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Original contribution

Increased SSTR2A and SSTR3 expression in succinate dehydrogenase–deficient pheochromocytomas and paragangliomas☆,☆☆

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Summary Many neuroendocrine tumors, including pheochromocytomas (PCs) and paragangliomas (PGLs), express one or more somatostatin receptors (SSTR1-5). A number of studies have reported SSTR expression in PCs and PGLs. However, receptor expression patterns have been conflicting, and until recently, specific monoclonal antibodies were not available against SSTR1-5. The aim of this study was to compare SSTR1-5 expression in succinate dehydrogenase (SDH)–deficient PCs and PGLs (defined as having absent SDHB immunostaining) to those tumors with normal SDHB staining. Immunohistochemistry for SDHB and SSTR1-5 was performed using specific monoclonal antibodies on archived formalin-fixed, paraffin-embedded tissue from patients who had undergone surgery for PC or PGLs. A total of 182 PC/PGLs were included (129 adrenal, 44 extra-adrenal, 9 metastases); 32 tumors were SDH deficient, whereas 150 tumors had positive SDHB staining. SDH-deficient tumors were more likely to demonstrate moderate or strong staining for SSTR2A and SSTR3 when compared with SDH-sufficient tumors (91% versus 49% [$P < .0001$] and 50% versus 21% [$P = .0008$], respectively). Immunostaining for the other SSTRs was not different between SDH-deficient and tumors with preserved SDHB staining. SSTR2A and SSTR3 are more likely to be expressed in SDH-deficient PC/PGLs as compared with tumors demonstrating normal SDHB staining pattern. These findings suggest that the role of somatostatin analogue therapy

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(unlabeled or radiolabeled) should be reexamined in the context of the underlying SDHB immunohistochemistry pattern.

1. Introduction

Pheochromocytomas (PCs) and paragangliomas (PGLs) are rare catecholamine-producing tumors arising from chromaffin cells in the adrenal glands or extraadrenal neural crest tissue. These catecholamine-secreting tumors are frequently hereditary, resulting from germline mutations in various tumor predisposition genes. Of these genes, *succinate dehydrogenase subunit B* (*SDHB*) is of particular interest due to increased malignant potential of associated PC/PGLs (metastatic rate of 30% versus 10% of all PC/PGLs) [1]. Determining whether PC/PGLs are benign or malignant in the absence of metastatic disease is limited by lack of reliable criteria to predict malignant behavior. Currently, the only effective treatment of PC/PGLs is surgery. Patients with inoperable malignant PC/PGLs may die of metastatic disease or from complications due to excess catecholamine production such as sudden death or heart failure. Nonsurgical treatment options for malignant disease are limited, and there is a lack of survival data available from randomized controlled trials using chemotherapy and radiolabeled therapies [2–4], in part resulting from the rarity of these tumors. Improved understanding of the biology of SDHB-associated and/or malignant PC/PGLs would assist in identifying new nonsurgical therapies.

Many neuroendocrine tumors, including PC/PGLs, express one or more somatostatin receptor subtypes (SSTR1-5). Somatostatin is a neuropeptide with affinity for all 5 receptor subtypes and can inhibit both hormone secretion and cell proliferation. Analogues of somatostatin (octreotide, octreotide LAR, and lanreotide) demonstrate high affinity for SSTR2 and, to a lesser extent, SSTR5. Although they are very successfully used in the treatment of some tumors types, such as SSTR2-expressing growth hormone–secreting pituitary tumors, and have been demonstrated to not only control symptoms but also increase progression-free survival in patients with metastatic small intestinal neuroendocrine tumors [5,6], treatment for patients with chromaffin cell tumors has been variable and overall disappointing [7–13].

A number of studies have assessed SSTR subtypes in PC/PGL [14–26]. Results have been conflicting as to the frequency of receptor subtype expression. Although SSTR3 has been detected in most tumors studied, SSTR2A expression has varied from less than 15% [15,16] to up to 100% of tumors [18,24]. Similarly, results for SSTR1 have varied, and when assessed, SSTR5 has been shown to be positive in less than 50% of tumors in most studies [14–16,18]. Differences in SSTR subtypes between tumors from patients with familial tumor syndromes and those with sporadic tumors have only been assessed in one study with only small numbers of hereditary tumors, and differences in SSTR expression were not identified [18]. A further study identified SSTR2a staining in 2 patients with a germline *SDHD* mutation, but there was no control group [25]. Studies assessing SSTR expression have used a variety of methods including reverse transcriptase polymerase chain reaction and immunohistochemistry (IHC). In addition to varying methods used, until recently interpretation has further been hampered by the lack of specific monoclonal antibodies against the 5 SSTR subtypes. Fischer et al [27] briefly reported the use of the monoclonal antibody UMB-1 against SSTR2A in a number of normal and neoplastic tissues including PCs, in which most tumors demonstrated positive staining. Use of monoclonal antibodies against the other SSTRs has not been reported in PC/PGLs. Recently, a novel somatostatin analogue has been developed, pasireotide, which has activity at a wider range of SSTRs than octreotide (all SSTRs with the exception of SSTR4) [28]. The role of pasireotide in patients with metastatic and/or inoperable PC/PGL is not known, but cell culture studies have suggested that it is more promising than octreotide [21]. Based on the results of SSTR status, evidence of expression of SSTRs other than SSTR4 would support a targeted trial of this agent (unlabeled and/or labeled to radionuclides) in patients with metastatic/inoperable PC/PGL.

The aims of this study were (1) to assess the somatostatin receptor status of PC/PGLs using specific monoclonal antibodies against somatostatin receptor subtypes 1 to 5 and (2) to determine whether somatostatin receptor subtype expression varies in SDH-deficient tumors when compared with tumors showing a normal pattern of SDHB staining.

2. Materials and methods

Patients who had undergone previous surgery for PC or PGL were identified from the Waikato Hospital endocrine unit, Hamilton, New Zealand, and the Royal North Shore Hospital anatomical pathology department, Sydney, Australia. Ethical approval was obtained from the Northern Y Regional Ethics Committee (NTY/11/05/049) and in accordance with the Human Tissue Act for the New Zealand and Australian samples, respectively. Results of germline genetic testing for PC/PGL predisposition genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *RET*, *VHL*, *TMEM127*) were available on selected

patients. Diagnosis of PC/PGL was confirmed by clinical, biochemical, and histologic assessment.

IHC for SDHB, SDHA, and SSTRs was performed on tissue microarrays containing duplicate 1-mm cores of tissue from archived formalin-fixed, paraffin-embedded tissue blocks using rabbit monoclonal antibodies (dilutions and clones provided in Table 1). Slides were stained using the LeicaBondIII autostainer (Leica Microsystems, Mount Waverley, Victoria, Australia). Heat-induced epitope retrieval was performed at 97°C for 30 minutes in the manufacturer's acidic retrieval solution ER1 (VBS part no. AR9961). SSTR immunostaining was scored using a scheme similar to that reported by Körner et al [29]. Briefly, cases were scored as 0 (negative) if no cells demonstrated positive staining, and then semiquantitatively if staining was present from 1+ (weak staining, in <10% of cells) to 2+ (moderate staining, eg, positive at low power but not circumferential at high power) to 3+ (moderate diffuse and strong staining including circumferential staining) to 4+ (intense diffuse and strong staining including circumferential staining). Islets of Langerhans from nonneoplastic pancreas were used as external positive controls, with other tissue from the same sections including pancreatic acinar cells acting as external negative controls. Immunostaining was evaluated by a single observer (A. J. G.) who was blinded as to the results of genetic testing and SDHB IHC at the time of scoring.

Statistical analysis was performed using nonparametric statistics due to the nonnormality of the data; Kolmogorov-Smirnov 2-sample tests and difference of proportion testing, where appropriate, were performed using Statistica version 11 (Statsoft Inc, Tulsa, OK).

Table 1 Details of the antibodies used for immunohistochemical studies

Antibody	Clone	Type	Supplier	Dilution
SDHA	2E	Mouse monoclonal	Abcam, Cambridge, MA	1:1000
SDHB	21A11	Mouse monoclonal	Abcam	1:500
SSTR1	UMB-7	Rabbit monoclonal	Epitomics Inc, Burlingame, CA	1:1000
SSTR2a	UMB-1	Rabbit monoclonal	Epitomics Inc	1:100
SSTR3	UMB-5	Rabbit monoclonal	Epitomics Inc	1:300
SSTR4	ACE29616	Rabbit monoclonal	Novartis, Basel, Switzerland	1:1000
SSTR5	UMB-4	Rabbit monoclonal	Epitomics Inc	1:100

3. Results

A total of 182 PC/PGL specimens were identified from 174 patients. Of the 182 tumors, 129 were adrenal, 44 extra-adrenal (of which 18 were head and neck), and 9 metastases (all from PC).

3.1. SDH staining

Thirty-two tumors had absent SDHB staining and 2 also had loss of SDHA staining. All patients with tumors that demonstrated negative staining for SDHB (ie, "SDH-deficient") who underwent germline mutation testing were shown to have a mutation in *SDHA*, *SDHB*, *SDHC*, or *SDHD*. Of the 2 patients with negative staining for SDHA, both were found to have a germline *SDHA* mutation.

Of SDH-deficient tumors, there were 32 specimens from 30 patients (6 adrenal lesions; 22 PGLs, of which 9 were arising from the head or neck; and 4 metastases). The median age of patients with an SDH-deficient tumor was 45 years (range, 19-79 years) as compared with 52 years (range, 19-84 years) in the SDH-sufficient patients ($P < .05$).

3.2. SSTR receptor status

SSTR IHC results are shown in Table 2 and illustrated in the Figure.

3.2.1. SSTR1

One hundred seventy-nine tumors had SSTR1 staining performed. Overall, 159 (89%) of 179 of all PCs and PGLs demonstrated strong (3+) staining for SSTR1. All SDH-deficient tumors (n = 32) demonstrated strong (3+) staining, as did 127 (86%) of 147 SDH-sufficient tumors. No significant difference in SSTR1 staining was present between SDH-deficient tumors and those with normal SDH staining ($P > .10$). No differences in SSTR1 status were identified within different lesions from the same patient.

3.2.2. SSTR2a

One hundred seventy-nine tumors had SSTR2a staining performed. Overall, 82 (46%) of 179 of all PCs and PGLs demonstrated strong (3+ or 4+) staining for SSTR2a. This staining was accentuated along the membrane, but was also cytoplasmic (Figure).

SSTR2a staining was moderate (2+) or strong (3+ or 4+) in 29 (91%) of 32 SDH-deficient tumors compared with 72 (49%) of 147 SDH-sufficient tumors ($P = .0000$). This differentiation was independent of the size of the 2 tumor groups ($\chi^2 = 19.58$, $P < .001$). No staining was observed in 54 (37%) of 147 of SDH-sufficient patients as compared with 3 (9%) of 32 SDH-deficient tumors.

Comparing adrenal versus extra-adrenal lesions, PCs demonstrated moderate (2+) or strong (3+ or 4+) staining in 61 (49%) of 128 tumors (5/6 SDH-deficient PCs and 56/122 SDH-sufficient PCs); moderate or strong staining was present in 36 (77%) of 47 PGLs (20/22 SDH-deficient PGLs and 14/21 SDH-sufficient PGLs), and all 9 head and neck PGLs (HNPGL). SDH-sufficient tumors showed very strong staining (4+), as did 5/9 SDH-deficient tumors. Of the

Table 2 SSTR immunohistochemistry results

		SDH deficient			SDH sufficient			
Receptor	Grade	A	EA	Met	A	EA	Met	Total
SSTR1	0	0	0	0	0	1/21	0	1/179 (0.6%)
	1	0	0	0	5/121	0	0	5/179 (3%)
	2	0	0	0	12/121	2/21	0	14/179 (8%)
	3	6/6	22/22	4/4	104/121	18/21	5/5	159/179 (89%)
SSTR2a	0	1/6	2/22	0	48/122	5/21	1/4	57/179 (32%)
	1	0	0	0	18/122	2/21	1/4	21/179 (12%)
	2	1/6	4/22	0	9/122	3/21	2/4	19/179 (11%)
	3	3/6	3/22	2/4	15/122	0	0	23/179 (13%)
	4	1/6	13/22	2/4	32/122	11/21	0	59/179 (33%)
SSTR3	0	3/6	8/22	1/4	79/121	16/22	5/5	112/180 (62%)
	1	2/6	2/22	0	17/121	1/22	0	22/180 (12%)
	2	1/6	7/22	2/4	18/121	1/22	0	29/180 (16%)
	3	0	5/22	1/4	7/121	3/22	0	16/180 (9%)
	4	0	0	0	0	1/22	0	1/180 (0.6%)
SSTR4	0	6/6	20/22	4/4	123/123	20/21	4/4	177/180 (98%)
	1	0	1/22	0	0	1/21	0	2/180 (1%)
	2	0	1/22	0	0	0	0	1/180 (0.6%)
SSTR5	0	6/6	20/20	4/4	118/119	20/21	4/4	172/174 (99%)
	1	0	0	0	0	0	0	0/174 (0%)
	2	0	0	0	1/119	0	0	1/174 (0.6%)
	3	0	0	0	0	1/21	0	1/174 (0.6%)

Abbreviations: EA, extra-adrenal (PGL); A, adrenal; Met, metastasis.

6 patients in whom multiple tumors were studied, SSTR2a status varied in only 1 SDH-sufficient patient (1 of 2 metastases assessed had weak (1+) staining, whereas both the other metastasis and primary lesion were negative).

All 4 metastases from SDH-deficient tumors demonstrated strong SSTR2a staining compared with none of the SDH-sufficient metastases.

3.2.3. SSTR3

SSTR3 immunostaining was performed in 180 tumors. Overall, 46 (26%) of 180 had moderate (2+) or strong (3+ or 4+) staining. Moderate or strong staining was present in 16 (50%) of 32 tumors from SDH-deficient tumors compared with 30 (20%) of 148 SDH-sufficient tumors ($P = .004$). This differentiation was also independent of the group sizes ($\chi^2 = 13.84$, $P < .01$).

PCs demonstrated moderate (2+) or strong (3+ or 4+) SSTR3 staining in 26 (20%) of 127 tumors (1/6 SDH-deficient PCs and 25/121 SDH-sufficient PCs). Moderate or strong staining was present in 17 (39%) of 44 PGLs (12/22 SDH-mutated PGLs and 5/22 non–SDH mutated PGLs). There was a significantly different proportion of moderate and strongly stained PCs compared with PGLs ($P = .012$).

All 5 metastases demonstrating SDH sufficiency had absent SSTR3 staining as compared with 1 of 4 with SDH deficiency. Six patients had more than 1 tumor included. Of these, 1 SDH-deficient patient had absent SSTR3 staining in 1 PC metastasis, whereas the other metastasis had strong (3+) staining. Two SDH-sufficient patients also demonstrated variation between PC tumors (0 versus 3+ and 0 versus 1+, respectively).

3.2.4. SSTR4

SSTR4 IHC was performed in 180 tumors. Overall immunostaining was positive in 3 PGLs (2 SDH deficient). All PCs and metastases demonstrated negative staining. No significant differences were seen between SDH-deficient and SDH-sufficient tumors ($P > .10$). SSTR4 status was identical when different lesions from the same patient were compared (including metastases).

3.2.5. SSTR5

SSTR5 IHC was performed in 174 tumors. Immunostaining was positive in 2 of 174 tumors (both SDH sufficient—1 PC and 1 PGL). No significant staining differences were seen between SDH-deficient and SDH-sufficient tumors ($P > .10$). No differences in SSTR5 status were identified within different lesions from the same patient.

4. Discussion

SSTR immunostaining of PC or PGLs varies according to receptor subtype. Most tumors demonstrated positive staining for SSTR1, whereas most tumors did not stain for SSTR4 or SSTR5, irrespective of whether tumors were SDH deficient or not. SSTR2a and SSTR3 expression patterns were more variable.

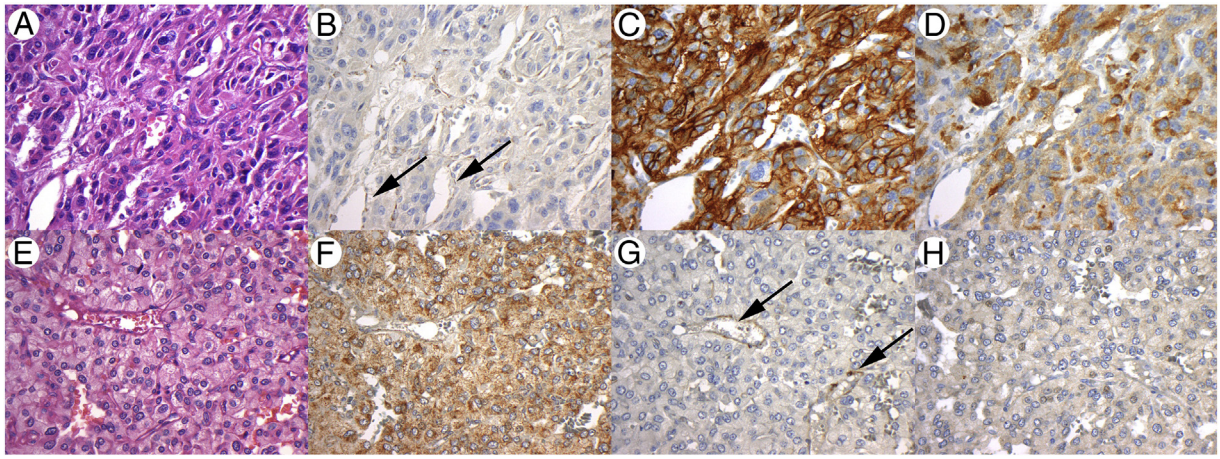

Figure Serial sections of PC/PGL stained with hematoxylin and eosin (A, E), SDHB (B, F), SSTR2A (C, G), and SSTR3 (D, H). Panels A to D show an *SDHB*-mutated PGL with completely negative staining for SDHB (B) but diffuse strong positive staining for SSTR2A (C), which is predominantly membranous, and SSTR3 (D), which is granular and cytoplasmic. In contrast, panels E to H show a PC that lacks *SDH* mutation and therefore shows positive staining for SDHB (F) and negative staining for SSTR2A (C) and SSTR3 (D). The presence of positive staining of endothelial cells (arrows) for SDHB (B) and SSTR2A (G) acts as a positive internal control in cases that otherwise show negative staining. Original magnifications ×400.

Both SSTR2A and SSTR3 staining was significantly different between SDH deficient and those with normal SDH staining patterns. We have found SDHB IHC to be a robust and reliable marker of *SDH* mutation [30,31], and all patients in our SDH-deficient cohort who underwent full genetic testing for the *SDH* genes as part of their clinical care have been shown to harbor a mutation in one of the *SDH* genes [30]. SDH-deficient tumors were more likely to demonstrate moderate or strong SSTR2a and SSTR3 staining when compared with tumors with preserved SDH staining. Of note, SSTR staining was not exclusively membranous in our cohort, as most cases demonstrated some cytoplasmic staining but with membranous accentuation (Figure)—a similar pattern to that reported by Körner et al [29] in meningiomas but not gastrointestinal neuroendocrine tumors.

Of the small numbers of patients with more than 1 tumor studied, there was some variation in SSTR3 staining, whereas the other SSTRs showed good correlation. Although data were limited, there was a suggestion that within the group of SDH-sufficient tumors, strong SSTR2a staining was more likely in HNPGLs than thoracoabdominal PGLs. Conversely, in SDH-deficient PGLs, strong SSTR2a staining was more likely in abdominal PGLs. These data would suggest that the role of somatostatin analogues, both labeled and unlabeled, should be reviewed in the context of the underlying SDH status.

The outcomes of treating patients with PCs and PGLs with somatostatin analogues have been variable and overall disappointing [7–13,32] despite in vivo data suggesting that these agents may be effective [33]. However, assessment of response to treatment based on specific SSTR subtype was not included in previous trials, as specific monoclonal antibodies against the SSTRs were not available at the time. Similarly, assessment of germline *SDH* mutation status was not reported in these trials of somatostatin analogues because the publications mostly predated widespread availability of genetic testing. A single case reported by Tonyukuk et al [12] with multiple somatostatin receptor scintigraphy (SRS)–positive HNPGLs, secretory disease, and a positive family history was most likely *SDH* associated. In this case, a partial tumor response, an improvement in performance status, and decreased frequency of "attacks" in response to octreotide therapy (short-acting, followed by depot preparation) were noted for 26 months of treatment [12]. A recent article reported a dramatic clinical and biochemical response of an SRS-positive noradrenaline- and dopamine-secreting HNPGL to high-dose long-acting octreotide, but neither the *SDH* mutation nor SSTR status was reported [34]. In articles in which SRS was performed, there appeared to be a trend to improved biochemical and/or clinical status (blood pressure, performance status) in some SRS-positive patients in response to either short-acting or depot preparations of octreotide [8,11,12], but the presence of mixed disease (both SRS-positive and SRS-negative tumors) may have affected response in some series [7]. There have been no reports specifically assessing the use of unlabeled SST analogues in *SDH* mutation–positive patients with positive SSTR2a expression. Safe, effective treatment options for patients with metastatic PC/PGL are currently very limited. The findings from the current study suggest that if a randomized controlled trial of SSTR agonists of patients with metastatic PC/PGL is performed, this should include the assessment of the underlying SSTR status and SDH status.

Pasireotide is a new somatostatin analogue, which has activity at all SSTRs apart from SSTR4 [28]. In vitro data suggest that it may be more promising than octreotide [21], but to date, there is no published literature assessing biochemical or tumor response to this agent. The current study demonstrates that *SDH*-associated tumors are more

likely to show SSTR3 immunostaining in addition to SSTR2a. If pasireotide is to be trialed in PC/PGL patients, it would seem prudent to select *SDH*-associated tumors with positive SSTR2a and SSTR3 staining because this group would seem the most likely to demonstrate benefit. Tumors with preserved SDH staining are less likely to express SSTR2A or SSTR3; therefore, pasireotide is less likely to be effective in this patient group.

Radiolabeled somatostatin analogues have been used in a number of small series of patients with metastatic or inoperable PC/PGLs [3,35–40]. Similar to the studies using unlabeled somatostatin analogues, most patients are not classified according to SSTR status and do not have their *SDH* status described. In a small case series of 4 *SDHD* germline mutation–positive patients with nonsecretory PGLs assessing the response to peptide receptor radionuclide therapy (PRRT) using (^{177}Lu) DOTATATE, there were 2 responses according to RECIST criteria and the other 2 individuals had reduced uptake on SRS [35]. At best some patients with progressive disease do appear to have partial response to PRRT [3,35–38]; however, description of secretory status of tumors is surprisingly lacking in most of these series.

SRS, using either traditional ^{111}In-octreotide or other octreotide derivatives, for example, DOTATATE, has been described to be more sensitive than metaiodobenzylguanidine scanning in patients with an underlying *SDH* mutation with a sensitivity of 69.5% versus 42.7% [41]. The higher rate of SSTR2a positivity demonstrated in this study supports the use of SRS, to complement anatomical imaging, in the diagnostic algorithm of patients with *SDH*-positive tumors.

There are a number of limitations of this study. This is a retrospective study, and fresh-frozen tissue was not available to confirm the findings using an alternative method such as Western blotting or in vitro autoradiography. No patients had received somatostatin analogues (radiolabeled or unlabeled) to see if the response correlated with the SSTR staining. Only a small number of patients had more than 1 lesion available, so we are limited in being able to assess the heterogeneity of SSTRs between tumors within the same patient. However, this study is the first to suggest that SSTR subtypes appear to vary according to SDH status. The mechanism for the difference in SSTR status is not known. It would seem prudent in those patients who are receiving PRRT to check SSTR status of all lesions in which tissue is available and to see if this correlates with treatment response (clinical, biochemical, and radiologic).

5. Conclusion

SDH-deficient tumors are more likely to demonstrate positive SSTR2a and SSTR3 immunostaining than tumors with a normal SDH staining pattern. These findings suggest that the role of somatostatin analogue therapy (unlabeled or radiolabeled) should be reexamined in the context of the underlying SDH status. Somatostatin analogue therapy may have a particular therapeutic role in patients with an underlying *SDH* germline mutation and for HNPGLs in patients without an underlying *SDH* mutation.

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