first report concerning the relationship between genetic variation of amylin gene and essential hypertension. Further studies in other ethnic groups will be required to clarify the association between essential hypertension and genetic variation of the amylin gene.

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