Absence of somatic mutations in natriuretic peptide receptor type A gene in human aldosterone-secreting adenomas

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Abstract

Somatic mutations of genes codifying for key regulatory proteins are the cause of different types of hormone-secreting adenomas. Natriuretic peptides (NP) are the strongest inhibitors of aldosterone secretion but aldosterone-secreting adenomas (aldosteronomas) are resistant to this inhibition and have reduced binding sites for NPs. The objective of this study was to sequence the entire coding region of the NP receptor type A (NPRA, codified by the Npr1 gene) to find loss-of-function somatic mutations.

Total RNA was extracted from eight aldosteronomas and cDNA was synthesized. NPRA mRNA expression was evaluated by Northern blot analysis and compared with 18S actin mRNA as the housekeeping gene. Twelve primer couples were designed on the basis of the Npr1 gene organization to amplify, by PCR, all 22 coding exons of the gene. The two strands of amplified DNAs were purified and directly sequenced by automated capillary sequencer.

NPRA mRNA expression did not differ among aldosteronomas. Npr1 open reading frame sequences obtained from eight aldosteronomas did not contain any mutation. The coding sequences of all 22 exons were identical in all samples and identical to published sequences. In the 3′-untranslated region (3′-UTR) a new length difference 3C/4C polymorphism was found at position 15 129 (three adenomas were 3C/4C and two were 3C/3C). Such a 3C/4C polymorphism was present in genomic DNA from 80 control subjects (25, 4C/4C; 40, 3C/4C; 15, 3C/3C).

Mutations in the coding exons of the Npr1 gene do not appear to be a common cause of aldosteronomas. Moreover, the exons of Npr1 encoding for the translated portion of mRNA do not appear to be prone to polymorphisms. The polymorphism identified in the 3′-UTR might affect mRNA stability resulting in lower receptor synthesis, but it is not likely to confer a predisposition to the development of aldosteronomas.

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Introduction

Aldosterone-secreting adenomas (aldosteronomas) are a common cause of primary aldosteronism in humans. These ‘benign’ endocrine tumours cause deadly cardiovascular complications due to hypertension and hypokalaemia, and arise from adrenal cortical cells by unknown molecular mechanisms.

It is well known that some hormone-secreting adenomas originate from somatic mutations of key regulatory proteins involved in the growth and function of endocrine cells. Activating mutations of the thyroid-stimulating hormone receptor in toxic thyroid adenomas (Parma et al. 1993), and gain-of-function mutations of G protein αs subunit in both toxic thyroid adenomas (Parma et al. 1997) and growth hormone-secreting pituitary adenomas.
Ballare et al. 1998 have been found. In these adenomas cAMP cascade permanent activation maintains cell differentiation, confers proliferative advantage and stimulates hormone secretion.

Following a similar reasoning, we (Sarzani et al. 1995) and others (Davies et al. 1997, Sachse et al. 1997) have analysed angiotensin II receptor 1 (AT1) gene expression and sequence in aldosteronomas according to the evidence that angiotensin II is the main stimulator of growth and function of normal aldosterone-secreting adrenal cells. The AT1 gene was expressed in aldosteronomas similarly to normal adrenals (Sarzani et al. 1995), explaining the angiotensin II responsiveness of some of these adenomas, but no somatic mutations were found. Other authors have focused on the adrenocorticotrophin (ACTH) receptor gene because of the role of ACTH in the stimulation of aldosterone secretion but, again, no mutations were found (Latronico et al. 1995).

Inactivating mutations of genes whose products mediate an inhibitory control on aldosterone secretion might also be involved in the molecular genesis of aldosteronomas. Cardiac natriuretic peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are potent inhibitors of aldosterone secretion (Anderson et al. 1986, Holmes et al. 1993), being effective antagonists of angiotensin II. The effects of both ANP and BNP on aldosterone secretion are mediated by natriuretic peptide receptor type A (NPRA, codified by the Npr1 gene), a membrane guanylyl cyclase with cGMP being the main intracellular messenger (Maack et al. 1996).

In patients with aldosteronomas, plasma levels of ANP (Yamaji et al. 1986, Opocher et al. 1992) and BNP (Mukoyama et al. 1990) are increased, but aldosterone secretion appears to be unaffected by these higher levels of natriuretic peptides. Moreover, ANP infusion in patients with aldosteronomas...
(Rocco et al. 1990) and ANP added to aldosteronoma cells in vitro (Higuchi et al. 1986a, Rocco et al. 1989) did not reduce aldosterone secretion, suggesting an unresponsiveness of aldosteronoma cells to natriuretic peptides.

The unresponsiveness of aldosterone-secreting adenomas to ANP might be due to reduced expression of NPRA and/or to its structural alterations. We have previously found that, in most cases, the genes for natriuretic peptide receptors are expressed in adrenals and in aldosteronomas without significant differences (Sarzani et al. 1999). Despite these findings, ANP binding was reduced in aldosteronomas in comparison with surrounding adrenal glomerulosa tissues (Sarzani et al. 1999).

We have therefore hypothesized that a somatic mutation of the NPRA gene might be responsible for the reduced ANP binding and/or reduced biological responsiveness, conferring a proliferative advantage and an increased aldosterone secretion to aldosteronoma cells.

The human Npr1 gene is located on chromosome 1q21–22 and is composed of 22 exons spanning an area of 16 kb (Fig. 1A) (Takahashi et al. 1998). The primary objective of our project was to analyse the coding sequences of the NPRA-encoding gene in search of functional inactivating mutations.

Materials and methods

 Patients and controls

Eight patients (five men and three women) with primary aldosteronism were found to have a secreting adrenal adenoma. The diagnosis of aldosterone-secreting adenomas was based on the full clinical picture and follow-up, including endocrine tests. Table 1 shows some of the clinical and humoral characteristics of the patients studied and histology of the sections is shown in Fig. 2. After surgery, all adenomas were sectioned and examined by an expert pathologist and a portion of each adenoma was quickly frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

A healthy portion of a human kidney with a localized upper polar renal carcinoma was obtained at nephrectomy and used as control RNA/cDNA. We also obtained, after written informed consent, blood samples from 80 control subjects (40 males and 40 females) to verify, at the genomic level, the presence and the frequency of the gene variants identified.

Extraction of RNA from adrenal tissue

Total RNA was extracted from tissue after homogenization in guanidine thiocyanate buffer and CsCl gradient as previously described (Sarzani et al. 1992). RNA was solubilized in RNase-free water and stored at −80 °C. First-strand cDNA was synthesized from 1·25 µg total RNA which together with oligo-deoxythymidine primer (0·25 µg) and random primers (0·25 µg) were kept for 5 min at 72 °C. Then, 5 × reaction buffer (Tris–HCl, 5 mmol/l, pH 8·3; KCl, 7·5 mmol/l; MgCl2, 150 µmol/l), deoxy(d)-NTP (1 mol/l), dithiothreitol (10 mmol/l), bovine serum albumin (2 µg), RNase inhibitor (15·7 U; Amersham Pharmacia Biotech, Milan, Italy) and Moloney murine leukaemia virus reverse transcriptase (400 U; Gibco-BRL, Life

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>K⁺ (mEq/l)</th>
<th>Blood pressure at admission (mmHg)</th>
<th>Plasma renin activity (ng/ml per h)</th>
<th>Plasma aldosterone (standing) (ng/dl)</th>
<th>Urinary aldosterone (µg/24 h)</th>
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<tr>
<td>A</td>
<td>59</td>
<td>M</td>
<td>3·8</td>
<td>165/110</td>
<td>0·2</td>
<td>47</td>
<td>9·2</td>
</tr>
<tr>
<td>B</td>
<td>45</td>
<td>F</td>
<td>2·2</td>
<td>170/100</td>
<td>0·001</td>
<td>21·8</td>
<td>11·9</td>
</tr>
<tr>
<td>C</td>
<td>56</td>
<td>F</td>
<td>3·5</td>
<td>165/105</td>
<td>0·3</td>
<td>23·1</td>
<td>20·1</td>
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<td>D</td>
<td>28</td>
<td>F</td>
<td>3·0</td>
<td>160/110</td>
<td>0·3</td>
<td>39·7</td>
<td>25·3</td>
</tr>
<tr>
<td>E</td>
<td>35</td>
<td>M</td>
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<td>170/110</td>
<td>0·2</td>
<td>31·2</td>
<td>34</td>
</tr>
<tr>
<td>F</td>
<td>38</td>
<td>M</td>
<td>2·2</td>
<td>170/120</td>
<td>0·2</td>
<td>47</td>
<td>26·1</td>
</tr>
<tr>
<td>G</td>
<td>54</td>
<td>M</td>
<td>3·2</td>
<td>180/100</td>
<td>0·2</td>
<td>18</td>
<td>39·8</td>
</tr>
<tr>
<td>H</td>
<td>39</td>
<td>M</td>
<td>3·6</td>
<td>180/120</td>
<td>0·1</td>
<td>36·9</td>
<td>8·1</td>
</tr>
</tbody>
</table>
Technologies, Inc., Gaithersburg, MD, USA) were added to obtain a final volume of 25 µl. Incubation was performed for 1 h at 38 °C.

**Extraction of genomic DNA**

Genomic DNA was isolated from the blood samples of 80 control subjects with a commercially available DNA extraction kit (QIAamp DNA Blood Midi kit; QIAGEN, Hilden, Germany). The quantity of DNA was evaluated by spectrophotometry and gel analysis.

**Northern blot analysis**

Aliquots of 15 µg total RNA were resolved by electrophoresis on 1% agarose gel under denaturing conditions (formamide/formaldehyde) and triplicate blots were performed as previously described (Sarzani et al. 1999). In brief, nucleic acid was transferred to nylon membrane (Hybond-N+; Amersham Pharmacia Biotech), cross-linked by ultraviolet irradiation and baked at 80 °C. Membranes were hybridized with 32P-labelled cDNA fragments corresponding to Npr1 exons (2, 3 and 4,

*Figure 2* (A) Macroscopic section of an aldosterone-secreting adrenal adenoma (patient H); the tumour is sharply demarcated and yellow-orange; the remaining adrenal gland is grossly normal. (B) Histology of the section shows tumour cells with a nesting pattern and lipid-rich cytoplasm (courtesy of M Scarpelli).
Table 2 Primer pairs used for amplification of the human NPRA cDNA

<table>
<thead>
<tr>
<th>Primer pair no.</th>
<th>Exons amplified</th>
<th>Position</th>
<th>Sequence</th>
<th>cDNA fragment size (bp)</th>
<th>Annealing (°C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (5'-UTR)</td>
<td>719–739</td>
<td>5'-GCCTGATGCTGCTGGACGGGACG-3'</td>
<td>493</td>
<td>62</td>
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<tr>
<td></td>
<td></td>
<td>1211–1185</td>
<td>5'-CGGGACCGCCAGTGGCGGTGAAGCG-3'</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>807–833</td>
<td>5'-ATGCCGCGGCCCCGCGGCCGGCTGGT-3'</td>
<td>405</td>
<td>62</td>
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<td></td>
<td>1211–1185</td>
<td>5'-CGGGACCGCCAGTGGCGGTGAAGCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1162–1180</td>
<td>5'-TGTACGCCGCGCCAGCAGT-3'</td>
<td>366</td>
<td>70</td>
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<tr>
<td></td>
<td></td>
<td>1527–1509</td>
<td>5'-CTCGGCCCTTTGCGCGCCAAGC-3'</td>
<td></td>
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<tr>
<td>4</td>
<td>2–3–4</td>
<td>1473–1493</td>
<td>5'-GACGAACCTCAGCCACTACACC-3'</td>
<td>543</td>
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<td></td>
<td></td>
<td>4233–4211</td>
<td>5'-ATCGCCACTGCTATCAATTTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5–6–7–8</td>
<td>3476–3499</td>
<td>5'-TGGGGGAACTGTTACTGAGGGA-3'</td>
<td>523</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7531–7508</td>
<td>5'-TACAGGGAGCCGTTAATGGAGCC-3'</td>
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<td></td>
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<tr>
<td>6</td>
<td>9–10–11–12</td>
<td>6717–6740</td>
<td>5'-GGACGTGAGCCCATGAGCCTTG-3'</td>
<td>429</td>
<td>64</td>
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<td></td>
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<td>8926–8903</td>
<td>5'-CAGATAGCCCCATTGTAGAAAC-3'</td>
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<tr>
<td>7</td>
<td>13–14–15</td>
<td>8755–8778</td>
<td>5'-GTACTCACAACATGACATCTG-3'</td>
<td>541</td>
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<tr>
<td></td>
<td></td>
<td>10 676–10 653</td>
<td>5'-CAGTTGCTCCAGGTGTTGCTC-3'</td>
<td></td>
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<tr>
<td>8</td>
<td>16–17–18</td>
<td>9856–9879</td>
<td>5'-GGACCACAGGAGAGCCACCATT-3'</td>
<td>439</td>
<td>60</td>
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<tr>
<td></td>
<td></td>
<td>11 537–11 514</td>
<td>5'-CCCTGACACCACTAAGTGGATC-3'</td>
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<tr>
<td>9</td>
<td>19–20–21–22</td>
<td>11 185–11 208</td>
<td>5'-GCTGTCAATAGACACACATTGATG-3'</td>
<td>487</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 124–15 101</td>
<td>5'-GTTTGAAGGAGATAGGAGGAGG-3'</td>
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<tr>
<td>10</td>
<td>22 (5'-UTR)</td>
<td>15 049–15 069</td>
<td>5'-CGGACCTACTGGCTCCTGGG-3'</td>
<td>490</td>
<td>62</td>
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<td></td>
<td></td>
<td>15 538–15 515</td>
<td>5'-TTGTGGCAAGGCAGCCCGAGATG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>22 (5'-UTR)</td>
<td>15 427–15 447</td>
<td>5'-GCTTGGATCTACGCTTGACCTA-3'</td>
<td>290</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 711–15 691</td>
<td>5'-TTCTGGCAACCTCCTCTCGCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>(3'-UTR)</td>
<td>15 622–15 639</td>
<td>5'-TGTATGCGCTCTTCCTCAGT-3'</td>
<td>189</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 810–15 793</td>
<td>5'-GTTAAGAATCGAGAAGCT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

UTR, untranslated region. Position number according to GenBank accession number AF190631.
amplified by primer pair number 4, see Table 2) under stringent conditions in a hybridization oven (HB-1 Hybridiser; Techne Ltd, Cambridge, Cambs, UK) at 68 °C. The radioactive probes were prepared with a Rediprime DNA labelling kit (Amersham Pharmacia Biotech) and Redivue \([\alpha-^{32}P]\)dCTP (10 mCi/ml, 3000 Ci/mmol; Amersham Pharmacia Biotech).

Membranes were washed and autoradiographed by standard procedures. Blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 4 h to 4 days at −80 °C. Bound cDNA probes were removed by boiling for 10 min in 0·1% SDS, and the same membranes were re-hybridized with a control probe synthesized from a \(\beta\)-actin cDNA.

A semiquantitative analysis of NPRA expression was performed using arbitrary density units (ADU) of the housekeeping \(\beta\)-actin gene.

**Amplification of Npr1 cDNA**

All 22 exons were amplified by PCR using the primers shown in Table 2. Primers were designed to amplify partially overlapping portions of NPRA cDNA (Fig. 1B). Each primer pair was used to amplify three or four exons, whereas exon 1, because of its length, was amplified with three primer pairs.

PCR was carried out in a total volume of 50 µl, from 50–100 ng cDNA or genomic DNA, 10 × reaction buffer \([\text{KCl}, 5 \text{ mmol/l; MgCl}_2, 150 \mu\text{mol/l;}

Tris–HCl, 1 mmol/l, pH 9), dNTPs (0.2 mmol/l each), Taq (2.5 U; Amersham Pharmacia Biotech), with 50 ng of each primer. Thirty-five amplification cycles were performed, consisting of denaturation at 95 °C for 1 min, annealing (see Table 2) and elongation at 72 °C for 1·5 min.

An initial denaturation step was carried out at 95 °C for 5 min and a final elongation was carried out at 72 °C for 7 min. These amplifications were performed using the DNA Thermal Cycler (Perkin Elmer Cetus) and PCR products were analysed by gel electrophoresis using 2% Seakem LE agarose (BioWhittaker Molecular Applications, Rockland, ME, USA).

Direct sequencing

The PCR products were purified by agarose gel electrophoresis and eluted using QIAquick Gel Extraction Kit (QIAGEN). Cycle sequencing (96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min, for 25 cycles) was carried out on both strands using DNA sequencing kit Big Dye terminator Cycle sequencing Ready Reaction kit (Applied Biosystems, Warrington, Cheshire, UK) and the thermal cycler PCR Sprint (Hybaid). The reactions were purified by Centri-Sep Spin Columns (ABI PRISM; Applied Biosystems, Foster City, CA, USA), dried, resuspended in TSR buffer (ABI PRISM; Applied Biosystems) and subjected to automated capillary sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Sequences were analysed on electropherograms with Sequencing Analysis (ABI PRISM 3·3 software; Applied Biosystems) and Sequence Navigator software for Macintosh.

Results

The NPRA gene was expressed in all aldosteronomas analysed as shown by PCR (data not shown) and Northern blot analysis (Fig. 3A); we also evaluated, by Northern blot analysis, the NPRA expression levels which were similar among the adenomas studied (Fig. 3B). A representative PCR of eight different portions of the Npr1 cDNA is shown in Fig. 4. We amplified and sequenced a total of 3988 Npr1 cDNA bp from each aldosteronoma. Npr1 open reading frame sequences obtained from the eight adenomas studied did not contain any mutation, being identical among all aldosteronomas, control kidney tissue and deposited sequence (GenBank, accession number

**Figure 4** PCR products obtained from eight of the twelve primer pairs. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. M.W.M., molecular weight markers (\(\Phi\times174\)RF Hae III digest).
Moreover, no mutations were detected with the first primers pair (Table 2) which spanned nucleotides –88 to 405 of the 5′p9′-UTR encoded by exon 1.

A length difference 3C/4C polymorphism was detected with primer pair number 10 at position 15 129 in the 3′p9′-UTR, encoded by exon 22, 30 bp downstream of the stop codon (Fig. 5). Sequencing analysis revealed that patients D and F were 3C/3C homozygous, whereas patients A, B, C, and the kidney sample were 3C/4C heterozygous and, finally, patients E, G and H were 4C/4C homozygous. To analyse whether this 3C/4C polymorphism was a somatic mutation in the adenomas or a polymorphic genomic site, we amplified and sequenced genomic DNA extracted from 80 healthy control subjects. This 3C/4C polymorphism was present in genomic DNA with a frequency of 50% for the heterozygous genotype, 31·25% for the homozygous (4C/4C) genotype and 18·75% for the homozygous (3C/3C) genotype, according to the Hardy–Weinberg equilibrium, indicating the hereditary genomic nature of this polymorphism.

**Discussion**

Some endocrine tumours arise from gain-of-function mutations of key proteins (receptors or coupling proteins) that stimulate hormone secretion and cell growth. We hypothesized that, as a mirror image, aldosteronoma might arise from loss-of-function mutation(s) of key inhibitory proteins. Thus, we analysed eight human aldosteronomas searching for somatic mutation(s) in the *Npr1* gene. This gene is a likely candidate in the molecular pathogenesis of aldosteronomas because it mediates the potent ANP-dependent inhibition of glomerulosa adrenal cell secretion and growth (Higuchi et al. 1986b). The full-length direct double-strand sequence of the cDNAs synthesized from eight...
aldosteronomas did not reveal any somatic mutation in \textit{Npr1} exons. On the contrary, a remarkable stability of this sequence was found.

The only polymorphic site was a genomic length difference 3C/4C polymorphism at position 15 129 in the 3'-UTR (encoded by exon 22). The functional significance of this polymorphism in aldosteronomas is unknown and, because of its frequency in healthy subjects, it appears highly unlikely that it is a cause of aldosteronoma. It might affect mRNA stability (Misquitta et al. 2001) resulting in lower receptor synthesis (Knowles et al. 2003) but it is not likely that it confers a predisposition to the development of aldosteronomas (Pitzalis et al. 2003).

The only coding sequences that were not analysed were the starting 21 bp of the published full-length sequence (GenBank, accession number AF190631) due to upstream primer annealing. Because NPRA was normally and equally expressed in aldosteronomas (Fig. 2), it is also unlikely that somatic mutations in the regulatory regions of the gene were present.

Our results suggest that NPRA gene somatic mutation is unlikely to be a common cause of aldosteronomas, even though the apparent lack of inhibition of aldosterone secretion by natriuretic peptides remains to be explained. Other genes involved in the post-receptor mediation of the biological effect of natriuretic peptides might be involved, and post-translational defects cannot be excluded.

Key proteins involved in steroidogenesis as well as the multiple endocrine neoplasia type 1 gene (Zenkert et al. 2000, Zwermann et al. 2000) have also been excluded as causes of aldosteronomas. One case of a somatic \textit{G}_{\alpha} mutation of uncertain functional significance among 30 functional adenomas was recently reported (Kobayashi et al. 2000). Overall, the search for functional somatic mutations as a cause of aldosteronomas is still wide open.

References


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