Absence of angiotensin II type 1 receptor gene mutations in human adrenal tumors

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Abstract

Regulatory actions of angiotensin II (AngII), which is involved in the pathophysiology of hypertension and also participates in cell proliferation and cell differentiation, are mainly mediated by AngII type 1 (AT1) receptor. Recently, activating mutations of receptors causing hyperfunctioning endocrine diseases have been described in the case of the TSH and LH receptors, implicating that such mutations might occur in other G-protein-coupled receptors. Furthermore it seems to be possible that genetic variations of AT1 receptor have an influence upon the action of AngII. Therefore, we searched by sequence analysis of the coding region of AT1 receptor gene for activating mutations and genetic polymorphisms in 56 human adrenal tumors (16 aldosterone-producing adenomas, 10 cortisol-producing adenomas, 1 aldosterone-producing carcinoma, and 29 incidentalomas). We were not able to identify any activating mutation in the coding region of AT1 receptor gene. We conclude that activating mutations of the AT1 receptor are not a major cause of the development of adrenal adenomas, if at all. In addition, polymorphic subtypes of AT1 receptor do not seem to play a major role in the pathogenesis of these tumors, even though a tendency towards a higher frequency of the polymorphic base substitution at position 573 (T573→C) in cortisol-producing tumors needs to be further evaluated.

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Introduction

Several lines of evidence suggests that angiotensin II (AngII) is not only a steroidogenic and vasoactive peptide but also a growth factor-like agent which can induce hypertrophy as well as hyperplasia on target cells through AngII type 1 (AT1) receptor activation (1, 2). Expression of a local renin–angiotensin system and AngII formation has been revealed in human adrenals and in aldosteronomas (3). In addition, AT1 receptor expression has been found in human adrenals and discordantly increased expression of AT1 receptor in functioning adrenal tumors also has been shown (4). In particular, the AT1 receptor proved to be activated in human aldosteronomas (5, 6). Therefore, AT1 receptors, besides stimulation of aldosterone secretion, may also transduce the growth factor-like activity of AngII on adrenal cells.

In the case of thyroid-stimulating hormone (TSH) and luteinizing hormone (LH) receptors constitutive activations caused by mutations in the third cytoplasmic loops, which are known to interact with G proteins and activate intracellular signaling, have been revealed to result in hyperfunctioning endocrine diseases (7, 8).

In this regard, to explain the discordantly increased expression of AT1 receptor in functioning adrenal adenomas, the third cytoplasmic loops of AT1 and adrenocorticotropic hormone (ACTH) receptors were examined in 20 various adrenocortical tumors without detection of any mutation (4). Also, analysis of the coding region of AT1 gene by PCR-SSCP method did not identify any mutation in 19 aldosteronomas, 1 adrenal carcinoma and 1 incidentaloma (9).

Genetic polymorphisms of AT1 gene have been described (10, 11), and one of those appears to be an important risk predictor for arterial stiffness in hypertensive subjects (12). It seems to be possible that genetic variations of AT1 have an influence upon the action of AngII.

In this study we examined DNAs from 56 human adrenal tumors (16 aldosterone-producing adenomas, 10...
cortisol-producing adenomas, 1 aldosterone-producing carcinoma, and 29 incidentalomas) for possible activating mutations or genetic variations of AT1 by sequence analysis of the coding region of AT1 gene. Our results indicate that mutations of AT1 gene are rarely, if ever, involved in the pathogenesis of adrenal tumors. In addition, we did not find any significant hint for particular typical polymorphic subtypes of AT1 receptor, even though a tendency towards a higher frequency of the polymorphic base substitution at position 573 (T573→C) in cortisol-producing tumors needs to be further evaluated.

**Materials and methods**

**Tissues and preparation of DNA**

Eighteen fresh surgical specimens of adrenal tumors and 38 specimens of paraffin-embedded sections of adrenal tumors were obtained from the Department of Surgery, and the Pathology Division of the University of Erlangen respectively. Histological identification of these tumors was performed in the Pathology Division of the University of Erlangen. Characterization of different tumors is shown in Table 1. High molecular weight DNAs from the fresh surgical specimens and from the paraffin-embedded sections of adrenal tumors after deparaffination with xylene were prepared according to the method of Blin and Stafford (13).

**PCR amplification of AT1 gene**

Primers were designed on the basis of the published data for the gene (14–17) to amplify the entire coding region of AT1 gene into four overlapping fragments (Table 2 and Fig. 1). In addition to the specific amplification sequence, forward primers also contain the M13 universal 5'-extension (TAAAACGACGGCCAGTCAG), and reverse primers contain the universal reverse M135'-extension (CAGGAAACAGCTATGACC). DNA samples (0.1 μg) were subjected to PCR in a 50 μl volume under the conditions described previously (10). Thirty-five cycles of the reaction at 95°C, 56°C and 72°C for 30 s, 45 s, and 45 s respectively, were performed in a DNA Thermal Cycler (Perkin-Elmer-Cetus, Überlingen, Germany). In the case of paraffin-embedded tumor tissue 1 μl of the PCR product of fragment 1 was reamplified by semi-nested PCR under the same conditions as described above.

**Direct DNA sequencing**

The reaction mixture of the PCR was diluted and deionized by a QiAQuick Kit (Qiagen, Hilden, Germany) and PCR products were subjected to direct automated sequence analysis by cycle sequencing (Taq Dye primer Cycle Sequencing Kit, Applied Biosystems, Forster, CA, USA) as described previously (10). Fluorescence dye-labeled sequencing products were analyzed using the ABI 373A machine (Applied Biosystems). Sequencing data were analyzed by the GeneWorks computer program (IntelliGenetics, UK).

**Results**

**PCR amplification of AT1 gene**

The entire coding region of AT1 gene was amplified into four overlapping fragments (Fig. 1) in 18 fresh surgical specimens of adrenal tumors. Representative results of agarose-gel electrophoresis of PCR products are shown in Fig. 2. Lane M contains size standard, lanes 1 to 4 contain fragments 1 to 4 from the fresh surgical specimen of tumor 1 (see later Table 3). In paraffin-embedded sections of adrenal tumors this PCR amplification of AT1 gene did not result in sequenceable products. Hence, in 38 specimens of paraffin-embedded sections of adrenal tumors we reamplified fragment 1 into two overlapping fragments (Fig. 1) by semi-nested PCR.

**Direct DNA sequencing of PCR products**

PCR products were subjected to direct sequencing. Polymorphic sites in AT1 gene, detected in leukocyte DNA from healthy subjects, are described at base pair
positions 9 (C9→A), 16 (T16→C), 87 (C87→A), 133 (G133→A), 186 (G186→A/C), 573 (T573→C), and 1062 (A1062→G), with two of them causing an exchange of the amino acid (amino acid codon 6: Ser→Pro, amino acid codon 45: Gly→Arg) (10, 11). In 18 fresh surgical specimens of adrenal tumors we found the original described sequence (14–17) at positions 9, 16, 87, 133, and 186. In no case were we able to identify any polymorphic base substitution at these positions. Results of sequence analysis at polymorphic sites 573 and 1062 are listed in Table 3. Frequencies of different alleles at position 573 differ in aldosteronomas and cortisol-producing adenomas with a tendency towards a higher frequency of the base C at this position in cortisol-producing adenomas (Fig. 3). Since the polymorphic base 1062 is located only seven base pairs upstream from the 3’-end of the reverse primer P8 we were not able to read the sequence at this position in all cases. We did not identify any mutation in the entire coding region of AT1 gene in 18 fresh surgical specimens of adrenal tumors.

In 38 specimens of paraffin-embedded sections of adrenal tumors we sequenced fragment 1 including polymorphic sites 9, 16, 87, 133, and 186 after reamplification into fragments 1a and 1b. At polymorphic site 16 we found homozygous T in 36 cases, homozygous C and heterozygous T/C in one case each (one aldosterone-producing adenoma, and one cortisol-producing adenoma respectively), resulting in an amino acid exchange Ser to Pro. This mutation has been
described previously as a genetic polymorphism found from healthy subjects and patients with hypertension, coronary heart disease or cardiomyopathy (10). It is located outside the gene regions characterized as being important for signal transduction. In positions 9, 87, 133, and 186 we were not able to identify any polymorphic base substitution. We did not identify any activating mutation in fragment 1 including codons 1 to 93 of AT1 gene in 38 specimens of paraffin-embedded sections of adrenal tumors.

**Discussion**

The molecular genetic events underlying the development of human adrenal neoplasm are still not well understood. Adrenocortical neoplasms were recently shown to be monoclonal in composition, leading to the hypothesis that they may arise by spontaneous transformation of adrenocortical cells because of somatic mutations (18, 19). Point mutations in guanine nucleotide-binding proteins were reported in two of ten adrenocortical adenomas, suggesting a potential role of this isoform in the genesis of these tumors (20). However, these findings could not be confirmed in two subsequent studies, concluding that these mutations are not determinant for human adrenocortical tumorigenesis (21, 22). Mutations of p53 tumor suppressor gene are important in the carcinogenesis of adrenocortical cancer, but do not seem to play a major role in the formation of adrenal adenomas (23). This result was supported by the finding of a consistent loss of heterozygosity (LOH) at chromosomal locus 17p in adrenal carcinomas but not in adrenal adenomas. Allelic losses at the 17p locus were shown in all adrenocortical carcinomas, whereas LOH at 11p and 13q loci were found in only half of the malignant tumors (24).

AngII has been shown to be involved in the pathophysiology of hypertension and also participates in cell proliferation and cell differentiation. Its regulatory actions are mediated by two pharmacologically distinguishable classes of G-protein-coupled cell-surface receptors (AT1 and AT2), of which the AT1 class has been now regarded as a mediator of the major pathophysiological actions of AngII (2). In the adrenals, locally formed AngII might play an important role as an autocrine or paracrine regulator of adrenal function, whereas an abnormal activity of the local renin–angiotensin system might be involved in the pathogenesis of abnormal adrenal growth, abnormal mineralocorticoid production, and hypertension (25). Therefore, similar to the constitutive activation of G-protein-coupled TSH and LH receptors by somatic mutations (7, 8), a constitutive activation or a genetic variant of AT1 receptor could lead to increased AngII actions on target cells. Sequence analysis of the third loops of AT1 and ACTH receptors, however, did not reveal any mutation in 20 different adrenal tumors (4). In addition, in two subsequent studies PCR-SSCP analysis of the coding region of AT1 gene was not able to detect any activating mutation in 17 (26) or 19 (9) aldosterone-producing adenomas. Although PCR-SSCP analysis is efficient, it may not detect all possible nucleotide substitutions and cannot rule out false-negative cases (27, 28). Therefore, in this study we analyzed AT1 DNA in 56 adrenal tumors by sequence analysis. We did not identify any activating mutation in the whole coding region of AT1 gene in DNA from 18 fresh surgical specimens and in codons 1 to 93 of AT1 gene in DNA from 38 paraffin-embedded sections of different adrenocortical tumors. We conclude that activating mutations of the AT1 receptor are rarely, if ever, involved in the pathogenesis of human adrenal tumors. In addition, we did not find any significant hint for typical polymorphisms in different adrenal tumors in the coding region of AT1 gene. However, the different frequency of the two polymorphic alleles at position 573 (T573 → C) in adosteronomas and cortisol-producing adenomas requires further investigation. Another explanation for the discordantly increased expression of the AT1 receptor in functioning adrenal adenomas (4), and in particular the activation of the AT1 receptor in aldosteronomas (5, 6), might be found in the promoter region of AT1 gene. The recent sequencing of the promoter region of the human AT1 receptor (29) will enable this region of the gene to be studied, which will yield additional information regarding the expression and regulation of this gene in patients with functioning adenomas.

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References


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