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LONG-PCR OF THE ANP GENE AND PCR-SSCP ANALYSIS OF THE PROXIMAL PROMOTER REGION OF THE ANP GENE IN PATIENTS WITH ALDOSTERONE PRODUCING ADENOMA

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SUMMARY: Previous studies have shown a significant association between allelic frequencies at the ANP gene locus and aldosterone responsiveness to angiotensin in aldosterone-producing adenoma (APA). We searched for any gross insertions or deletions in the ANP gene in APA and any associations between allelic frequencies at the *Hpa* II and *Sca* I RFLP sites within the ANP gene and angiotensin-responsive and unresponsive APA and normal subjects. We also searched for possible point mutations in the promoter region of the ANP gene (-595 to transcription start site) in peripheral blood and tumor DNA from 59 patients with APA and in peripheral blood DNA from 39 normal subjects by polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) analysis.

No large alterations in the ANP gene were observed, and no difference in allelic frequencies at the RFLP sites were seen between the two tumor subtypes, angiotensin-responsive and angiotensin-unresponsive APA, or between the APA group and normal subjects. SSCP analysis, however, did reveal mutations in the promoter region of the ANP gene (-375 to -595) in both peripheral blood and tumor DNA from 8 of 59 (14%) patients with APA, compared with only one of 39 normal controls (2.6%). This study suggests that alterations in the proximal

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promoter region of the ANP gene in APA may be important in the regulation of ANP transcription and may be involved in the underlying pathophysiology of aldosterone-producing adenoma in at least some patients.

Primary aldosteronism is a potentially curable form of hypertension resulting from adrenocortical adenoma or bilateral adrenal hyperplasia which is of unknown aetiology except for a familial variety with a well described genetic defect[1]. Apart from this glucocorticoid-suppressible variety of primary aldosteronism, where the biochemical defects can be explained by a single genetic mutation, namely the presence of a hybrid gene which encodes for the enzyme aldosterone synthase and is which is abnormally regulated by adrenocorticotrophic hormone, a number of other genetic factors may be involved in the development and phenotypic expression of primary aldosteronism[2,3].

APAs can be divided into two subtypes based on their aldosterone responsiveness to infused angiotensin II (2ng/kg/min for 60min)[4], angiotensin-responsive (AII-R APA, greater than 50% increase in plasma aldosterone levels over basal) and angiotensin-unresponsive (AII-U APA).

There is evidence suggesting a paracrine role for natriuretic peptides in extra-atrial tissues including the adrenal[5], with the presence of brain natriuretic peptide and atrial natriuretic peptide transcripts in many tissues. Expression of the ANP gene is controlled by a number of positive and negative cis-acting regulatory elements in the 5' flanking sequences[6]. We have recently reported significant differences in the allelic frequencies of the *BglI*, *TaqI* and *XhoI* polymorphic sites at the ANP gene locus between angiotensin-responsive and angiotensin-unresponsive aldosterone-producing adenomas (APA)[7]. The first 600 base pairs 5' of the initiating transcription site for the ANP gene are highly conserved across species[8], and contain major regulatory elements; firstly, an activator protein-1

(AP1) like binding site and secondly, a cyclic AMP regulatory element recognition site[9]. Hence, the examination of this area is of interest.

In the current study, we examined: (1) the ANP gene using long-PCR based techniques, searching for any gross insertions or deletions in peripheral blood and tumor DNA from patients with AII-U APA and AII-R APA, (2) compared the allelic frequencies at the *Sca* I and *Hpa* II restriction fragment length polymorphic (RFLP) sites within the ANP gene in peripheral blood leucocyte and tumor DNA from patients with AII-U and AII-R APA and compared both to normal subjects, (3) examined by PCR-SSCP analysis, proximal elements (-595 to transcription start site) in the 5' flanking region of the ANP gene in 59 patients with APA (31 AII-U APA) and 39 normal subjects.

METHODS

Genomic DNA was extracted from peripheral blood leucocytes and adrenal aldosterone-producing tumors.

Long PCR of ANP Gene

The ANP gene (2.7kb) was amplified by a long-PCR based method employing 1μg (2μl) of genomic DNA, 2 μl rTth polymerase (Perkin Elmer, New Jersey, USA), 10mM dNTP's 8μl, 1.6μl forward oligonucleotide primer (TGG CTG CCT GCC ATT TCC TCC TCT) and 1.6μl reverse oligonucleotide primer (AAC GCA GGC ATT TGT CTT CTG TCC ATG) primers, 6.5μl of 25mM $MgCl_2$, 48.3μl H_2O, 30μl XL buffer, with a final reaction volume of 100μl. PCR run times were: 1min 20 sec at 93°C (denaturation), 1min at 93°C, 10min at 68°C (18cycles), 10min at 68°C 12 cycles with 15sec annealing extension for each cycle, 10min at 72°C. Amplification products were electrophoresed on 0.7% agarose and visualized under ultraviolet illumination. A negative control lane containing all reagents except DNA was run under identical PCR conditions for all gels.

Restriction Enzyme Digests at Polymorphic Sites within the ANP Gene

Analysis of two known RFLP sites within the ANP gene were examined. Amplified product (12.5μl) was digested at 37°C overnight using restriction enzymes *Hpa* II and *Sca* I. Digests were deactivated by placing in a waterbath at 65°C for 20 min. Digests were electrophoresed on 0.7% agarose and viewed under UV illumination. Allelic frequencies at the RFLP sites in the ANP gene produced by *Hpa* II[10], and *Sca* I[11] were examined.

PCR-SSCP Analysis of the Proximal Promoter Region of the ANP Gene

Single strand conformation polymorphism analysis was performed according to the method first described by Orita and coworkers[12]. Polymerase chain reaction (PCR) was performed on three overlapping fragments of the promoter region of the ANP gene using the following three specific primer sets to amplify the first -595bp 5' flanking region of the ANP gene.

Primer sequences:
1) forward primer: GCT TCT CAC TTG GCA GCT TT; reverse primer: AGG AAG CTC ACG GTG GTG, fragment size 212bp.
2) forward primer: CAC GGC GGT GAG ATA ACC; reverse primer: ATA AAG CTG CCA AGT GAG AAG C, fragment size 247bp.
3) forward primer: CAG GTG TGA GGC CAG CTT; reverse primer: GGT TAT CTC ACC GCC GTG, fragment size 211bp.
Each PCR reaction mixture (25 μl) contained $Mg(OAc)_2$ 25mM, 10X buffer, 2.0μl of 25mM dNTPs, 0.5μl of oligonucleotide primers, Taq polymerase 0.3μl (Perkin Elmer, New Jersey, USA), 0.3μl of ^{33}P-dATP, 15.9μl of deionized water and 2μl of DNA template. PCR run cycle conditions were 94°C for 1min, 55°C for 1min, 72°C for 1min, 35 cycles, 72°C for 5min.
30μl of denaturing solution (98% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) was then added to amplified product, 4μl added to a 6% non-denaturing polyacrylamide gel (37.5:1), electrophoresised at 10W for 4-6h, and exposed to autoradiographic film for 2 days.

Data was compiled according to genotype and allelic frequencies calculated. Gross insertions and deletions were looked for, and allelic and genotypic frequencies were calculated and tested by χ^2 analysis. Hardy-Weinberg equilibrium was satisfied.

RESULTS

No gross insertions or deletions were observed in the amplified ANP gene in patients with aldosterone-producing adenoma (APA) (Figure 1). No variation in

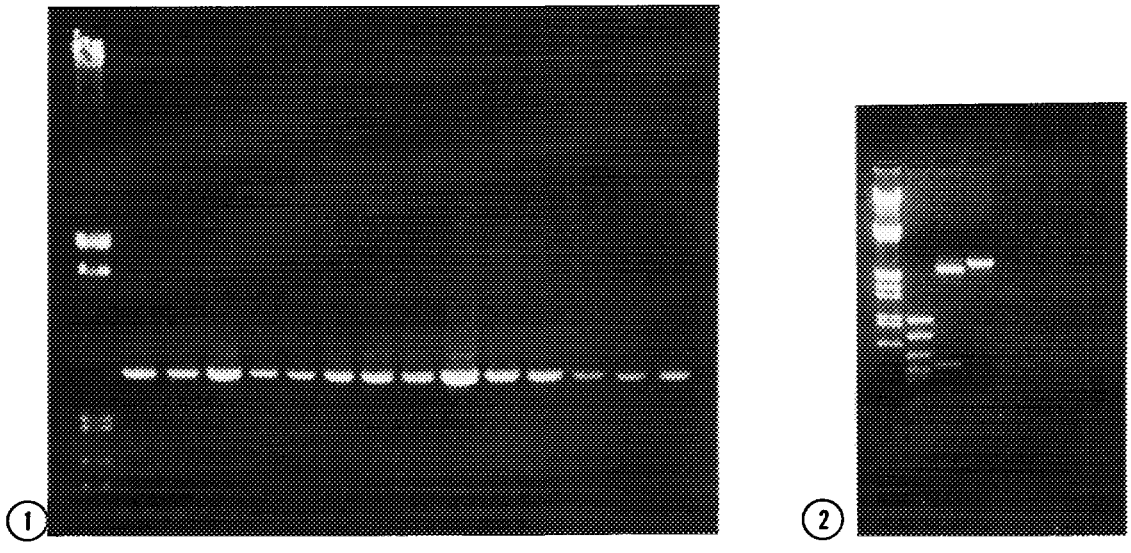

Figure 1. Representative long-PCR amplification of the ANP gene (2.7kb) in AII-U APA (lanes 2-6), AII-R APA (lanes 7-11) and normal subjects (lanes 12-15), marker (lane 1).

Figure 2. Characteristic bands for RFLP sites generated by *Hpa* II (lane 2) and *Sca* I (lane 3) in the ANP gene, undigested (lane 4), marker (lane 1).

allelic frequencies in peripheral blood DNA was observed for the *Hpa* II and the *Sca* I RFLP sites (Figure 2) within the ANP gene between 28 patients with AII-U APA and 27 patients with AII-R APA. No difference in allelic frequency was seen between the two subtypes and normal subjects (n=38). No loss of heterozygosity was observed at the two RFLP sites, between peripheral blood and tumor DNA in 18 patients with AII-U APA and 10 patients with AII-R APA .

However, PCR-SSCP analysis demonstrated significant alterations in the promoter region of the ANP gene, -375 to -595, in peripheral blood DNA from 8 (5 AII-R APA, 3 AII-U APA) patients (14%) with aldosterone-producing adenoma, 5 patients with mutation A, 2 with mutation B and one with mutation C (Figure 3). The same alterations were also observed in DNA extracted from the patient's matched tumor tissue. An alteration in this region, mutation A, was observed in only one of 39 normal subjects examined (2.6%).

DISCUSSION

SSCP analysis has been employed to detect single point mutations in a number of diseases[13,14] in humans. Wu and coworkers[6] reported that the major positive elements in the controlling region for atrial expression of the ANP gene were between -1150 and -222 relative to the transcription start site, and major negative regulatory elements in non-myocardial cells in the distal -2593 to -410

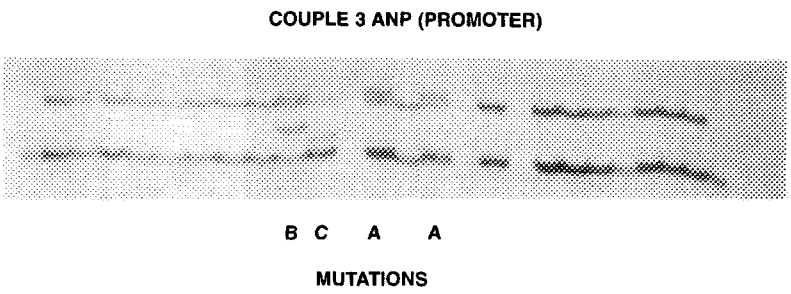

Figure 3. Mutations in the promoter region of the ANP gene (-375 to -595) examined by PCR-SSCP analysis are shown in patients with aldosterone-producing adenoma.

region and in the proximal -222 to transcription start site. The presence of an important negative regulatory silencer element between -2593 and -410 was suggested[6]. Silencer elements have been reported to be involved in the control of tissue-specific gene expression in a number of systems[15,16].

This study suggests that no gross insertions or deletions in the ANP gene are present in patients with APA, but smaller alterations in the gene are not excluded. However, alterations in the regulatory sequences of the proximal promoter region of the ANP gene (-375 to -595) were detected in some patients with APA and may contribute to altered transcription of ANP. The mutations in this area may not be specific for patients with APA as one normal subject also had an alteration in this region. Mutations in this region were however much more frequent in patients with APA and may still be an important factor in the adrenal expression of ANP in at least some patients with APA.

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