行政院國家科學委員專題研究計畫成果報告

計畫名稱：人類第四型多巴胺受器在腎上腺皮質醛酮瘤的表現及其多形性的臨床意義
計畫編號： NSC 88-2314-B002-234
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一、中英文摘要

臨床及實驗室的證據顯示，皮質醛酮的分泌受到多巴胺的抑制。雖然這種調節可能是經由類似第二型的多巴胺受器（dopamine receptor, DR**）的作用，但是並無研究證明是何種的** DR**。目前已知類似第二型的多巴胺受器至少有三種**: D2, D3, D4**。為探討是何種** DR **存在腎上腺的皮質，吾人以逆轉化-多聚胸鏈鎖反應法（**RT-PCR**），和原位雜交（**in situ hybridization**），來檢視腎上腺以及其腫瘤。吾人發現** D2 **和** D4 **表現在腎上腺的皮質和髓質；且兩者的分布並無明顯差異。在皮質的表現兩者主要在小球區（**zona glomerulosa**），少部份在網狀區（**zona reticularis**），但束狀區（**zona fasciculata**）則幾無訊號。**D3 **無論在皮質或髓直皆無表現。**
D2 **和** D4 **表現於嗜鉻細胞瘤（**pheochromocytoma**），且兩者在分布及量並無明顯差異。雖然這兩種受器也存在皮質醛酮瘤（**aldosterone-producing adenoma, APA**），但** D2 **的表現並不一致，有些** APA **並不表現** D2**。反之，**D4 **在所有的腎上腺和** APA **皆有明顯的表現。**D4 **和** D2 **在** APA **的表現主要是在類非束狀區細胞（**nonzona fasciculata-like cells**），這個發現符合吾人過去認為，**APA **細胞的類非束狀區細胞可能表現較多的** D2-like **受器。總之，吾人的這項研究結果，可能是第一次發現** D2 **和** D4**，尤其是** D4**，表現在人類的腎上腺皮質，特別是在小球區。嗜鉻細胞瘤同時表現** D2 **和** D4**。但在** APA **中，**D4 **的表現較明顯且一致，雖然，部份** APA **也有** D2 **的表現。而** D4 **的表現主要在類非束狀區細胞。**

關鍵字：多巴胺受器、皮質醛酮瘤、腎上腺、多形性

Abstract

The aldosterone secretion is evidently regulated by a dopaminergic inhibitory mechanism. Pharmacological characterization and autoradiographic studies revealed a D2-like receptor in the adrenal cortex, especially in the zona glomerulosa (ZG). However, the subtype of the dopamine receptors (DR) involving this regulation is not elucidated, although a D2-like receptor was suspected. To investigate which subtype of DR expresses in the adrenal cortex, we examined the messages of D2-like receptors, D2, D3 and D4, by RT-PCR and in situ hybridization of adrenal glands and adrenal neoplasm. Both D2 and D4 receptors were expressed in normal adrenal gland, pheochromocytoma and aldosterone-producing adenoma (APA). However, D2 receptor was not universally expressed in all adrenal samples in contrast with D4 receptor that was detected in all cases of APA and adrenal remnant. No D3 message was detected by RT-PCR in all adrenal samples. In the adrenal cortex, the messages of D2 and D4 were mainly localized in the ZG and, to a lesser extent, in the zona reticularis. In APA, the non-zona fasciculata-like cells expressed D4 and D2 receptors more abundantly than zona fasciculata-like cells. In summary, we demonstrated the existence of both D2 and D4 receptors in the human adrenal gland and adrenal neoplasm. The expression of both DR in the adrenal cortex is mainly localized in the ZG. This observation indicates that both DR may play a role in the regulation of aldosterone secretion.

二、計畫緣由與目的

There are evidences that aldosterone secretion is subjected to a dopaminergic inhibitory mechanism [McKenna et al., 1979; Carey et al., 1980; Ganguly, 1984; Porter, et al., 1992]. Administration of dopaminergic antagonists, such as metoclopramide (MCP), causes a rise in plasma aldosterone level, but not plasma cortisol level, in several animal species as well as human [Carey et al., 1980; Carey, 1982; Hsieh et al., 198?; Fraser et al., 1989]. Although there is argumentation of the mechanism of MCP-induced aldosterone secretion [Rizzi, et al., 1997], dopamine binding sites in the adrenal zona glomerulosa (ZG) has been well recognized [Bevilacqua, et al., 1982; Stern, et al., 1986; Missale, et al., 1986; Amenta et al., 1994]. Additionally, a substantial amount of dopamine (DA) has been found around the ZG (Pratt, 1987, Vizi, et al., 1993). The adrenocortical dopamine is suggested to be uptaken and released by local noradrenergic endings limited around ZG (Vizi, et al., 1993). Therefore, dopaminergic modulation of steroid secretion and/or biosynthesis seems limited to the ZG. Since MCP also increases plasma aldosterone concentration in patients with aldosterone-producing adenoma (APA), we speculate that dopaminergic receptor (DR) which regulates aldosterone secretion is present in APA (Wu et al., 1995).

Based on pharmacological and molecular biological studies, there are five subtypes of human DR, D1-D5 [Civelli et al., 1991]. The subtype of DR regulating aldosterone secretion is speculated to be D2 or D2-like receptors [Barrett et al., 1987; Missale et al., 1987; Lombardi, et al., 1988; Gallo-Payet et al., 1991]. However, at molecular level, the presence of this DR has not been proved. The mRNA of D2 receptor has been detected in adrenal medullae and pheochromocytoma (Schalling, et al., 1990; Pupilli et al., 1994; Barili, et al., 1996), but not observed in the adrenal cortex. Autoradiography showed that DR in the adrenal cortex, mainly in the zona glomerulosa, possessed the highest affinity to clozapine [Amenta et al, 1994; Amenta & Ricci, 1995] which binds to the D4 receptor with an affinity 10 times higher than to D2 and D3 receptors (Van Tol, et al., 1991). In speculation of D4 receptor expressing in the adrenal cortex, the transcript of D4 receptor was not demonstrated in rat adrenal glands, however (O'Malley, et al., 1992). In contrast, D4 receptor mRNA was observed in mouse adrenal gland (Suzuki et al., 1995), but the localization was not studied. Accordingly, the subtypes of DR expressing in adrenal cortex may be species-dependent.

To investigate which subtype human DR expresses in the adrenal cortex, we used RT-PCR and in situ hybridization to examine the expression of human D2-like receptors, i.e. D2, D3 and D4, in adrenal gland and adrenal neoplasm.

三、結果與討論

1. Materials and Methods

A human D4 receptor cDNA clone was generously given by Dr. Mitsuyuki Matsumoto [Ibarakoi, Japan]. The cDNA contains two tamden repeats in exon 3. The probes used in this study were derived from this clone.

Isolation of Poly A+ RNA Poly A+ RNA was isolated from APA (6 cases) and the remnant adrenal (3 cases), pheochromocytoma (2 cases), normal adrenal glands from radical nephrectomy of renal cell carcinoma (2 cases), human kidney, and human brain tissue (from

Clontech Laboratories). Five micrograms of poly A+ RNA from each sample was reversely transcribed by M-MLV reverse transcriptase () as described previously (Wu, et al., 1995). The reverse transcription mixture was finally diluted to 100 µl.

RT-PCR amplification The primers for amplification of human D2 receptor were 5'-TTCACCATCTCCTGCCCACT-3' and 5'-GTGCTGGAGAGCATCTCCAT-3', corresponding the nucleotides of 589-608 and 941-960, respectively. The primers amplify both the short and long splicing variants, 283 and 372 bp respectively. No product will be amplified from genomic DNA because the primers flank a large intron (13kb). The primers for amplification of human D3 receptor were 5'-CAGAATCTATGTGGTGCTGA-3' (627-646) and 5'-GGGAGAAGAAGGCAACCCAA-3' (968-987).

The names and sequences of primers for human D4 receptor, M1 and M3, were identical to those reported by Mitsuyuki et al.; M1 (204-223; cDNA sequence by Hubertt et al., 1991), 5'-CACCAACTCCTTCATCGTGA-3', and M3 (602-583), 5'-AAGGAGCACACGGACGA-GTA-3'. The conditions for PCR amplification reported by Mitsuyuki et al. were followed. Briefly, 3 µl of the diluted reverse transcription mixture or 1 pg human D4 receptor cDNA clone was added in the presence of 10 pmol of primers, 200 µM dNTPs, 1.5 mM $MgCl_2$, 5% DMSO and 2.5 U of Taq DNA polymerase (Gibco). For examination of tandem-repeat polymorphism of D4 receptor, D4I3a and D4I3b primers were used. The corresponding nueclotides were 5'-TCTACTGGGCCACGTTCCGCGGCCTGC-3' (632-658) and 5'-ACCGGCCGGGAGC-GCAAGGCCATGAGGG-3' (968-995). The amplified products will be 321 bp and 417 bp for 2- and 4-repeat variants, respectively.

Southern blots Digoxigenin-labeled cDNA probe was synthesized by Klenow enzyme according to the manufacture's protocol. Probe for D2 receptor was the long fragment from RT-PCR described above. Probe for D4 receptor was the fragment (234-538 nucleotides) derived from the human D4 receptor clone digested by Not I.

The products of PCR were electrophoresed in 5% ployacrynamide gel of 0.5X TBE and then transferred to positively charged nylon mambrane (Boehringer Mannheim) electrically. The membrane was denatured in 0.4N NaOH for 10 minutes, followed by rinsing in 2X SSCP for 5 minutes and UV crosslinking. Hybridization with digoxigenin-labeled cDNA probe was performed according to the manufacture's protocol (Boehringer Mannheim). The signal was detected by using alkaline phosphatase-conjugated antibody and **DSA.**

Synthesis of cRNA probes The antisense and sense digoxigenin-labeled cRNA probes for in situ hybridization of human D2 and D4 receptors were synthesized with RNA polymerase from the corresponding clones. For D4 receptor, a fragment (663-1081 nucleotides) digested by Hinc II and Pst I was ligated to KS- Bluescript plasmid (Stragene); for D2 receptor, the PCR product amplified by primers, 5'-AATGGGCATGCCAAAGACCA-3' (1147-1166), and 5'-CATCCTGAACATACACTGTG-3' (1344-1363), was ligated to pGEM-T (Promega). Riboprobes used for in situ hybridization were filtered through Chroma Spin™-100 columns (Clontech,USA) to separate unincorporated digoxigenin-11-UTPs from digoxigenin-11-UTP-labelled riboprobes.

In situ hybridization The procedures were modified from previous methods (Chen et al., 1999). Eight-micrometer cryosections of 4% PFA-immersed tissues were cut and mounted on siliconized glass slides. Sections were then permeabilized with Proteinase K at 1 µg/ml for 20

min at room temperature. After permeabilization, sections were treated sequentially with 0.2% Triton-100, 0.2 M HCl and acetylated with 0.1 M triethanolamine containing 0.25 % acetic anhydride for 10 min of each step at room temperature. Sections were subsequently incubated with pre-hybridization buffer which containing 4 x SSC with 50% formamide at 48 °C for at least 1 hr.

After prehybridization, sections were incubated with hybridization buffer containing 50% formamide, 10% dextran sulfate, 1 x Denhardt's solution, 4 x SSC, 1mg/ml sperm DNA, and 7 µg/ml of digoxigenin-labeled RNA probe at 48°C overnight in a humid chamber. The sections were then washed 3 x 10 min with 0.1 x SSC at 50°C and subjected to immunological detection. Blocking was performed with 10% skim milk for 30 min at room temperature, and followed by anti-DIG-antibody conjugated with alkaline phosphatase for 1 h at room temperature. The sections are finally visualized with the enzyme substrate containing NBT and BCIP and counterstained with methylene blue.

2. Results

By PCR with M1/M3 primers, a product with expected size of 399bp was observed when human D4 receptor cDNA clone as template. This fragment was also detected in the normal adrenal gland, brain tissue, kidney, APA, and pheochromocytoma. We examined the specificity of this amplification by digesting the PCR products with Bgl I. Two fragments with the expected size of 131 and 267 bp were observed. Therefore, human D4 receptor was expressed in cells derived from the adrenal cortex as well as adrenal medullae. There was no signal observed of human D3 receptor of all adrenal samples (data not shown).

Two fragments were amplified by the D2 primers, which indicated the long and short splicing variants of D2 receptor. The short variant was of much less abundance in all samples. The expression of D2 receptor message was observed in human brain, normal adrenal gland, APA, and pheochromocytoma, but not in kidney. The expression of D2 in APA was not universal, however. Fig 1 showed the results of RT-PCR of D2 and D4 receptors in all samples of APA and 3 remnant adrenal glands. D4 receptor was detected in all samples, although different amounts were noted. In contrast, the amounts of D2 receptor mRNA were much variable in either APA and remnant adrenal glands. The levels of D2 receptor expressed in the remnant adrenals were much higher than those in their respective APA. There was no D2 receptor mRNA detected in 3 cases of APA. Polymorphism of D4 receptor was observed in APA. Three cases of APA were homozygote of 4-tandem-repeat alleles (D4(4/4)) and the other 4 cases were heterozygote of 2-and 4-tandem-repeat alleles (D4(2/4)).

To investigate the localization of D2 and D4 receptors in adrenal gland, we performed in situ hybridization. Both human D2 and D4 receptors were expressed abundantly in the adrenal medullae. More than 50% of medullar cells expressed these dopamine receptors. Accordingly, a significant portion of cells express both dopamine receptors. The expression was evenly distributed in the medullae (Fig 2a). In the adrenal cortex, both subtypes of dopamine receptors were expressed, mainly in the ZG (Fig 2c, 2d). Cells of zona reticularis also expressed the dopamine receptors, but in a less abundance (Fig 2c). There was no or very little expression in the zona fasciculata. Pheochromocytoma expressed both D2 and D4 receptors (Fig 3a, 3b). The expression of D2 or D4 in APA was not in homologous pattern. The messages of both DR were more abundant in non-Zona-fasciculata-like cells, and less or

no expression of these DRs in zona-fasiculata-like cells (Fig 3c, 3d).

四、計畫成果自評

In pharmacological and autoradiographic studies, D2-like receptor in the adrenal cortex has been well-demonstrated (Missale, 1986; 1988; Stern 1986; Amenta, 1994). However, the subtype of DR has not been determined at molecular level. In the present study, we demonstrated that both D2 and D4 receptors expressed in the adrenal cortex. These two DRs are mainly localized in the ZG. The cells of zona reticularis also expressed both receptors, but in less abundance. The signals were hardly detected in the zona fasiculata. This distribution pattern of these two D2-like receptors is compatible with the observation in autoradiographic study (Amenta et al., 1994). It has been shown that a substantial proportion of enzymes responsible for DA metabolism is present around zona glomerulosa (Vizi, et al., 1993). The localization of these D2-like receptors, therefore, indicates that D2 and/or D4 receptors in ZG may play an important role in the regulation of aldosterone secretion. However, it is not known whether both receptors play equally.

Pharmacological characterization suggested that D4 receptor was the predominant subtype of dopamine D2-like receptors expressing in the adrenal cortex. Amenta et al demonstrated that clozapine, a D4-specific antagonist, was the most powerful displacer of spiroperidol. To our knowledge, there is no data that clozapine can increase aldosterone level. Their result can not exclude the presence of other D2-like receptors in the adrenal cortex, however. We have observed that the cultured cells (NCI-H295R), which secret aldosterone, also express human D2 and D4 receptors (unpublished data). In the present study, the mRNA levels of these two receptors were not quantified. However, D4 receptor was universally expressed as compared with D2 receptor. The latter was much less abundant in APA in comparison with its expression in the remnant adrenals (Fig 2). We speculate that D4 receptor is probably a “house-keeping” DR and D2 receptor can be regulated physiologically.

The expression of DR in different tissues is discrepant among species. As shown in the present study, D4 receptor mRNA was detected in human adrenal gland as well as in mouse (Suzuki et al., 1995), but not in rat (O'Malley, 1992). In the kidney, D2-like receptors have been identified by radioligand binding study (Felder et al, 1989). By RT-PCR, different subtypes of D2-like receptors were detected in the kidney, including D_{2L} (Gao et al, 1994) and D3 (Sokoloff et al, 1990) in rat, and D4 in human (Matsumoto et al, 1995). The present study shows that human kidney expresses D4 receptor, but not D2 receptor.

Our previous study showed that the increment of plasma aldosterone concentration by MCP in patients with APA correlated positively with the percentage of non-ZF-like cells of adenoma (Wu, 1995). We speculate that aldosterone secretion from non-ZF-like cells of APA is inhibited in a greater extent by dopamine, which may result from these cells express more D2-like receptors, than ZF-like cells. This speculation is supported by the present results that both D2 and D4 receptors in APA are mainly expressed in non-ZF-like cells.

The human D4 receptor exists with different insertions in the third intracellular loop, which contains repeat sequences of 16 amino acids (Van Tol et al, 1992). The clinical significance of the polymorphism of human D4 receptor is not clear yet, although different properties between shorter and longer variants were observed with respect to clozapine binding (Van Tol et al, 1992). In our 7 cases of APA, three cases were D4(4/4) and four cases

of D4(2/4). These two variants are the most common in our population, with 68 % of D4(4/4) and 29 % of D4(2/4), respectively (data unpublished). Although the case number in the present study is limited, APA does not seem expressing specific variants of D4 receptor. Further studies will elucidate whether different responsiveness of aldosterone secretion to MCP in APA results from different variants of D4 receptors.

Several studies have demonstrated the expression of D2 receptor in adrenal medulla and pheochromocytoma (Schalling, et al., 1990; Pupilli et al., 1994; Barili, et al., 1996). The present study revealed that D2 as well as D4 receptor expressed in these chromaffin cells. In situ hybridization did not show obvious difference of the distribution and abundance between these two D2-like receptors in either the adrenal medulla or pheochromocytoma. Activation of D2-like receptor can inhibit catecholamine release from chromaffin cells of the adrenal gland (Mannelli et al, 1988). Again, the presence of both D2 and D4 receptors makes it difficult to clarify which receptor is mediated through. Although different characteristics of D2-like receptor between the adrenal cortex and adrenal medulla (Dunn, 1981; Quik M, 1987) were suspected, our results do not support this speculation.

In summary, this is the first study, to our knowledge, demonstrating the existence of both D2 and D4 receptors in the human adrenal gland and adrenal neoplasm. The expression of both DR in the adrenal cortex is mainly localized in the ZG. This observation indicates that both DR may play a role in regulation of aldosterone secretion.

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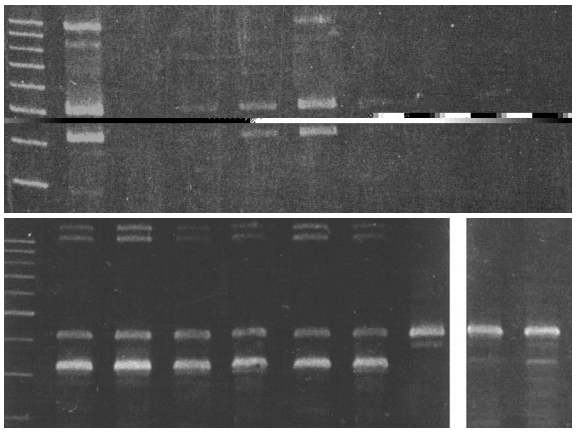

Fig 1. The results of RT-PCR of dopamine receptor types 2 (D2) and type 4 (D4) from 6 patients with APA and their remnant adrenals. The two fragments of D2 products (sized 510, 410 bp) indicate the long and short variants. The two fragments of D4 indicate the 4- and 2-tandem repeats of D4 polymorphism. Lane 1, the 100-bp DNA marker; lanes 3, 5, 7, 8, 9, and 10, APA; lanes 2, 4, and 6, the remnant adrenal glands of APA from 3, 5, and 7, respectively.

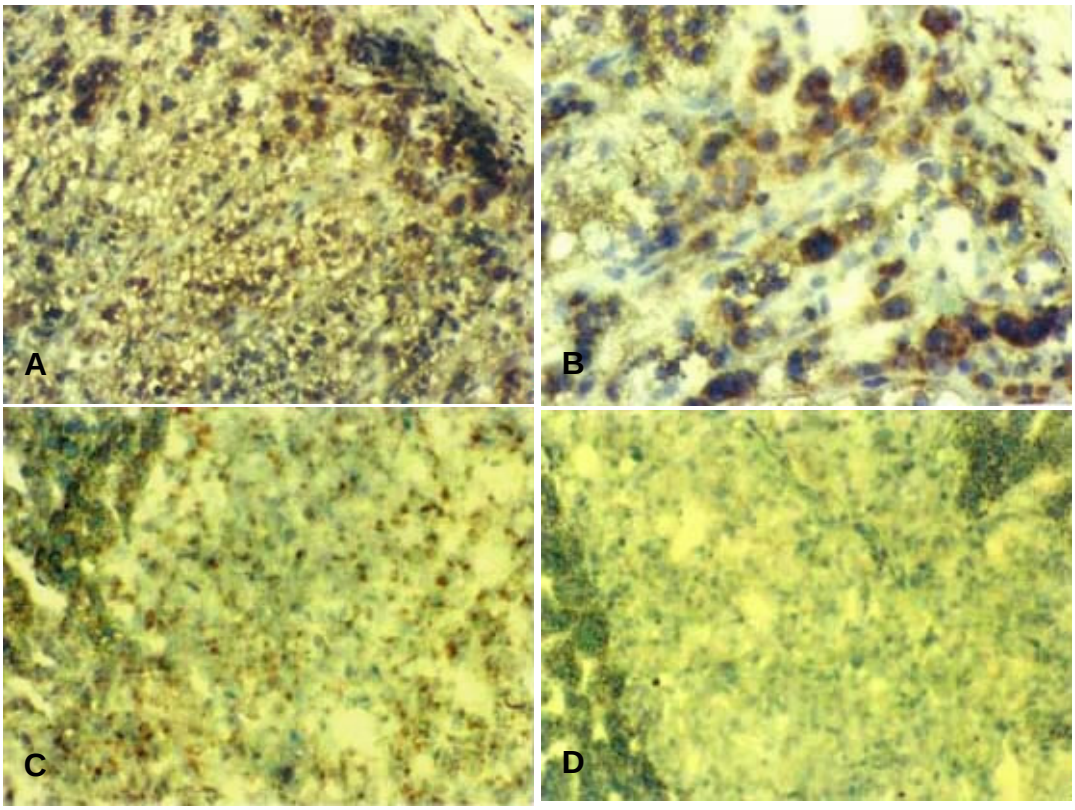

Fig 2. In situ hybridization of human D4 receptor in the cortex (A and B) and the medullae (C and D, sense probe) normal adrenal gland.

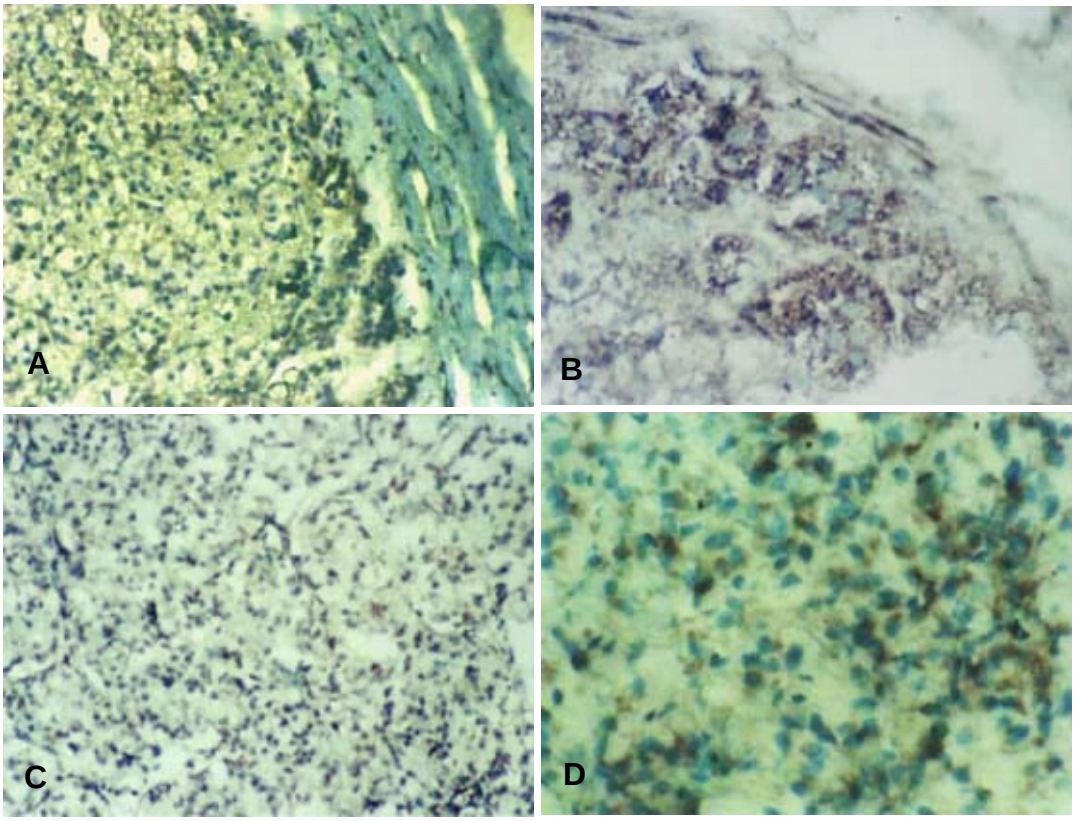

Fig 3. In situ hybridization of human D4 receptor in aldosterone-producing adenoma (A and B) and pheochromocytoma (C and D).