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Catecholamine synthesizing enzymes in 70 cases of functioning and non-functioning phaeochromocytoma and extra-adrenal paraganglioma

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Summary. Immunohistochemical localization of the catecholamine synthesizing enzymes, tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), dopamine-β-hydroxylase (DBH) and phenylethanolamine *N*-methyltransferase (PNMT), was investigated in 70 cases of functioning and non-functioning phaeochromocytomas comprising 52 of adrenal and 18 of extra-adrenal origin. Of 59 functioning tumours, 30 were mixed epinephrine and norepinephrine-producing (mixed type) and 29 were norepinephrine-producing tumours. TH, AADC and DBH were detected in all functioning phaeochromocytomas, but PNMT was limited to the mixed-type phaeochromocytomas. Non-functioning phaeochromocytomas were divided into two groups, comprising a complete type, which induced neither elevated plasma catecholamines nor their metabolites in urine, and an incomplete type which exhibited no elevated plasma catecholamines, but showed a slightly high urinary vanillylmandelic acid level. In the non-functioning complete-type tumours, immunoreactive TH was negative, but the incomplete tumours of the adrenal medulla had all four enzymes, and corresponded to a mixed-type phaeochromocytoma. AADC and DBH were present universally in all functioning and non-functioning tumours, including TH-negative tumours. TH is a rate-limiting enzyme of catecholamine biosynthesis and deficiency of TH is an important feature of extra-adrenal non-functioning phaeochromocytomas.

Key words: Phaeochromocytoma – Tyrosine hydroxylase – Aromatic L-amino acid decarboxylase – Dopamine β-hydroxylase – Phenylethanolamine N-methyltransferase

Introduction

Phaeochromocytoma is derived from the paraganglia and usually produces catecholamines; clinical symptoms depend on the plasma levels of the catecholamines produced by the tumour. Our previous study revealed that plasma catecholamine levels are directly correlated to the catecholamine content of the tumours (Kimura et al. 1984). When a patient with phaeochromocytoma has neither clinical symptoms nor elevated serum or urinary catecholamine levels, that tumour is described as non-functioning. Non-functioning pheochromocytomas have similar histology to those of the functioning type and although the size of the neuroendocrine granules is, by comparison, smaller and the number of granules is somewhat less than those of the functioning phaeochromocytoma, no essential differences have been found between the histopathology.

Tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), dopamine-β-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT) are the enzymes needed to synthesize catecholamines for dopa, dopamine, norepinephrine and epinephrine respectively (Verhofstad et al. 1983). TH is the rate-limiting enzyme in the synthesis of catecholamines in neural tissues and the adrenal medulla (Han et al. 1987). If both DBH and PNMT reactions are positive, then it can be concluded that the cells synthesize epinephrine. Alternatively, if the cells are immunoreactive only to DBH and negative for PNMT, it can be deduced that they synthesize norepinephrine (Verhofstad et al. 1983). We performed an immunohistochemical study of those enzymes on functioning and non-functioning phaeochromocytomas and extra-adrenal paragangliomas to clarify whether there is a close correlation between immunoreactive expression of catecholamine-synthesizing enzymes and tumour types classified by plasma and/or urinary catecholamine levels, such as mixed epinephrine and norepinephrine-producing tumours and norepinephrine-producing tumours, and also to determine if there is an abnormality in the process of catecholamine synthesis in non-functioning tumours.

Materials and methods

Seventy cases of phaeochromocytoma were examined. Multiple catecholamine measurements of blood and urine were performed

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pre-operatively in all except 7 cases. Surgically operated tumours were divided into three groups: functioning, non-functioning and unknown types, on the basis of catecholamine levels in the plasma and/or urine. The location of the tumours is summarized in Table 1.

There were 59 functioning tumours, including 30 of the mixed epinephrine and norepinephrine-producing type (mixed type) and 29 of the norepinephrine-producing type.

In the non-functioning type, there were 3 tumours of adrenal origin and 1 from the urinary bladder. These non-functioning adrenal phaeochromocytomas had a normal plasma level of catecholamines, but an increased pre-operative urinary vanillylmandelic acid (VMA). Intra-operative palpation of the tumour induced high blood pressure and arrhythmia. These tumours were called non-functioning incomplete phaeochromocytomas in the present study.

In a case of malignant phaeochromocytoma of the urinary bladder with multiple metastasis in the lymph nodes, pelvis and mediastinum, the levels of both serum catecholamines and urinary VMA were always within the normal range; epinephrine: 16 pg/ml (normal <100 pg/ml); norepinephrine: 60 pg/ml (normal <300 pg/ml); VMA 2.0 mg/day (normal <5 mg/day) in spite of frequent measurements. This type of tumour was called non-functioning complete phaeochromocytoma. Thus we divided non-functioning phaeochromocytomas into two groups: complete and incomplete, the former tumour without evidence of excessive catecholamine production determined by analysis of serum catecholamine and urinary VMA and the latter with a normal range of serum catecholamine levels, but a slight increase in urinary VMA. Although 1 patient did not complain of clinical symptoms associated with hypercatecholaminaemia, stimulation (intra-operative palpation) induced catecholamine release into the blood as well as hypertension and tachycardia. This case was included among those classed as incomplete. There were 3 cases of incomplete, and 1 case of complete tumour in the present study.

Seven cases, including 3 carotid body tumours, 1 spinal cord paraganglioma, 1 urinary bladder paraganglioma and 2 adrenal phaeochromocytomas, were operated on without pre-operative catecholamine analysis. They had no symptoms of hypercatecholaminaemia at the time of operation.

Four malignant cases with multiple distant metastases, including 3 norepinephrine-producing phaeochromocytomas and 1 non-functioning phaeochromocytoma, were examined.

Tumour tissue was fixed immediately in 10% buffered formalin and embedded in paraffin. For immunohistochemical procedures, the streptavidin-biotin-peroxidase complex method was employed using a Histofine SAB-PO kit (Nichirei, Tokyo) as described elsewhere (Kimura et al. 1988, 1990). The source and working dilution of the primary antibodies were as follows. TH for rat phaeochromocytoma (Chemicon, El Segundo, Calif., 1:200), AADC for bovine adrenal medulla (Dr. I. Nagatsu, Toyoake, 1:1000), DBH for bovine adrenal medulla (Dr. I. Nagatsu, Toyoake, 1:50), PNMT for bovine adrenal medulla (Bioclone, Marrickville NSW, Australia, 1:2000). All of these antibodies were raised in rabbits. The specificity of the antibodies for AADC and DBH has been described elsewhere (Nagatsu et al. 1988). Primary antibodies were incubated at 4° C for 18 h. Indicative reaction for peroxidase was performed using 0.03% 3,3-diaminobenzidine 4 hydrochloride. Sections were counterstained with methyl green.

For negative controls, normal rabbit serum diluted 1:300 was used. For the positive controls, human adrenal medulla was used.

Results

All the enzymes TH, AADC, DBH and PNMT were detected in the normal adrenal medulla of the positive controls. Immunoreactive TH and AADC were diffusely demonstrated in the cytoplasm of almost all medullary cells. In contrast, immunoreactive DBH was shown as rough granules located mainly in the periphery of the cytoplasm. PNMT was shown diffusely in the cytoplasm. Cells immunoreactive to PNMT tended to be located in the sub-cortical region. Negative controls did not exhibit immunoreactivity.

Table 1. Immunohistochemical localization of catecholamine-synthesizing enzymes in phaeochromocytomas and paragangliomas

	Cases examined	Cases positive for			
		TH	AADC	DBH	PNMT
I. Functioning	*59*				
1) Mixed epinephrine and norepinephrine-producting type	30				
a) Adrenal gland	29	29	29	29	29
b) Retroperitoneum	1	1	1	1	1
2) Norepinephrine-producing type	29				
a) Adrenal gland	18	18	18	18	0
b) Extra-adrenal gland	11	11	11	11	0
II. Non-functioning	*4*				
1) Incomplete, adrenal gland	3	3	3	3	3
2) Complete, urinary bladder	1	0	1	1	0
III. Unknown	*7*				
Carotid body	3	1	3	3	0
Spinal cord	1	0	1	1	0
Urinary bladdar	1	1	1	1	0
Adrenal gland	2	2	2	2	2
Total	70	66	70	70	35

TH, tyrosine hydroxylase; AADC, Aromatic L-amino acid decarboxylase; DBH, dopamine-β-hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase

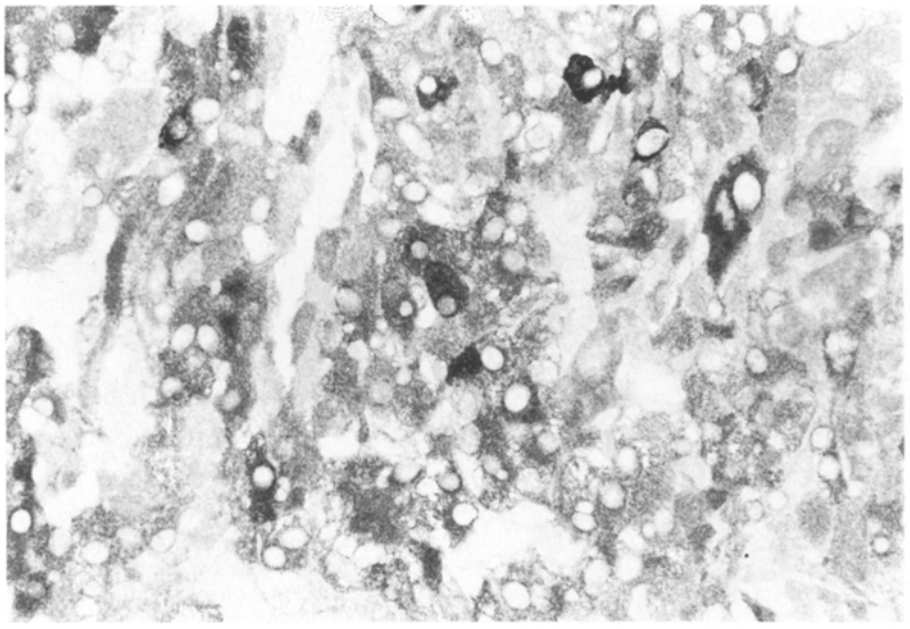

Fig. 1. Although there is some heterogeneity in immunoreactivity, almost all tumour cells are immunoreactive to tyrosine hydroxylase (TH) in a functioning adrenal phaeochromocytoma. ×400. (Case: 49-year-old female; plasma epinephrine; 890 pg/ml; plasma norepinephrine: 5800 pg/ml; tumour size: 65 × 55 × 55 mm, 112 g)

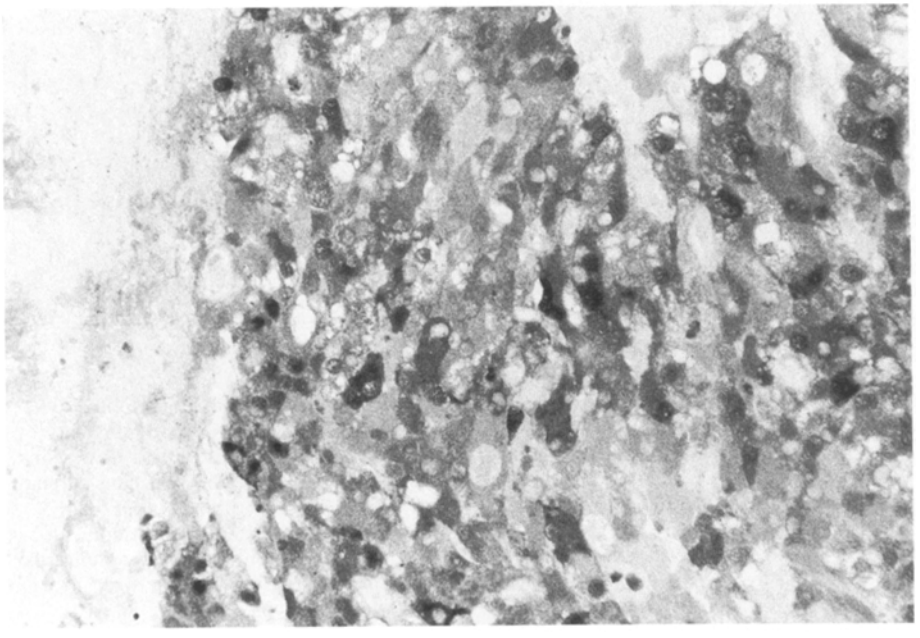

Fig. 2. All tumour cells are strongly immunoreactive to aromatic L-amino acid decarboxylase (AADC) in a functioning adrenal phaeochromocytoma. ×200. (Case: 41-year-old female; plasma epinephrine: 1900 pg/ml; plasma norepinephrine: 2080 pg/ml; tumour size: 57 × 46 × 38 mm, 60 g)

In the functioning phaeochromocytomas, almost all tumour cells were diffusely stained with TH (Fig. 1) and AADC (Fig. 2). Immunoreactive DBH was observed in all tumours, but there were fewer cells immunoreactive to DBH (Fig. 3) than to TH and AADC. PNMT immunoreactivity was limited to the mixed tumours (Fig. 4), and no norepinephrine-producing type had cells immunoreactive to PNMT (Fig. 5).

There was no histological difference between functioning and non-functioning tumour tissue stained with haematoxylin and eosin (Fig. 6).

In the non-functioning phaeochromocytomas, TH immunoreactivity was observed in 3 of 4 tumours. All positive tumours were of the incomplete type. Only the tumour of complete type in the urinary bladder was TH negative (Fig. 7). AADC immunoreactivity was strongly demonstrated in all non-functioning tumours including the TH-negative, complete type (Fig. 8). Cells immunoreactive to DBH were observed in all 4 tumours; however the number of DBH-positive cells was much less than that of functioning phaeochromocytomas (Fig. 9). Immunoreactive PNMT was observed in all 3 adrenal tumours, but not in a complete-type tumour of the urinary bladder.

In the unknown group, adrenal phaeochromocytomas were immunoreactive to all four enzymes, corresponding to mixed phaeochromocytoma. Extra-adrenal tumours located in the carotid body, spinal cord and

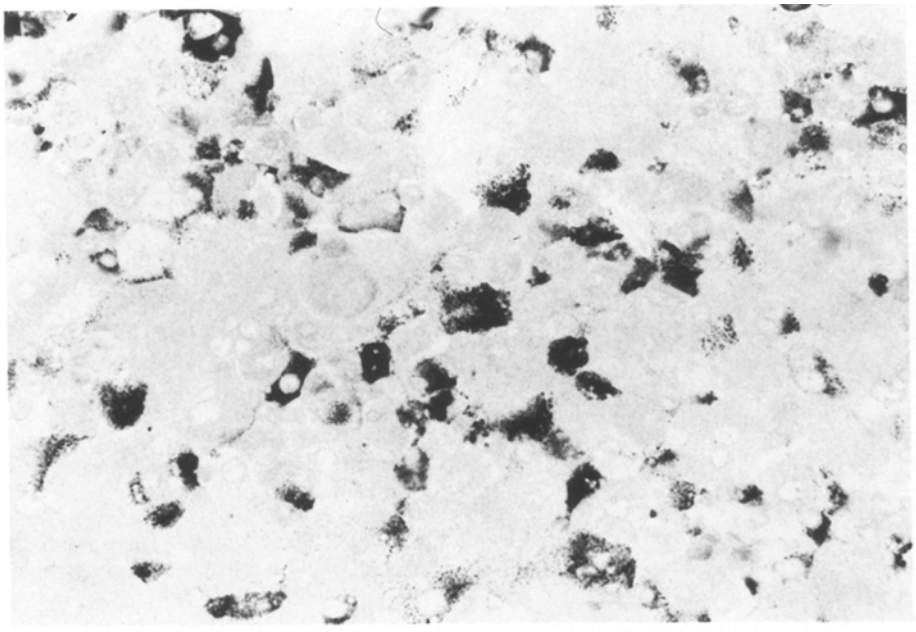

Fig. 3. Dopamine-β-hydroxylase (DBH) immunoreactivity is sporadically but strongly observed in a functioning adrenal phaeochromocytoma ($\times 400$). (Same case as Fig. 1)

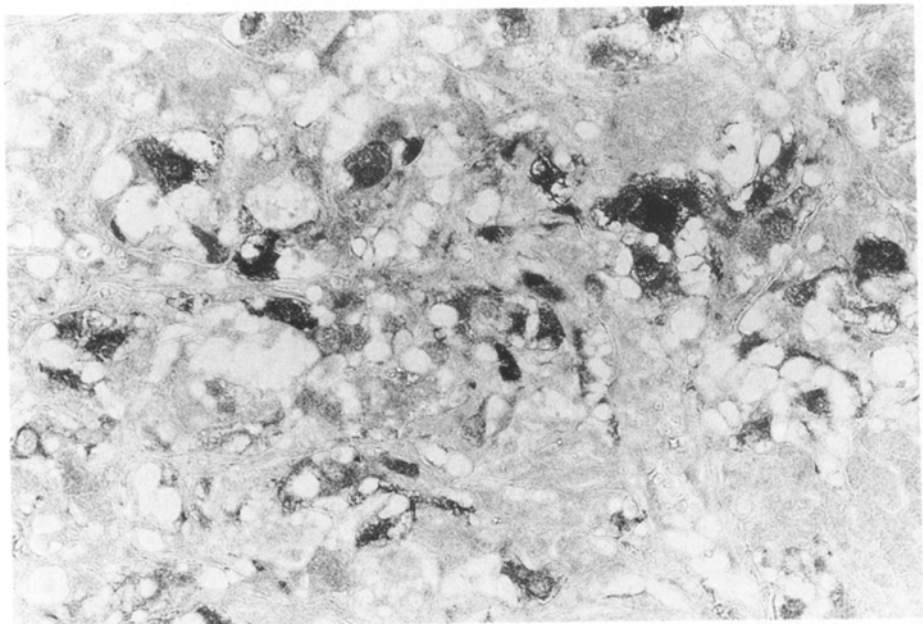

Fig. 4. Phenylthanolamine-*N*-methyltransferase (PNMT) immunoreactivity is limited in an mixed type epinephine and norepinephrine producing adrenal phaeochromocytoma. $\times 400$. (Case: 61-year-old female; plasma epinephrine: 1860 pg/ml; plasma norepinephrine: 2192 pg/ml; tumour size: $42 \times 33 \times 35$ mm, 23 g)

urinary bladder showed strong immunoreactivity to AADC, but TH was detected in only 1 of 3 carotid body tumours and a urinary bladder tumour. Two carotid body tumours and a spinal cord tumour were negative for TH. These TH-negative tumours were larger than the positive ones, with a maximum diameter of 3.0 cm versus 0.5 cm. DBH was sporadically positive in all 5, but PNMT was negative in all cases.

In 4 malignant tumours with multiple distant metastases, a tumour of the urinary bladder, non-functioning, complete type, had no cells immunoreactive to TH, but cells immunoreactive to AADC and DBH were observed. The other 3 tumours, which were of the norepinephrine-producing type, were immunoreactive to TH, AADC and DBH. The results are summarized in Table 1.

Discussion

Since the report of Goldstein et al. (1971), there have been many investigations of catecholamine-synthesizing enzymes in the adrenal medulla (Eränko et al. 1966; Wurtman and Axelrod 1966; Nagatsu and Kondo 1974; Verhofstad et al. 1979), the extra-adrenal paraganglion system (Eränkä et al. 1966), and brain (Nagatsu et al. 1988). However, immunohistochemical analysis of these enzymes in phaeochromocytoma and extra-adrenal

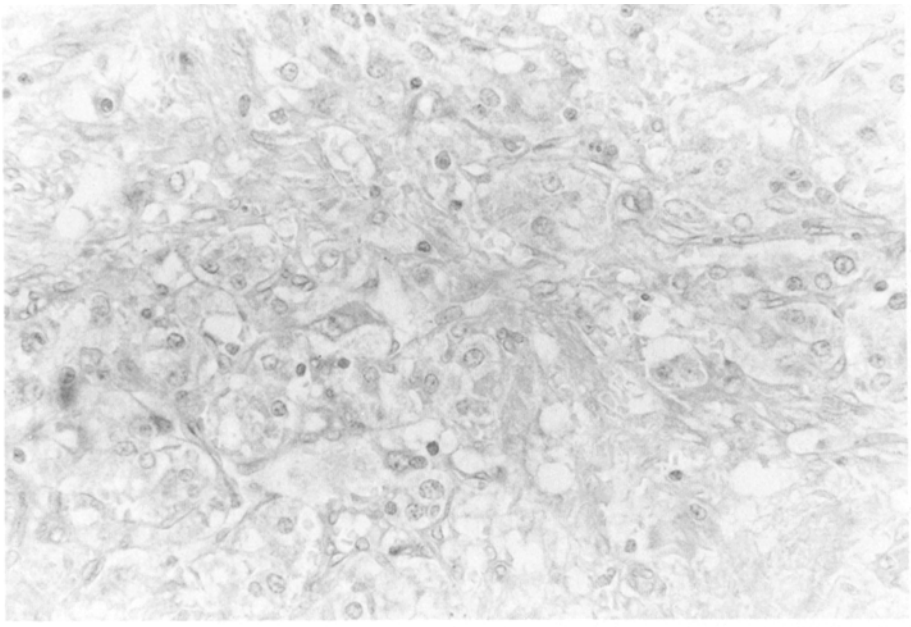

Fig. 5. There are no immunoreactive cells to PNMT in a norepinephrine-producing adrenal phaeochromocytoma. ×400. (Case: 51-year-old female; plasma epinephrine: 75 pg/ml; plasma norepinephrine: 4100 pg/ml; tumour size: 55 × 45 × 40 mm, 53 g)

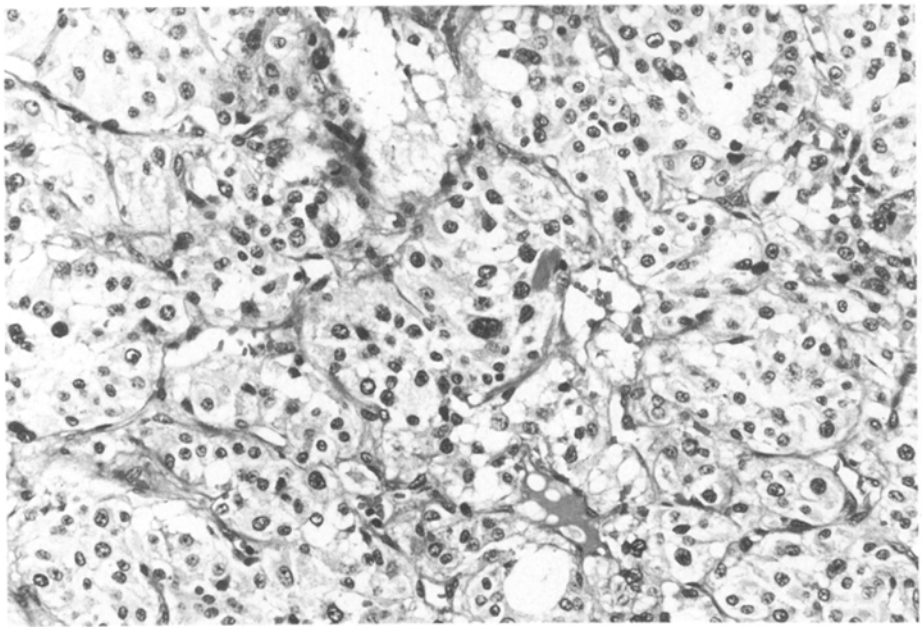

Fig. 6. Phaeochromocytoma of the urinary bladder, non-functioning, complete type. This tumour is indistinguishable from a functioning phaeochromocytoma. H&E, ×200. (Case: 23-year-old male; plasma epinephrine: 16 pg/ml; plasma norepinephrine: 60 pg/ml; tumour size: 75 × 55 × 45 mm, 60 g. Tumour metastases to regional lymph nodes were confirmed)

paraganglioma has been very limited (Lloyd et al. 1986; Takahashi et al. 1987; Osamura et al. 1990; Schröder et al. 1990; Kimura 1991; Kimura et al. 1991).

We divided non-functioning phaeochromocytomas into two groups: complete and incomplete types. The former was defined as a tumour with no evidence of catecholamine production which was determined by analysis of both plasma catecholamines and their metabolites in urine. The latter was defined as a tumour with a normal level of plasma catecholamine and no clinical symptoms associated with hypercatecholaminaemia, but with a slightly increased urinary VMA. Some stimulation such as intra-operative palpation may induce catecholamine release into the blood and hypertension. In the unknown group, which had not been examined by catecholamine analysis pre-operatively, all 5 extra-adrenal cases had neither hypertension nor other symptoms associated with hypercatecholaminaemia. These cases may be included with the non-functioning tumours of the complete or incomplete type.

There were no cells immunoreactive to TH in a non-functioning phaeochromocytoma, a complete tumour of the urinary bladder, 2 carotid body tumours and a spinal cord tumour of the unknown group. TH-negative tumours were larger than the positive, with a mean size of 3.0 cm versus 0.5 cm in maximum diameter. However, three non-functioning incomplete type phaeochromocytomas of the adrenal gland had many cells positive for

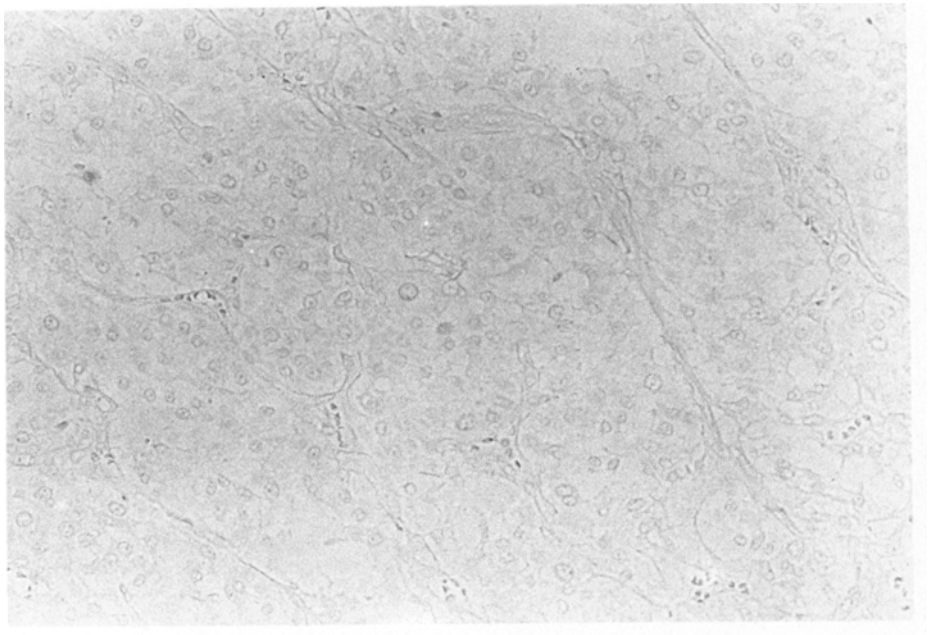

Fig. 7. There are no cells immunoreactive to TH in non-functioning phaeochromocytoma, complete type (same case as Fig. 6, ×400)

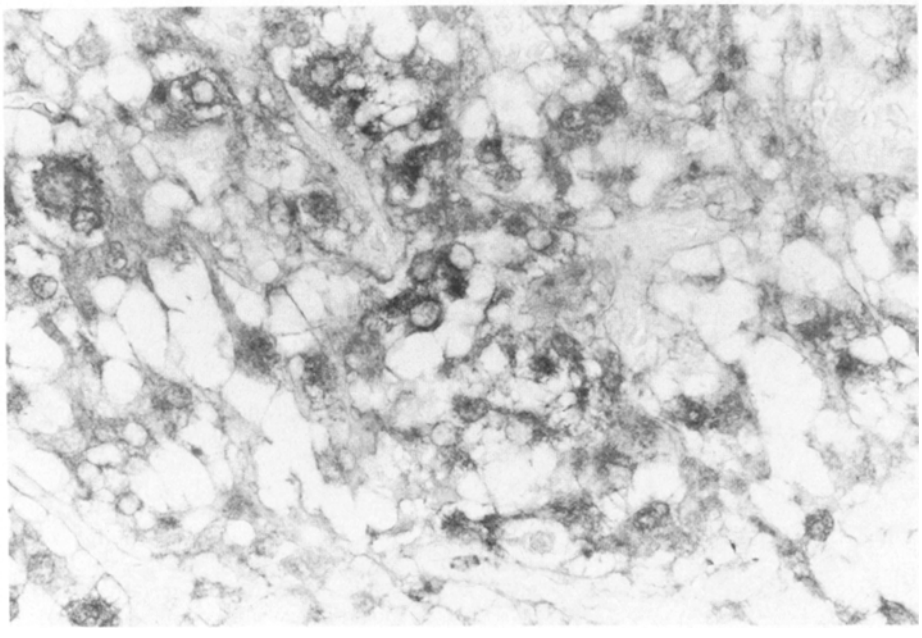

Fig. 8. Even in TH-negative case, AADC immunoreactivity is observed in non-functioning tumour (same case as Fig. 7, ×400)

TH. Non-functioning complete-type phaeochromocytomas had no or very few cells immunoreactive to TH but the incomplete type had no abnormal expression of TH immunoreactivity. The TH-negative cases were all extra-adrenal.

TH is the rate-limiting enzyme of catecholamine biosynthesis and if it is deficient or small in amount, the tumour might be unable to produce excessive catecholamines despite the presence of enzymes such as AADC, DBH and PNMT. Thus, we believe that TH deficiency is a characteristic feature of the non-functioning, complete-type tumours, which were all extra-adrenal tumours. Osamura et al. (1990) reported that 3 of 4 cases with a low level of plasma catecholamines (non-functioning cases) showed equivocal staining for TH and the remaining case was weakly positive, although TH immunoreactivity was strongly positive in tumours producing high catecholamine levels. Furthermore, 2 extra-adrenal malignant phaeochromocytomas were negative for TH (Osamura et al. 1990). Schröder et al. (1990) also reported that positivity for TH in the adrenal phaeochromocytomas was strictly correlated with the presence of endocrine symptoms. In the cases of the craniocervical region (Takahashi et al. 1987), all 3 tumours examined were positive for TH, but 2 of the above 3 cases had no immunoreactive cells for TH in metastatic sites in cervical lymph nodes. Takahashi et al. (1987) have speculated that the TH-negative tumour cells were imma-

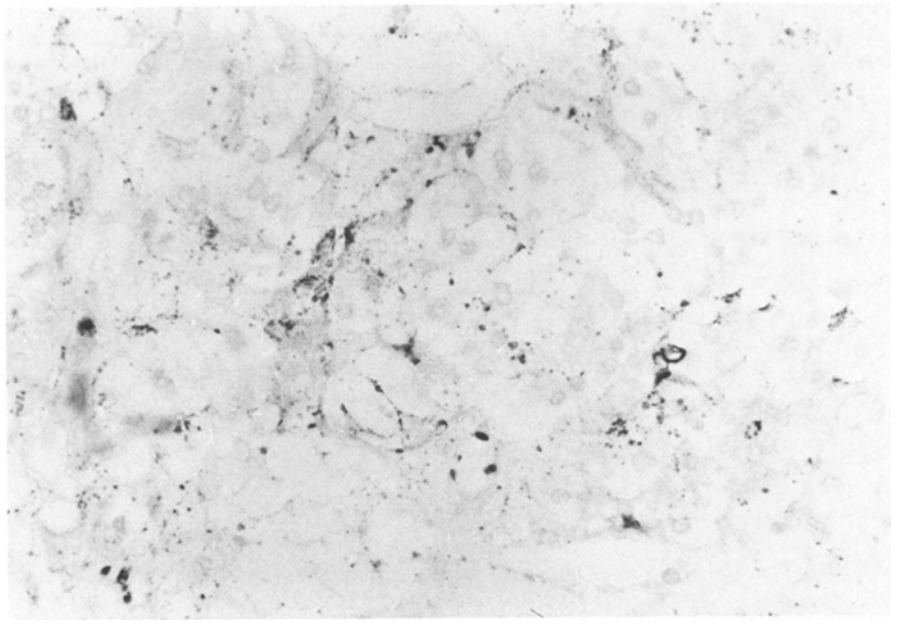

Fig. 9. The intensity of immunoreactivity and the number of DBH-positive cells are less than those of functioning phaeochromocytoma (same case as Fig. 8, ×400)

ture in nature and tended to show malignant behaviour. However, in the present study, all functioning tumours, including malignant cases with multiple distant metastases, showed strong immunoreactivity to TH even at the metastatic sites. Thus, deficiency of TH immunoreactivity itself is not reliable evidence of malignancy, but it may be an expression of cell immaturity and may correlate with malignant transformation.

Despite considerable research in this field, a definition of malignancy in phaeochromocytomas is still controversial. Distant metastasis seems to be the only acceptable criterion for malignancy. We have investigated 38 benign and 26 malignant phaeochromocytomas which had distant multiple metastases (Kimura and Sasano 1990) and reported that malignant phaeochromocytomas could be classified into four types: spindle-shaped cell type, small round cell type, pseudo-rosette-forming type and mixed neuroendocrine-neural type. Malignant tumours were shown to be composed of significantly smaller cells with a higher nuclear/cytoplasmic ratio when compared into that of benign tumours. Nuclear DNA measurement also revealed a high mode with a peak at 3C and narrow variance in malignant tumours, and a low mode with wide variance in benign tumours. Immunohistochemical studies of intermediate filaments, peptide hormones, S100 protein etc. have not contributed to the definition of malignancy in phaeochromocytomas (Kimura and Sasano 1990; Schröder et al. 1990).

AADC is an essential enzyme for the formation of both serotonin and catecholamine and widespread distribution of AADC has been found in most of the neuroendocrine system (Gazdar et al. 1988). An extensive study of AADC in phaeochromocytomas, however, was performed for the first time in the present study. AADC immunoreactivity was observed in all tumours, even in TH-negative non-functioning lesions.

In functioning phaeochromocytomas all three enzymes, TH, AADC and DBH, were present in both norepinephrine-producing and mixed tumours. PNMT, however, was localized only in the cytoplasm of tumour cells of the mixed-type tumours of the adrenal gland and retroperitoneum. There was a close correlation between localization of PNMT and epinephrine production in phaeochromocytoma. Neither norepinephrine-producing nor extra-adrenal non-functioning tumours expressed PNMT.

All tumours of the non-functioning, incomplete type and unknown group in the adrenal medulla had four enzymes and were considered to be mixed phaeochromocytomas. Epinephrine-producing phaeochromocytoma (mixed type) does not necessarily lead to development of hypertension. Why some tumours of the non-functioning, incomplete type having all four enzymes do not secrete excessive norepinephrine or epinephrine into blood remains to be clarified.

In situ hybridization is another powerful approach to the study of regulation of these enzymes (Han et al. 1987; Schalling et al. 1987). Whether TH mRNA is absent or significantly reduced in non-functioning tumours of the complete type should be investigated further.

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