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Catecholamine-Synthesizing Enzymes Are Expressed in Parasympathetic Head and Neck Paraganglioma Tissue

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Key Words
Aromatic L-amino acid decarboxylase · Tyrosine hydroxylase · Dopamine β-hydroxylase · Head and neck paragangliomas

Abstract
Background/Aim: Increased dopamine production may be a feature of head and neck paraganglioma (HNPGL). ^{18}F-fluorodihydroxyphenylalanine positron emission tomography scintigraphy has a high sensitivity for detecting HNPGLs. These observations strongly suggest that HNPGLs have the capacity for L-3,4-dihydroxyphenylalanine uptake and conversion towards dopamine. Therefore, our aim was to demonstrate the presence of catecholamine-synthesizing enzymes, i.e. tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC) and dopamine β-hydroxylase (DBH) in HNPGL tissue. ***Methods:*** A single-center study was performed among patients who underwent surgery for HNPGL at a single university referral center between 1994 and 2012. HNPGL tissue was immunohistochemically stained for TH, AADC and DBH. Data on paraganglioma-associated germline mutations, preoperative biochemical phenotype and imaging studies were retrieved. Catecholamine excess was defined as preoperative plasma and/or urinary levels of metanephrine, normetanephrine or 3-methoxytyramine above the upper reference limit. ***Results:*** Nineteen HNPGLs from 18 patients were evaluated. All tumor tissues (100%) stained positive for AADC, 6 (32%) for TH and 2 (11%) for DBH. Of 3 HNPGLs staining positive for DBH, 2 were also positive for AADC and TH. Catecholamine excess was only present in 1 patient (5%). The HNPGLs of this single patient only showed positive staining for AADC. ***Conclusions:*** Catecholamine-synthesizing enzymes, in particular AADC, are expressed in the majority of HNPGL tissues.

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Introduction

Paragangliomas (PGLs) of the head and neck (HNPGLs) are rare neuroendocrine tumors that arise from parasympathetic paraganglia in the head, neck and mediastinal region [1]. These tumors are designated according to their anatomical location as carotid body PGLs, jugulotympanic PGLs or vagal PGLs [1]. HNPGLs are often

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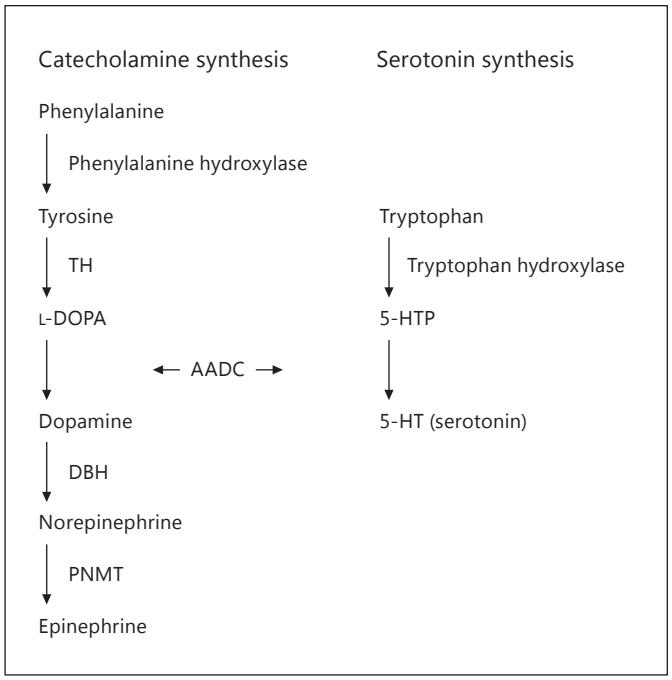

Fig. 1. Metabolic pathways in catecholamine and serotonin synthesis. AADC converts both L-DOPA to dopamine and 5-HTP to 5-HT or serotonin.

associated with germline mutations in the von Hippel-Lindau *(vHL)*, succinate dehydrogenase *(SDH)*, subunit A *(SDHA)*, subunit B *(SDHB)*, subunit C *(SDHC)*, subunit D *(SDHD)*, assembly factor 2 *(SDHAF2)* or transmembrane protein *(TMEM)* 127 genes [2–5].

HNPGLs lack the characteristic norepinephrine and epinephrine production of pheochromocytomas, but excess dopamine secretion, as reflected by elevated dopamine and/or 3-methoxytyramine (3-MT) in urine or plasma, has been demonstrated in 19–28% of patients with a HNPGL [6–9]. The first step of catecholamine biosynthesis is the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH; EC 1.14.16.2), which is the rate-limiting step in catecholamine synthesis. This is followed by the conversion of L-DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28), as illustrated in figure 1. In addition, AADC converts 5-hydoxytryptophan (5-HTP) to 5-hydroxytryptamine (5-HT, serotonin). Conversion of dopamine into norepinephrine and epinephrine is catalyzed by the activity of dopamine β-hydroxylase (DBH; EC 1.14.17.1) and phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28), respectively [10, 11]. Of interest, recent studies have suggested that 6-[^{18}F]-fluoro-L-3,4-dihydroxyphenylalanine positron emission tomography (^{18}F-DOPA-PET) has a high sensitivity for the detection of HNPGLs [12]. Uptake of this tracer by the large amino acid transporter 2 into the neuroendocrine cells is followed by decarboxylation by AADC to ^{18}F-fluorodopamine, which is subsequently stored in intracellular vesicles by the vesicular monoamine transporter [12, 13, 15–18]. Thus far, little is known about the presence of AADC in HNPGL tissue. In one report, AADC was immunohistochemically demonstrated in 3 carotid body PGLs [19].

The aim of the present study was to examine whether the catecholamine-synthesizing enzymes TH, AADC and DBH are expressed in HNPGL tissue. Based on the biochemical secretion profile, one would expect expression of TH and AADC, but not of DBH. Demonstration of AADC would support the contention that these tumors are capable of synthesizing dopamine.

Materials and Methods

Study Population

Paraffin-embedded tumor samples were obtained from HNPGL patients who had undergone surgery for biopsy or tumor resection at the University Medical Center Groningen between 1994 and 2012. The preoperative diagnosis of a HNPGL was based on clinical symptoms, the original report of anatomical imaging studies (i.e. computed tomography or magnetic resonance imaging) or nuclear imaging studies [i.e. ^{111}In-octreotide, ^{123}I-metaiodobenzylguanide (MIBG) or ^{18}F-DOPA-PET]. Preoperative data on catecholamine excess were obtained from medical charts. The tissue samples used in this study were obtained from archival material. Therefore, no further Institutional Review Board approval was required, according to the Dutch Medical Research Involving Human Subjects Act. Informed consent for the tissue staining of catecholamine-synthesizing enzymes was obtained from all patients who were still alive.

Laboratory Analysis

Isotope dilution mass spectrometry-based measurements were used for urinary and/or plasma metanephrine, normetanephrine and 3-MT levels. Urinary deconjugated metanephrine concentrations were determined by isotope dilution gas chromatography mass spectrometry, as described by Kema et al. [20]. Urinary deconjugated metanephrine concentrations were normalized to the urinary excretion of creatinine, measured using a picric acid-based method before 2005, or measured using an enzymatic method after 2005 (Roche Diagnostics, Almere, The Netherlands), and expressed in units of micromoles per mole creatinine. Reference intervals for urinary metanephrines were as follows: metanephrine 33–99 μmol/mol creatinine, normetanephrine 64–260 μmol/mol creatinine and 3-MT 45–197 μmol/mol creatinine, as previously reported by Willemsen et al. [21].

The plasma free metanephrine assay was performed with a High-Performance Liquid Chromatography tandem mass spec-

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trometric technique (HPLC-MS/MS) with automatic solid-phase extraction sample preparation, as described by de Jong et al. [22]. Established reference intervals for plasma free metanephrines were as follows: metanephrine 0.07–0.33 nmol/l, normetanephrine 0.23–1.07 nmol/l and 3-MT <0.17 nmol/l [22]. Measurement of plasma and urinary metanephrine concentrations was performed without prior dietary restrictions.

Excess catecholamine secretion was defined as preoperative plasma and/or urinary metanephrine, normetanephrine or 3-MT exceeding the upper reference limit.

Immunohistochemistry

Immunohistochemistry was performed using the Envision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (No. K4065; Dako, Glostrup, Denmark), as previously described [23]. In brief, paraffin-embedded sections, mounted on 3 aminopropyltriethoxysilane, were deparaffinized according to standard procedures, which was followed by a 20-min microwave pretreatment in Tris-EDTA buffer (pH 9.0). Then, the slides were incubated overnight at 4°C with anti-TH polyclonal antibodies (raised in rabbits with the use of rat TH; Chemicon International; 1:1,000 dilution), anti-AADC polyclonal antibodies (raised in rabbits; Chemicon/Millipore AB136; 1:100 dilution) and anti-DBH polyclonal antibodies (AB63939; ABCAM; 1:100 dilution). As controls we used healthy human adrenal medulla for each of the respective staining procedures for TH, AADC and DBH. Negative controls were performed by omission of the primary antibody.

Assessment of Immunohistochemistry

The immunohistochemical sections were evaluated by an expert pathologist (R.R.d.K.). In addition to determining the site of intracellular staining within the tumor, the proportion of immunoreactive cells was categorized into 4 groups: 1–25, 26–50, 51–75 or 76–100% positive cells. The intensity of staining was classified using a 3-level scale, ranging from negative to weakly positive or strongly positive. The assessments were made without knowledge of the catecholamine secretory profile. For all samples, the diagnosis (HNPGL) was evident from the available tissue specimens.

Statistical Analysis

Data are presented as mean ± standard deviation or as median with interquartile range where appropriate. Descriptive analyses were performed with SPSS statistics (version 22.0; IBM/SPSS, Armonk, N.Y., USA).

Results

Patient Characteristics

In 18 patients, a total of 19 HNPGLs were resected. Information about the location of tumors, catecholamine secretion and results of nuclear imaging studies are provided in table 1. HNPGLs were classified as sporadic in case of a negative family history and the absence of a germline mutation in one of the susceptibility genes. All patients with a sporadic mutation were negative for *SDHB* and *SDHD* germline mutations. In addition, a proportion of patients with a sporadic HNPGL were also found to be negative for a germline mutation in one of the following susceptibility genes: *SDHC* (patient No. 1, 5–7, 13–15, 17, 18), *SDHA* (No. 5–7, 14, 15, 18), *SDHAF2* (No. 1, 5–7, 14, 15, 18), *TMEM127* (No. 7, 14, 18), *MAX* (No. 7, 14, 18), *vHL* (No. 7, 14, 18) and *RET* (No. 7, 14, 18).

Table 1. Characteristics of the 18 patients

Sex, male/female	5/13
Age at first operation, years	55±15
Tumor location	
Vagal PGL	3
Jugulotympanic PGL	13
Carotid body PGL	3
Germline mutations	
SDHB (del exon 3/c.292T>C)	5 (4/1)
SDHD (c.292T>C)	1
Sporadic	12
Nuclear imaging (positive/negative)	
^{123}I-MIBG	4/9
^{111}In-octreotide	12/1
^{18}F-DOPA-PET	5/1
Preoperative biochemistry	
Urinary MN, µmol/mol creatinine	47 [35–69]
Urinary NMN, µmol/mol creatinine	126 [89–172]
Urinary 3-MT, µmol/mol creatinine	86 [59–107]
Plasma MN, nmol/l	0.2 [0.14–0.28]
Plasma NMN, nmol/l	0.73 [0.59–0.88]
Plasma 3-MT, nmol/l	0.06 [0.06–0.07]

Values are expressed as number, mean ± SD or median [interquartile range]. MN = Metanephrine; NMN = normetanephrine.

Immunohistochemistry

Results for the immunohistochemical staining of TH, AADC and DBH are listed in table 2. All tumor tissues (100%) were positive for the AADC enzyme (13 strongly positive and 6 weakly positive), 6 (32%) stained positive for the TH enzyme (all strongly positive), and 2 (11%) for the DBH enzyme (all strongly positive). Representative examples of immunohistochemical staining for these three catecholamine-synthesizing enzymes are shown in figure 2. The percentage of tissue staining per enzyme category is illustrated in figure 3. One of the 2 tissues staining positive for DBH was also positive for TH and AADC. All 6 HNPGLs that stained positive for TH also stained positive for AADC (100%). In contrast, there were 13 HNPGLs staining positive for AADC (65%) that were negative for TH.

There was 1 patient in whom 2 HNPGLs (No. 4 and 18) had been surgically removed (1 jugulotympanic and

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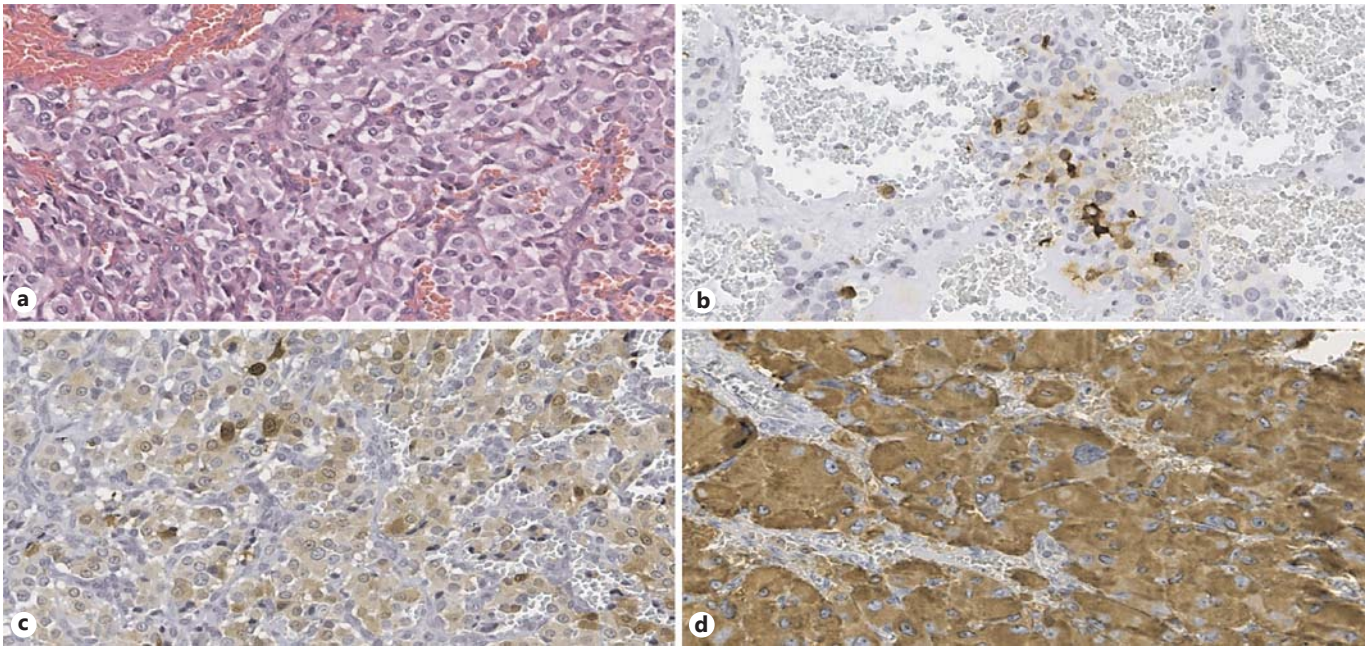

Fig. 2. Immunohistochemical staining for catecholamine-synthesizing enzymes in HNPGL of case 1 (see table 2). **a** HE staining. **b** Positive TH staining, 1–25% positive cells. **c** Positive AADC staining, 26–50% positive cells. **d** Positive DBH staining, 25–50% positive cells.

Table 2. TH, AADC and DBH staining in the different tissue samples

Tissue No.	Tumor localization	Genetic mutations	TH staining		AADC staining		DBH staining		Catecholamine	
			intensity	positive cells	intensity	positive cells	intensity	positive cells	sampled	excess, µmol/mol creatinine
1	carotid body PGL	sporadic	+	1	+/–	2	+	2	urine	–
2	carotid body PGL	SDHB	–	–	+	4	–	–	plasma, urine	–
3	carotid body PGL	SDHB	+	1	+	4	–	–	plasma	–
4*	jugulotympanic PGL	SDHB	+	4	+	4	–		urine	–
5	jugulotympanic PGL	sporadic	–	–	+/–	2	–	–	plasma, urine	–
6	jugulotympanic PGL	sporadic	–	–	+/–	4	–	–	urine	–
7	jugulotympanic PGL	sporadic	+	4	+/–	4	–	–	urine	MN 107
8	jugulotympanic PGL	SDHD	–	–	+	4	–	–	plasma, urine	–
9	jugulotympanic PGL	sporadic	–	–	+	3	–	–	plasma, urine	–
10	jugulotympanic PGL	sporadic	–	–	+	4	–	–	plasma, urine	–
11	jugulotympanic PGL	SDHB	+	4	+	4	–	–	plasma, urine	–
12	jugulotympanic PGL	SDHB	–	–	+/–	3	–	–	plasma	–
13	jugulotympanic PGL	sporadic	–	–	+	4	–	–	plasma	–
14	jugulotympanic PGL	sporadic	–	–	+	4	–	–	urine	–
15	jugulotympanic PGL	sporadic	–	–	+	4	–	–	NA	NA
16	jugulotympanic PGL	sporadic	–	–	+/–	4	–	–	urine	–
17	vagal PGL	sporadic	–	–	+	4	+	1	urine	–
18*	vagal PGL	SDHB	+	2	+	4	–	–	urine	–
19	vagal PGL	sporadic	–	–	+	4	–	–	urine	–

+ = Strongly positive staining of the tissue; +/– = weakly positive staining; – = negative staining of the tissue/no catecholamine excess. Categories: 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% positive-staining cells. MN = Metanephrine; NA = preoperative urinary and plasma metanephrine data were not available for this patient. * Tissue samples obtained from the same patient.

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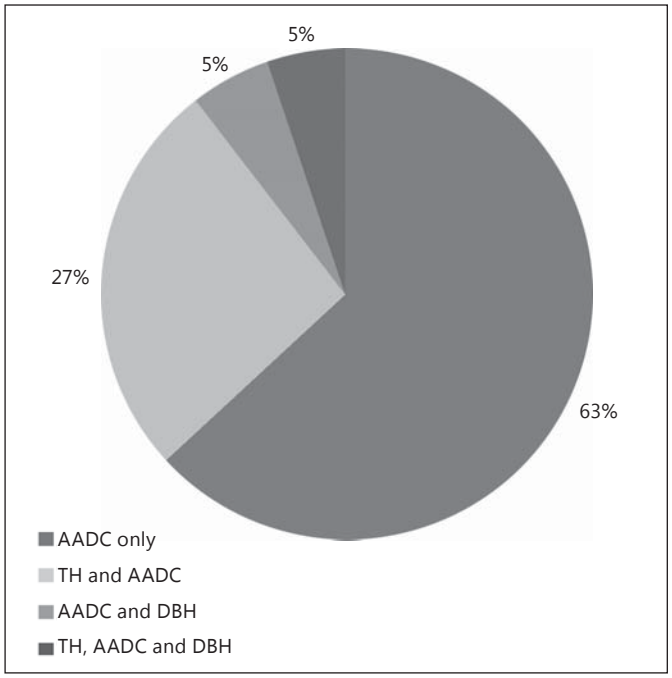

Fig. 3. The percentage of tissues with positive staining for each enzyme.

1 vagal PGL), and there was 1 patient in whom a biopsy was performed before surgical excision of the tumor (No. 7). The biopsy tissue (not included in table 2) stained strongly positive for TH and AADC in 76–100% of cells and strongly positive for DBH in 1–25% of cells. In this patient, there was a discrepancy in DBH staining between the biopsy and tumor.

Immunohistochemistry in Relation to Biochemistry and Imaging

Results of preoperative metanephrine measurement were available in 17 of the 18 patients (table 2). In all patients with a positive ^{18}F-DOPA-PET scan (n = 5), the tumor tissue stained positive for the AADC enzyme. There was 1 patient (No. 10) with a negative ^{18}F-DOPA-PET scan in whom the tumor tissue stained positive for AADC. This patient had a small tumor with an average diameter of 0.5 cm, which was below the detection limit of the PET camera at the time of evaluation.

There was 1 patient (No. 7) with an elevated urinary metanephrine excretion. The tumor tissue of this patient stained positive for TH and AADC, but was negative for DBH. Of notice, preoperative imaging studies with ^{123}I-MIBG and ^{111}In-octreotide did not demonstrate any lesion outside the head and neck region.

Discussion

In the present study, we show for the first time in a relatively large cohort the presence of catecholamine-synthesizing enzymes in HNPGL. AADC, TH and DBH were immunohistochemically detectable in 100, 32 and 11%, respectively, of the HNPGLs studied.

Immunoreactivity for catecholamine-synthesizing enzymes in HNPGLs has previously been studied in only four small case series [19, 24–26]. The study by Lloyd et al. [24] included 5 HNPGL tissues and showed immunoreactivity for TH and focal staining of DBH in 2 carotid body PGLs, whereas immunoreactivity for TH and DBH was negative in 3 jugular PGLs. Takahashi et al. [25] found TH immunoreactivity in 2 carotid body PGLs and 1 jugular PGL from 3 different patients. In that study, no other catecholamine-synthesizing enzymes were examined. In another case series, positive staining for TH in HNPGL tissue was demonstrated in 5 out of 8 patients [26]. Finally, in a small series of 3 patients with carotid body PGLs, 1 stained positive for TH, whereas all 3 were positive for AADC and DBH [19].

In pheochromocytomas, known for their catecholamine secretion, the presence of catecholamine-synthesizing enzymes has been demonstrated in large series by both immunohistochemical enzyme staining as well as by measurement of enzyme-specific mRNA expression. Kimura et al. [19] showed positive immunohistochemical staining of all these 3 catecholamine-synthesizing enzymes in 50 pheochromocytoma tissue samples, whereas Meijer et al. [27] described positive TH staining in all 20 (100%) and DBH in 15 out of 20 (75%) pheochromocytoma tissues samples. AADC staining, however, was not performed in that study.

We did not analyze catecholamine or metanephrine content in the tumor tissue. Previously, a positive correlation has been demonstrated between epinephrine and norepinephrine contents in pheochromocytoma tissue and metanephrine and normetanephrine levels in plasma or urine [28]. However, data on catecholamine content in HNPGLs are very limited. There are only a few cases reporting the presence of norepinephrine with or without epinephrine in HNPGL tissue [29].

The immunohistochemical staining pattern of AADC, DBH and TH in HNPGL is compatible with the biochemical catecholamine secretion profile generally found in these tumors, i.e. predominantly hypersecretion of dopamine, whereas hypersecretion of norepinephrine only occurs in rare instances [6–8]. In our study, the immunohistochemical staining did not correlate with the bio-

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chemical staining profile, as none of our patients had an elevated plasma 3-MT. This is at variance with a previous report by van Duinen et al. [8], who found an elevated plasma 3-MT in 28% of patients with HNPGL. Notably, the plasma metanephrines in that study were determined in our laboratory using exactly the same assay. This variation in plasma 3-MT concentrations might be explained by differences between the populations studied. The study by van Duinen et al. [8] included mainly *SDHD* mutation carriers (n = 86; 69%), whereas most patients in our study had sporadic tumors (n = 12; 67%). Dopamine production has been shown to be particularly prevalent among *SDHD* mutation carriers [30]. This might result from an indirect stimulatory effect of SDHx mutations on the TH enzyme activity. The *SDHA*, *-B*, *-C* and *-D* genes encode the four subunits of SDH, which together form the mitochondrial complex II. A mutation in one of these subunits has an inhibitory effect on prolyl hydroxylase domain proteins and diminishes the degradation of hypoxia-inducible factor α. This might have an effect on the dopamine secretion [31, 32].

The normal plasma 3-MT concentrations in combination with the positive AADC staining of HNPGL tissue in all our patients suggests that the locally produced dopamine is not released into the circulation but is mainly effective in an autocrine and/or paracrine fashion. Alternatively, it could be related to a lack of sufficient sensitivity of the biochemical methods used to measure dopamine production. Free circulating dopamine is rapidly incorporated by circulating platelets and metabolized by circulating COMT. Therefore, the biochemical diagnosis of HNPGL might be improved through development of a highly sensitive dopamine or plasma 3-MT assay.

Another discrepancy was found in a patient (No. 7) with an elevated urinary metanephrine excretion, despite negative immunohistochemical staining for DBH of the HNPGL tissue. Scintigraphy with ^{123}I-MIBG and ^{111}In-octreotide in this patient did not demonstrate a PGL/pheochromocytoma location outside the head and neck region, but the results of these imaging modalities might have been false-negative. In contrast, the absence of catecholamine secretion despite the presence of DBH staining in 15% of cases could be caused by a low catalytic activity of DBH. We can only speculate about the factor(s) that contributed to this discrepancy, but a lack of vitamin C, the cofactor for DBH, could be one possible explanation.

The presence of AADC in HNPGL cells is in agreement with the notion that these cells are part of the amine precursor uptake and decarboxylation (APUD) concept as postulated in the late 1960s by Pearse [29]. Three cases were reported of a carcinoid APUDoma arising within a carotid body PGL or jugulotympanic PGL [30–32]. APUDomas decarboxylate biogenic amines, which could imply that HNPGLs have the ability for uptake and decarboxylation of L-DOPA or 5-HTP and to store the resultant dopamine or serotonin into intracellular vesicles (fig. 1). Serotonin production, measured as its metabolite 5-hydroxyindolacetic acid, has not been shown in patients with HNPGLs [33]. However, platelet serotonin level is a more sensitive method to detect the serotonin secretion [34]. This has not yet been studied in these patients. In addition, the presence of AADC in HNPGL is in agreement with the high sensitivity of ^{18}F-DOPA-PET in patients with HNPGL [12, 13, 15, 17]. Intracytosolic conversion of ^{18}F-DOPA into ^{18}F-dopamine by AADC enhances the efficacy of ^{18}F-DOPA as a tracer [35].

Our study has several limitations. The immunohistochemistry results might have been affected by sampling error, as illustrated by the different staining patterns in biopsy material compared with the primary tumor that was observed in 1 HNPGL patient (No. 7). Alternatively, this might reflect the presence of heterogeneous cell populations in a single patient with HNPGL. We were not able to examine the relationship between specific germline mutations and tissue staining pattern for catecholamine-synthesizing enzymes, due to the few patients with a hereditary HNPGL in our series.

In conclusion, catecholamine-synthesizing enzymes, especially AADC, are present in HNPGLs. As AADC catalyzes the conversion of L-DOPA to dopamine, our findings raise the possibility that the development of a more sensitive assay for the detection of dopamine overproduction might improve the biochemical diagnosis of HNPGLs.

Disclosure Statement

The authors have nothing to disclose.

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