Pathophysiological roles for cardiac steroid hormone biosynthesis

ホルモン産生腺腫の成因に関する遺伝子学的研究

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内分泌臓器によるホルモン産生腫瘍の成因に関して近年遺伝子解析も含め多方面から研究がなされ、家族性甲状腺機能亢進症の一部にG蛋白結合レセプターファミリーであるTSHレセプターの遺伝子異常や、成長ホルモン産生下垂体腺腫の一部にG蛋白の遺伝子異常が報告されているが、多くは不明である。原発性アルドステロン症はアルドステロン過剰産生、高血圧、低カリウム血症を示す疾患であり、心疾患等の合併症を高率に有し、予後不良である。その中でアルドステロン産生腺腫は70%以上を占めるが、その成因は不明である。本研究においてアルドステロン産生腺腫(APA)及び特発性アルドステロン症におけるアルドステロン合成酵素遺伝子(CYP11B2)の異常を検討するためにDNAを抽出し、PCRにて増幅後直接シークエンス法によるDNA解析を行ったが、異常が見られなかった。またグルココルチコイド抑制性アルドステロン症の原因遺伝子であるCYP11B1/CYP11B2キメラ遺伝子の有無について検討したが、APA、IHAにおいて異常な遺伝子は検出されなかった。アンジオテンシンIIレセプター遺伝子や副腎腺腫を合併する多発性内分泌腺腫症I型の原因遺伝子であるMENIN遺伝子に関してもシークエンスを行ったが、異常が発見できなかった。APA、非機能性副腎腺腫、正常副腎組織におけるCYP11B2 mRNAの発現を競合的PCR法にて定量を行うとAPAではCYP11B2 mRNAの発現が高値を示した。しかしIHA、APA、非機能性副腎腺腫を有する患者から単球を分離し、単球におけるCYP11B2 mRNAの発現を同様に競合的PCR法により検討すると、IHAにおいてCYP11B2 mRNAの発現が有意に増加していた。アルドステロン産生腺腫に特異的に発現する遺伝子を解明すべく蛍光ディファレンシャルディスプレイ法を用いて検討した。候補遺伝子をスクリーニングし、ノーザンブロットによるメッセンジャーRNAの発現を検討するとアルドステロン産生腺腫で強く発現し、原因遺伝子の一つである可能性が示唆された。

研究組織

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研究発表

(1) 学会誌 (Yoshiyu Takeda, Isamu Miyamori, et al. Genetic analysis of aldosterone synthase in patients with idiopathic hyperaldosteronism. J Clin Endocrinol Metab 84巻, 1999年5月)

(2) 口頭発表 (武田仁勇、宮森勇、他　特発性アルドステロン症患者の単球におけるアルドステロン合成酵素遺伝子CYP11B2メッセンジャーRNAの発現について　第72回日本内分泌学会　1999年6月1日)
(Yoshiyu Takeda, Isamu Miyamori, et al. Genetic analysis of aldosterone synthase in patients with idiopathic hyperaldosteronism. 第81回アメリカ内分泌学会, 1999年 6月 13日)

Genetic Analysis of Type 1 Angiotensin II Receptor of Aldosterone-Producing Adenoma

Introduction

Primary aldosteronism is defined as overproduction of aldosterone independent of its normal chronic regulator, angiotensin II. In practice, plasma renin or plasma renin activity is a more robust and therefore more clinically useful measure than angiotensin II. Since aldosterone is being produced independently of angiotensin II conncetrations, resulting salt and water retention and hypertension will suppress renin/angiotensin. Suppressed renin concentrations are the hallmark of primary aldosteronism. Once aldosterone production is a autonomous and increasing, there is a tendency for blood volume to expand and for blood pressure to increase and the normal positive regulations of renin secretion are turned off. The resulting fall in renin is therefore an early sign of evolving primary aldosteronism., long before the aldosterone finally rises out of the normal range.

The most common clinical subtypes of primary aldosteronism are aldosterone-producing adrenocortical adenoma (APA) and bilateral adrenal cortical hyperplasia. Bilateral adrenal cortical hyperplasia is sometimes micronodular but occationally macronodular or apparently unilateral. Adrenal carcinoma is very rare and only a few such carcinomas produce aldosterone in excess (1).

Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system, exerts a wide variety of actions on the cardiovascular, renal, endocrine, metabolic and neuronal systems. The existence of two distinct subtypes of Ang II receptors, type 1 (AT1) and type 2 (AT2), has been confirmed using their selective antagonists. The AT1 receptor is now regarded as the major mediator of Ang II effects on blood pressure, fluid volume and adrenal aldosterone secretion (2).

Recently, it has been revealed that some hyperfunctioning endocrine diseases are caused by constitutive activation of G protein-coupled receptor by mutations in the third cytoplasmic loops, which are known to interact with G proteins and activate intracellular signaling (3). In the present study, to clarify this issue, we investigated the prevalence of germline and somatic mutations of human AT1 receptor gene in APA as well as the relative messenger RNA (mRNA) expression of AT1 receptor gene.

Subjects and Methods

Subjects

Ten patients with (4 male and 6 female, ages 35 to 62 years) were studied. All patients were diagnosed from characteristic biochemical abnormalities including hypokalemia, suppressed PRA, and increased aldosterone production. GRA was excluded by administering 2 mg/day of dexamethasone to patients for 3 days. If dexamethasone failed to suppress plasma aldosterone to the normal range and serum potassium and blood pressure did not normalize, a clinical diagnosis of primary aldosteronism was made. High concentrations of plasma aldosterone in the adrenal vein and presence of a solitary adenoma on CT scan of the adrenal glands confirmed a diagnosis of APA. Serum potassium concentrations (2.6 ± 0.3 mmol/L) were low and PRA (0.61 ± 0.17 ng/L·s) were seppressed, and plasma aldosterone concentrations (1530 ± 290 pmol/L;) were high in APA.

Competitive polymerase chain reaction assay AT1 receptor mRNA

Total cellular RNA isolated from aldosteronoma and adjacent adrenal tissues was amplified by a reverse-transcribed polymerase chain reaction (RT-PCR) as previously described (4). Briefly, 500 ng of total RNA was incubated at 42°C for 60 min with 2.5 U of M-MLV reverse transcriptase (Takara, Tokyo, Japan) in a 20 μL reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, each dNTP at 1 mM, and 2.5 mM random hexanucleotide primer (Takara). This mixture was incubated for 5 min at 99°C. The single-stranded cDNA was used for competitive PCR. The sequences of sense and antisense primers for AT1 receptor gene were 5'-AGATGATTGTCCCAAAGCTG-3' and 5'-GCTTCTTGGTGGATGAGCTT-3', following the sequences published by Takayanagi et al. (5). The competitive templates for AT1 receptor gene were made as previously described (6). After quantification, a serial dilution was used as an internal standard for competitive PCR, which was performed using 2.5 μL of the reverse-transcribed DNA, 2 μL of various concentrations of the competitive template, 0.5 μM each of sense and antisense primers, and 0.5 U of Taq DNA polymerase (Perkin-Elmer Japan, Tokyo, Japan) in 50 μL of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2

mM $MgCl_2$ with each dNTP at 0.2 mM. The reactions were performed for 1 min at 94°C, 1 min at 59°C and 2 min at 72°C for 30 cycles. Aliquots of 10 μL of amplification products were electrophoresed on a 3.0% agarose gel, which then was stained with ethidium bromide and photographed. Staining intensity was quantified by computer-assisted densitometry using the BIO-PROFIL BIO-1D system (Compak, Tokyo, Japan). Intensities of each product from cDNA and those from competitive templates were plotted as a function of the known amounts of the competitive templates. Intraassay and interassay variabilities of this competitive PCR were 11.5% and 14.8%, respectively. The concentration of AT1 receptor gene mRNA was expressed as attomoles per 100 ng of total RNA. To test the yield and efficiency of the reverse transcriptase reaction, 1 μg of total RNA was subjected to reverse transcription as above, with 5 μmole/L of radioactively labeled [^{32}P] dCTP (New England Nuclear, Tokyo, Japan) added to the reaction as previously reported (7).

Southern blotting of RT-PCR products

The RT-PCR products were electrophoresed in 10-μL aliquots on a 3% agarose gel and transferred to nylon membranes. Hybridization was performed as previously reported (8), using an oligoprobe specific for AT1 receptor gene that had been end-labeled with [^{32}P]ATP (6000 Ci/mmol, New England Nuclear) using a 5'-end oligonucleotide labeling kit.

Sequence analysis of AT1 receptor gene

Genomic DNA was extracted from EDTA blood and tumor tissue of patients with APA as previously reported (9). Sequence analysis of the genomic and somatic AT1 receptor gene in patients with APA was performed by PCR amplification of exons using both intron- and exon-derived primers as previously reported (8). Primers for AT1 receptor gene were shown in table 1. PCR products were cycle-sequenced with Taq polymerase FS dye-terminator sequencing kits (Perkin-Elmer Japan, Tokyo, Japan) on a model 377 automated DNA sequencer (Perkin-Elmer Japan).

TABLE 1. Sense and antisense primers

Primer sequence	Position	
5'-CCCCAGGTGTATTTGAT-3'	-53	sense
5'-CTACAAGCATTGTGCGTCGA-3'	414	antisense
5'-TGTAAGATTGCTTCAGCCAGCG-3'	301	sense
5'-ATTTGGTGGGGAATCCAGGA-3'	755	antisense
5'-GGCCCTAAAGAAGGCTTATG-3'	661	sense
5'-CTGCAGAGGAATGTTCTCTTG-3'	1124	antisense

Results

The levels of mRNAs for AT1 receptor in APA

Figure 1 shows that increasing concentrations of each competitive template for AT1 receptor from 0 to 80 $\times$ 10^{-3} attomole/μL increasingly inhibited the amplification of endogenous AT1 receptor in aldosteronoma tissue. There were no significant differences in AT1 receptor mRNA levels between APA and normal tissue adjacent to APA.

Sequencing analysis of AT1 receptor gene in patients with APA

Figure 2 shows the data of sequencing of AT1 receptor gene in the tissue of APA. The conversion of C^{573} to T^{573} was detected in three aldosteronoma tissues. However, this change was polymorphism.

Discussion

In this study, we could not detect any mutations in AT1 receptor gene in aldosteronoma. Parma et al. (3) reported that somatic mutation in the third loop of TSH receptor in hyperfunctioning thyroid adenomas and have proposed an very interesting idea that constitutive activation of G protein-coupled receptor by mutations in some hyperfunctioning endocrine adenoma activate intracellular signaling. Shenker et al. (10) also reported a constitutive activating mutation of the luteinizing hormone receptor in familiar precocious puberty. Nawata et al. previously reported no mutation in the third loops of AT1 receptor gene (9). We analyzed the coding region

including the intron-exon junctions of AT1 receptor gene. We also could not find any differences of the expression of mRNA of AT1 receptor between aldosteronoma and normal tissue. Clinically, plasma aldosterone in patients with APA exhibits a poor response to administered Ang II compared with normal subjects. Our results appear consistent with this clinical observations.

Steroid 21-hydroxylase mutations in APA were reported by Beuschlein et al. (11). However, this mutation can not explain the phenotype of mineralocorticoid hypertension. Recently, Carroll et al. (12) have reported that APA do not contain glucocorticoid-remediable aldosteronism chimeric gene duplications. Further study is necessary to find candidate gene responsible for the clinical and biochemical phenotype of APA or are oncogenic.

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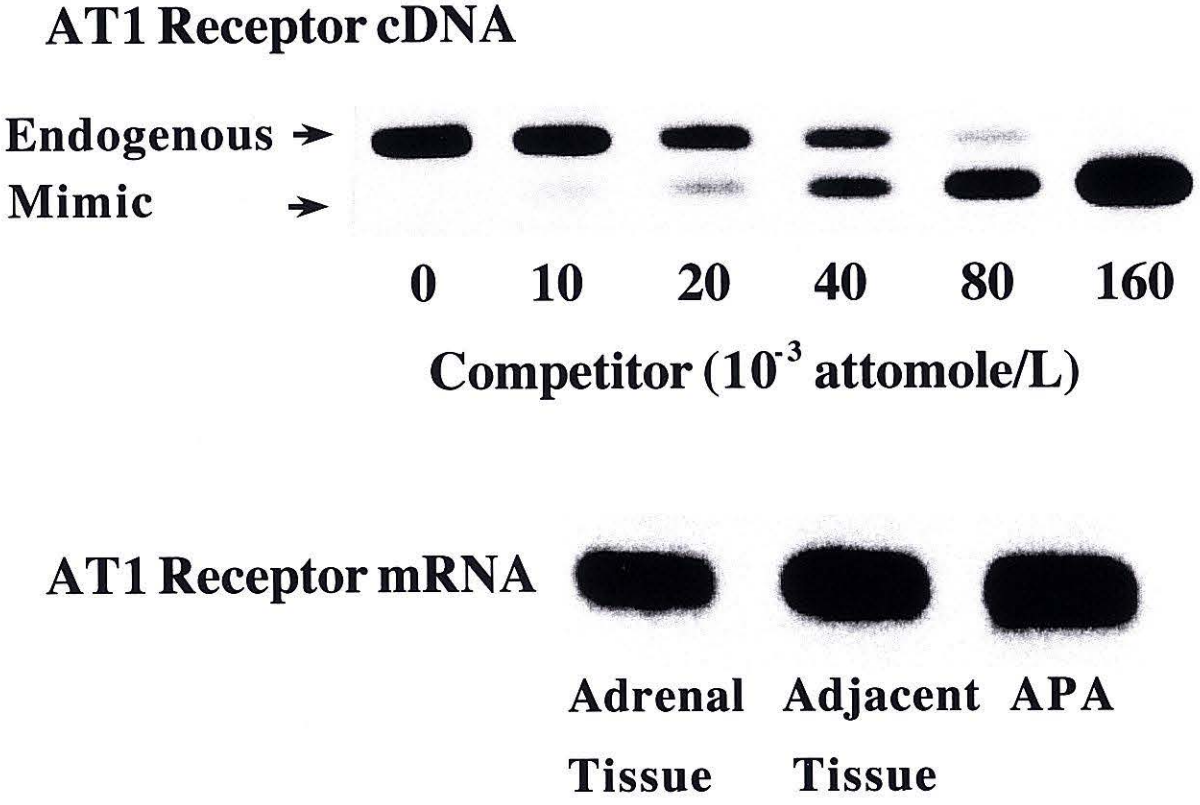

Figure 1. Upper panel shows the analysis of relative changes in AT1 receptor mRNA concentrations by competitive polymerase chain reaction. Increasing the concentration of competitive template for AT1 receptor from 0 to 160 $\times$ 10^{-3} attomoles/μL increasingly inhibited the amplification of endogenous AT1 receptor cDNA in the aldosteronoma. Lower panel shows that the expression of AT1 receptor mRNA using Southern blotting of reverse-transcriptase polymerase chain reaction in normal adrenal tissue which was obtained from a patient with renal cell carcinoma, adjacent adrenal tissue, and aldosterone-producing adenoma (APA). There were no significant differences of AT1 receptor mRNA in between adrenal tissue, adjacent tissue of aldosteronoma, and APA.

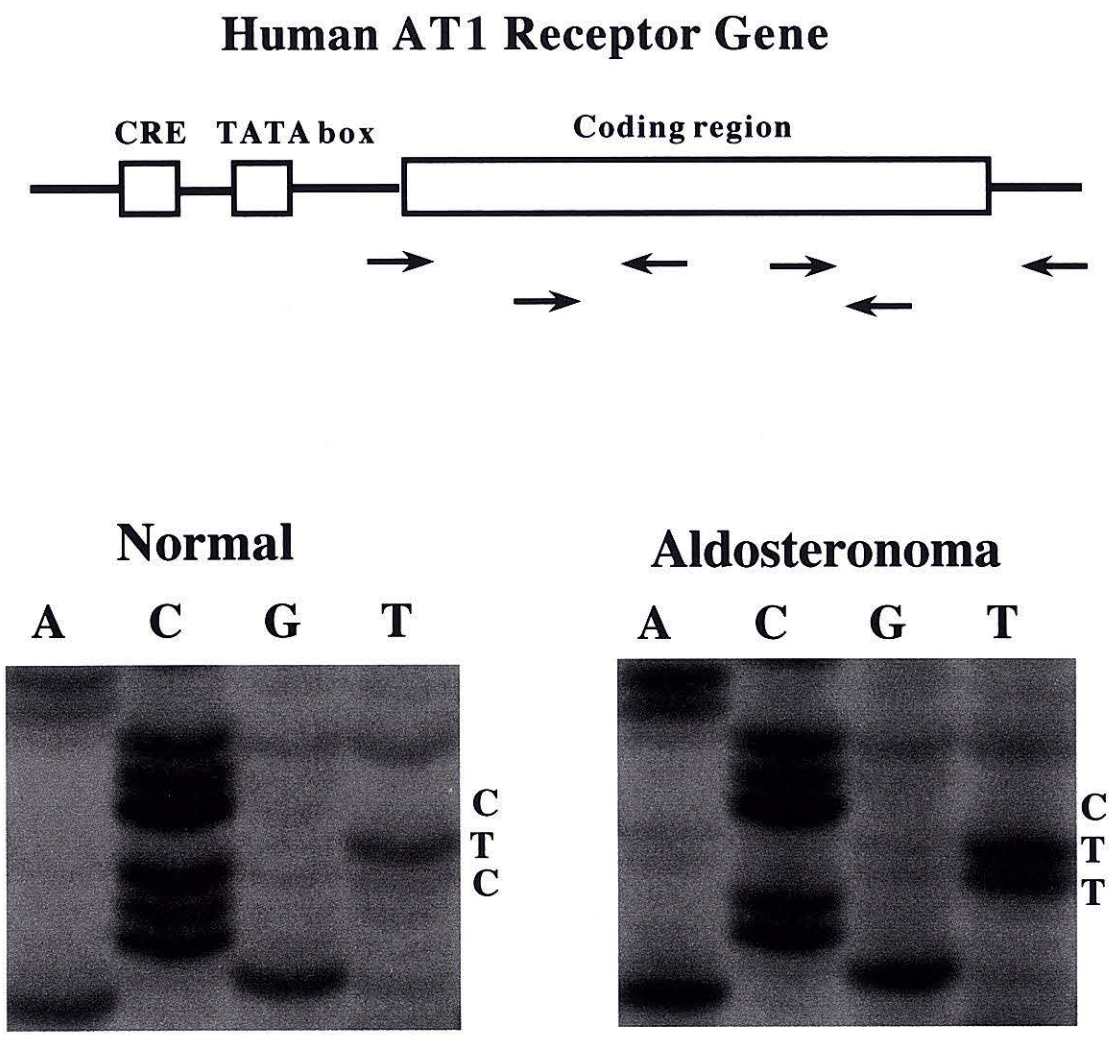

Figure 2. Schematic representation of coding region and the 5'-flanking region of human AT1 receptor gene. Arrows indicate each primer (upper panel). Lower panel shows the sequencing data of AT1 receptor gene in normal and aldosteronoma tissue. The conversion of C^{573} (Leu^{191}) to T^{573} (Leu^{191}) were detected.

Isolation of Preferentially Expressed Genes in the Aldosterone-Producing Adenoma

Introduction

The syndrome of primary aldosteronism is characterized by hypertension with excessive production of aldosterone, potassium loss and suppression of the renin-angiotensin system. Although primary aldosteronism is not a frequent cause of hypertension in unselected hypertensive patients, the distinction between surgically correctable subtypes can be accurately made in most cases. Over 70% of primary aldosteronism cases are due to a unilateral adenoma in Japan (1).

Glucocorticoid-suppressible hyperaldosteronism (GSH) is a disease in which the normal pattern of synthesis of the mineralocorticoid hormone, aldosterone, is disturbed. It is characterized by hypersecretion of aldosterone, in the face of a low plasma renin activity, and the production of two normally rare steroids, 18-hydroxycortisol and 18-oxocortisol, which require the simultaneous presence of a 17α-hydroxylase activity and the two C18 activities typical of the CYP11B2 enzyme. All synptoms of the disease are normalized by the administration of glucocorticoid analogues and are exacerbated by administration of ACTH (2). The genetic basis of this disease has been reported the presence of a hybrid CYP11B1/CYP11B2 gene segregating with the disease in a large pedigree (3). The hybrid gene is assumed to be transcribed in the zona fasciculata of the adrenal cortex under the control of ACTH, as a result of contanining 5'-sequences from CYP11B1, and to encode a protein with 18-hydroxylase and 18-oxidase activities, due to the presence of 3'-coding sequence from CYP11B2.

We tested whether a chimeric GSH-like gene was present in APA and found no such a mutation gene. Carroll et al. also reported the same result as ours (4). Gordon et al. have examined the abnormalities of renin, angiotensin II receptor, atrial natriuretic peptide and p53 genes in tumor and constitutive DNA in APA and have found no mutations (5).

In order to clarify the candidate gene in aldosteronoma, we isolated highly expressed gene I aldosteronoma compared with normal adrenal tissue or non-functioning adrenal adenoma using a fluorescent differential display method.

Materials and Methods

RNA isolation

Total RNA was extracted from aldosteronoma, normal adrenal tissue and non-functioning adrenal adenoma by using RNAzol B method. Samples were treated with Rnase-free DNase I (BRL, WI) to remove residual contaminating DNAs. RNA was dissolved in diethyl pyrocarbonate (DEPC)/deionized water, quantified and stored at –80 °C until use.

First strand cDNA synthesis

Total RNAs (2.5 μg) were mixed with 50 pmol of the Fluorescein isothiocyanate (FITC)-labeled 3'-anchored oligo-dT primer in 10 μL of DEPC-treated water, heated at 70 °C for 10 min and chilled by immersing tube in ice-water. To this solution, 10 μL of 2 × RT solution (1 × RT solution=20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM, $MgCl_2$, 10mM DTT, 0.1 mg/nL BSA and 0.5 mM each dNTPs) containing 200 uints of SuperScript II reverse transcriptase (BRL) was added, mixed well and incubated at 25 °C for 10 min and at 42 °C for 50 min. Following the incubation at 90 °C for 5 min, the reaction mixture was diluted 5-fold by addition of 80 μL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at –20 °C until use.

Polymerase chain reaction

The reaction mixture for the second strand synthesis and subsequent amplification was prepared in each tube containing 1 nmol of each dNTP, 1 unit of Taq DNA polymerase, 10 pmol of arbitary primer, 5 pmol of FITC-labeled 3'-anchored oligo-dT primer and 2 μL of DNA solution described above in 20 μL of 1 × PCR buffer supplemented with 1.5 mM $MgCl_2$. Thermal cycling protocol was done as previously described (6). Arbitrary decamers were obtained from Operon Technologies (CA).

Gel electrophoresis

Each PCR product was mixed with the same amount of dye solution (98% formamide, 10 mM EDTA and 0.01% Methyl violet), denatured at 90 °C for 2 min and chilled on ice. Two μL of each sample was applied to each well of a 6 % polyacrylamide/7M urea gel in 1 ×TBE buffer cast between the half-length gel plates. The gel was run on a HITACHI SQ-3000 DNA sequencer (Hitachi, Japan) as previously described (6). For the detection on a FluorImager 575 (Molecular Dymanics, CA), 5-6 μL of

samples containing the dye solution was applied to each well of a 0.35 mm thick polyacrylamide gel plate. Following the removal of upper glass plate, the gel was put on the sample tray and scanned at the high sensitivity mode.

Cloning of the bands interest

Since the initial analysis on the DNA sequencer revealed the size of the band of interest, one can choose such gel concentration that optimizes the separation of the target for preparative electrophoresis. The fingerprinting pattern is visualized by the FluorImager 575 and printed on a transparency film by its actual size, onto which the gel with lower glass plate was overlaid and the gel piece containing the band of interest was excised using a blade. The gel can be scanned to confirm the precise excision. For the re-amplification of the excised band, we found that the direct addition of the gel piece to a 50 μL PCR reaction gave satisfactory results. Neither tedious electroelution procedures nor even boiling steps were found necessary. Usually, 30 cycles of PCR yielded enough amount of the fragment for subsequent subcloning into a "T-vector" as well as direct sequencing.

Northern blot analysis

Total RNA (20 μg) was subjected to electrophoresis through a 1% agarose formamide gel and transferred to a Hybond-N^+ membrane (Amersham Japan) by the method previously reported (7). Probes were labelled by random priming in the presence of [α-^{32}P] dCTP with the Rediprime DNA labelling system (Amersham Japan) using the cloned candidate DNA. The membranes were prehybridized for 4 h then hybridized at 55 °C for 16 h. Filters were washed at high stringency at 55°C and autoradiography as previously reported (8).

Results

Ten cDNAs showing aberrant expression in aldosteronoma in comparison with the normal adrenal tissue or non-functioning adrenal adenoma were detected (data not shown). Northern blot analysis probed with one cDNA fragment confirmed that one gene was selectively overexpressed in aldosteronoma tissue compared with normal adrenal tissue or non-functioning adrenal adenoma (Fig 1).

Sequence analysis and data base search demonstrated that this gene

was a novel one (data not shown).

Discussion

The incidence of APA is reported to be about 10 %. Specific surgical or medical treatment of APA always results in significant improvement in hypertension. When treated with non-specific drugs, however, APA may go on to severe, resistant hypertension.

Gordon et al. have reported that the renin gene was overexpressed in the aldosteronoma tissue compared with normal adrenal tissues (9). They concluded that this phenomenon is to be expected in tumors of glomerulosa or hybrid morphology, in comparison with those of fasciculata morphology, since the components of the renin-angiotensin system have been demonstrated more readily in glomerulosa than fasciculata zones of the adrenal cortex. Of great interest in regard to etiology of APA is the observation of associations of APA with manifestations of multiple endocrine neoplasia (MEN) I such as hyperparathyroidism, pituitary tumors, carcinoids and multinodular goiter (10). Nodular adrenal cortical hyperplasia is common in MEN I. Recently the candidate gene for MEN I (menin) have been cloned (11). We analyzed the sequence of menin gene in tumor DNA and peripheral blood DNA of patients with APA and found no mutations in both DNA (unpublished data).

Dominantly inherited cancers are often associated with multiple genes. It is not unreasonable to suppose that there may be more than one genetic mutation which can predispose to hyperplasia and neoplasia of the adrenal cortex. Hyperplasia and neoplasia can also be the result of unrestrained stimulation, as in the thyroid in association with chronic iodine deficient diets. Such unrestrained stimulation could also result from defective genetic regulation of a second or third messenger system. When genetic change leads to hyperplasia or neoplasia of the adrenal cortex without recognized overproduction of steroid hormones, the resultant mass lesion is called an "incidentaloma or non-functioning adenoma". We have isolated a supposed candidate gene for aldosteronoma. Further study is necessary to clarify the expression of the recombinant protein and immunoblotting using the antibodies.

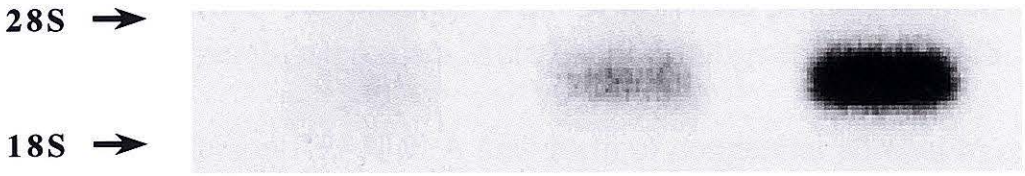

Figure 1. Northern blot analysis confirming up-regulated band of fluorescent differntial display. Total RNA (20 μg per lane) obtained from aldosteronoma (APA), normal adrenal tissue and non-functioning adrenal adenoma were subjected to Northern blot analysis using ^{32}P-labeled probe.

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