Prevalence of succinate dehydrogenase deficiency in paragangliomas and pheochromocytomas at Tygerberg Hospital: a retrospective review.

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Thesis presented in fulfilment of the requirements for the degree of Master of Medicine (MMed Anat Path) in the Faculty of Medicine and Health Sciences at Stellenbosch University

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DECLARATION

I, the undersigned, hereby declare that the work contained in this assignment is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Signature: Date: 18/03/2020

DECLARATION OF CONTRIBUTIONS

I, Cassandra Bruce-Brand, hereby declare that as the candidate/principal investigator (PI) my contributions to this project included performance of the literature review, writing of the protocol and ethics application, the interpretation of the immunohistochemical stains, data capturing of findings, interpretation of the results and the production of this final manuscript.

The contributions of the supervisor, Dr AC van Wyk, were in the conception of the idea for this study, assisting with the acquisition of funding and ethical approval, the acquisition of the immunohistochemical stain, interpretation of the stain and assistance with the formulation of the final manuscript.

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ABSTRACT

Introduction

Pheochromocytomas (PC) and paragangliomas (PGL) are rare neural crest-derived tumours that occur at adrenal and extra-adrenal sites. These tumours may be sporadic but a significant proportion are caused by familial syndromes due to germline mutations. Mutations of the succinate dehydrogenase (SDH) complex make up the bulk of syndromic cases in the international literature. SDH mutated cases are now known to have higher rates of metastatic disease, a younger age of onset, an association with other SDH mutated tumours as well as implications for first degree relatives. An immunohistochemical stain for SDHB that has excellent correlation with SDH mutation status has been developed and is routinely used in many centres to infer SDH mutation status. Loss of staining is seen when there is a mutation of any of the SDH subunit complexes. The prevalence of SDH mutated tumours is not known in the South African setting.

Methods

A retrospective laboratory-based study was conducted at Tygerberg Hospital in South Africa to assess the prevalence of SDH deficiency in all PC and PGLs between 2005 and 2015. These tumours were further stratified by other characteristics: tumour site, patient age, sex and presence of metastatic disease. Fifty-two cases met the inclusion criteria and the SDHB immunohistochemical stain was performed on these cases. Germline testing or sequencing of these cases was not performed.

Results

Thirty-six percent of cases showed loss of staining of SDHB by immunohistochemistry. Head and neck PGLs made up the bulk of cases (50%) and females were strongly represented, particularly at head and neck sites (73%). Loss of staining was significantly correlated with a younger age at presentation (z = -3.59, p < .001). There was no correlation between loss of staining and tumour site or patient sex. The inter-observer agreement in interpretation of the immunohistochemical stain was excellent (*Cohen's kappa* = 0.917, p < .001).

Conclusion

The prevalence of SDH deficiency in our setting, as shown by loss of immunohistochemical staining for SDHB, is comparable to the literature and makes up a significant proportion of our PC/PGL cases. This highlights the need for performance of this stain in our setting in order to recognise these syndromic cases. Many patients in South Africa do not have access to genetic testing upon diagnosis of a PC or PGL as this is costly and not widely available. Many studies have shown excellent correlation of the immunohistochemical stain with underlying SDH mutation status. Immunohistochemistry is widely available in South African pathology laboratories and is relatively affordable. Although interpretation of this stain can be challenging, we report excellent inter-observer agreement in a generalist pathology practice when following published guidelines for interpretation. We therefore advocate for routine use of this stain in all PC/PGL cases diagnosed in our setting.

AFRIKAANSE OPSOMMING

Inleiding

Feochromositome (FC) en paragangliome (PGL) is seldsame neurale kruin-afgeleide tumore wat voorkom in die byniere en buite die byniere respektiewelik. Hierdie gewasse kom meestal sporadies voor, maar 'n beduidende aantal gevalle word veroorsaak deur familiële sindrome as gevolg van kiemlynmutasies. Mutasies van die suksinaatdehidrogenase (SDH) -kompleks is verantwoordelik vir die meeste familiële sindrome in die internasionale literatuur. Dit is bekend dat SDH-gemuteerde gevalle ‘n hoër insidensie van metastases het, 'n jonger ouderdom van aanvang, geassosieer is met ander SDH-gemuteerde gewasse, en ook implikasies mag inhou vir eerstegraadse familielede. 'n Immunohistochemiese kleuring vir SDHB wat 'n uitstekende korrelasie met SDH-mutasie-status het, is ontwikkel en word roetinegewys in baie sentrums gebruik om die SDH-mutasie-status af te lei. Verlies van kleuring word gesien as daar 'n mutasie van enige van die SDH-subeenheidskomplekse is. Die prevalensie van SDH-gemuteerde tumour is nie bekend in die Suid-Afrikaanse konteks nie.

Metodes

'n Retrospektiewe laboratoriumgebaseerde studie is in die Tygerberg-hospitaal in Suid-Afrika gedoen om die prevalensie van SDH-gebrek in alle FC en PGL tussen 2005 en 2015 te bepaal. Hierdie tumore is verder gestratifiseer deur ander eienskappe: anatomiese verspreiding, pasiëntouderdom, geslag en teenwoordigheid van metastatiese siektes. Twee-en-vyftig gevalle het aan die insluitingskriteria voldoen, en die SDHB-immunohistochemiese kleuring is op hierdie gevalle

uitgevoer. Daar is nie 'n kiemlyntoetse of nukleotied-volgorde bepaling van hierdie gevalle gedoen nie.

Resultate

Ses-en-dertig persent van die gevalle het verlies van kleuring van SDHB deur immunohistochemie getoon. PG van die kop en nek het die grootste gedeelte van gevalle uitgemaak (50%) en die vroulike geslag was die meeste verteenwoordig, veral in die kop- en nektumore (73%). Die verlies van kleuring was beduidend gekorreleer met 'n jonger ouderdom van presentasie (z = -3.59, p <.001). Daar was geen verband tussen verlies van kleuring en anatomiese verspreiding of geslag nie. Die ooreenkoms in die interpretasie van die immunohistochemiese kleuring tussen waarnemers was uitstekend (Cohen se kappa = 0,917, p <0,001).

Gevolgtrekking

Die voorkoms van SDH-mutasies in ons konteks, soos aangetoon deur die verlies van immunohistochemiese kleuring vir SDHB, is vergelykbaar met die literatuur en maak 'n beduidende deel van ons FC / PGL-gevalle uit. Dit beklemtoon die behoefte aan die uitvoering van hierdie kleuring in ons praktyk om hierdie sindroomgevalle te herken. Baie pasiënte in Suid-Afrika het nie toegang tot genetiese toetsing tydens die diagnose van 'n FC of 'n PGL nie, aangesien dit duur is en nie algemeen beskikbaar is nie. Baie studies het uitstekende korrelasie van die immunohistochemiese kleuring met die onderliggende SDH-mutasie-status getoon. Immunohistochemie is algemeen beskikbaar in Suid-Afrikaanse patologie laboratoriums en is relatief bekostigbaar. Alhoewel interpretasie van hierdie kleuring

uitdagend kan wees, rapporteer ons 'n uitstekende ooreenkoms tussen waarnemers in 'n algemene patologiepraktyk wanneer gepubliseerde riglyne vir interpretasie gevolg word. Ons stel dus voor dat hierdie kleuring roetinegewys gedoen word in alle FC / PGL-gevalle wat in ons praktyk gediagnoseer word.

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LIST OF ABBREVIATIONS

GIST	–	Gastrointestinal stromal tumours
HNPGLs	–	Head and neck paragangliomas
IHC	–	Immunohistochemistry
NHLS	–	National Health Laboratory Service
PGL	–	Paraganglioma
PC	-	Pheochromocytoma
PA	–	Pituitary adenoma
PI	–	Principal investigator
RCC	–	Renal cell carcinoma
SDH	–	Succinate dehydrogenase
SDHB	–	Succinate dehydrogenase B subunit
SOP	–	Standard operating procedure
TAPGLs	–	Thoraco-abdominal paragangliomas
WHO	–	World Health Organisation

1. INTRODUCTION

Background information

Paragangliomas (PGLs) and pheochromocytomas (PCs) are neural crest-derived tumours that occur at adrenal and extra-adrenal sites. These tumours may be sporadic or associated with familial syndromes. Mutations of succinate dehydrogenase, a mitochondrial complex, has been demonstrated to be the genetic basis for the familial pheochromocytoma/paraganglioma syndrome. This syndrome makes up the majority of familial syndromic cases and tumours with mutations in specific subunits of the SDH complex occur at younger ages, are more likely to metastasize and may be associated with other syndrome associated tumours. Recognition of these cases is therefore important for prognostic and management purposes. Germline genetic testing is expensive and not widely available. An immunohistochemical stain against the SDHB complex was developed and loss of staining has shown excellent correlation with SDH mutation status.

Purpose of Study

The purpose of this study was to demonstrate what proportion of cases of PG/PGL in our setting are associated with SDH mutation by using IHC as a surrogate marker for SDH mutation status. We also aimed to describe the characteristics of these tumours in our setting including patient age, sex, tumour site and presence or absence of metastatic disease. Since this immunohistochemical stain was not yet available in our setting we aimed to acquire and optimise the stain and then assess the inter-observer variability in interpretation of the stain in order to infer its potential usefulness in the future.

2. LITERATURE REVIEW

2.1. Introduction

PC and PGL are rare neural crest-derived tumours that arise in the adrenal medulla and sympathetic or parasympathetic ganglia.[1,2] The World Health Organisation (WHO) 4th edition classification of endocrine tumours (2017) defines PC as a tumour of chromaffin cells that arises in the adrenal medulla.[1] Extra-adrenal PGLs are defined by the WHO as tumours originating from neural crest-derived paraganglion cells in the region of the autonomic nervous system ganglia and autonomic nerves.[1] Sympathetic PGLs are catecholamine secreting tumours and include those in the adrenal gland (PC) as well as extra-adrenal sites, predominantly the thorax and abdomen (thoraco-abdominal PGLs - TAPGLs).[1,2] Parasympathetic PGLs are extra-adrenal, do not secrete catecholamines and occur predominantly in the head and neck region (head and neck PGLs - HNPGLs).[2]

PGLs and PCs can occur sporadically or as hereditary tumours with up to 40% occurring as a result of germline mutations in susceptibility genes.[2,3] Up to 15% of apparently sporadic tumours have somatic mutations.[4,5] Currently ten such susceptibility genes have been described, all of which function as tumour suppressor genes with tumours showing loss of heterozygosity in combination with germline inactivating mutations.[5–9] PC/PGLs resulting from any of these mutations are histologically indistinguishable and there are no reliable histological features that predict malignancy.[10] Malignancy in PC and PGLs has been difficult to define and predict. Currently, metastases are the only well accepted criteria for malignancy.

According to the most recent WHO guideline, the preferred terminology is now 'metastatic' PGL/PC rather than 'malignant'.[1]

2.2. Hereditary syndromes associated with PC/PGL

Research conducted in the 19th and 20th centuries led to the recognition of three PC/PGL-associated syndromes.[11] These include von Hippel-Lindau (VHL) disease, Multiple Endocrine Neoplasia type 2 (RET) and Neurofibromatosis type 1 (NF1).[12–16] Between 2000 and 2010, the molecular basis for hereditary PC/PGL syndrome was discovered to be due to mutations in succinate dehydrogenase (SDH) subunits and related genes.[6–10,17,18] Following this discovery of the genetic basis of hereditary PC/PGL syndrome, other tumours with a lower penetrance including gastrointestinal stromal tumours (GIST), renal cell carcinomas and pituitary tumours, were found to be part of the full tumour spectrum of this genetic defect.[11] New susceptibility genes causing hereditary PC/PGL syndrome discovered over the past ten years include MAX, TMEM127, EGLN, HIF2α, MET and KIF1B.[2]

Currently these susceptibility genes are grouped into two categories; Major susceptibility genes including NF1, VHL, RET and SHDB/D and minor susceptibility genes including SDHA/C, SDHAF2, MAX, TMEM127.[5] The major susceptibility genes account for up to 90% of the hereditary tumours, the minor group accounts for the other 10%.[5]

2.3. Significance of determination of the genetic phenotype

The importance of determining the genetic phenotype of these tumours is two-fold: detection of syndromic cases and prognostication. Tumour genotypes have now been linked to specific tumour phenotypes such as their biochemical behaviour, site, clinical presentation, potential to metastasize as well as therapeutic responses.[5] Knowledge of the specific tumour genotype can therefore aid in further management of the patient in terms of prognosis, screening for other tumours, choice of therapy, genetic counselling for first degree relatives and perhaps most importantly assessing the risk of malignancy.[5,19] Although there are as yet no large trials showing data to support genotype specific therapy for metastatic disease, it has been shown that patients with mutations of SDHB respond well to CVD (cyclophosphamide, vincristine and dacarbazine) chemotherapy.[5]

Due to the above mentioned factors it is the current view of many authors that genetic testing should be offered to all patients with PC/PGL.[5] The major limiting factor in many settings is the cost of performing such broad genetic screening. Research which demonstrates links between genotype and clinical/biochemical phenotype therefore provides a means for clinicians to stratify patients based on the available data in order to more selectively perform genetic testing according to algorithmic approaches.

2.4. Succinate dehydrogenase deficiency

The succinate dehydrogenase enzyme complex (mitochondrial complex II) catalyses the conversion of succinate to fumarate in the Krebs cycle.[18] Loss of heterozygosity

with inactivating germline mutations results in lack of the SDH enzyme and destabilisation of the SDH protein complex leading to an accumulation of succinate.[18,20–22] This results in reactive oxygen species with free radical damage as well as disturbance of hypoxia inducible factor alpha (HIFα).[18,20–22] The complex consists of four subunits (*see Figure 1*) – SDHA, SDHB, SDHC and SDHD, any of which may be causative in hereditary PC/PGL.[5,17,23] SDHAF2, a mitochondrial protein which flavinates SDHA, is essential for formation of the SDH complex and is also implicated in familial PC/PGLs when mutated.[18,24]

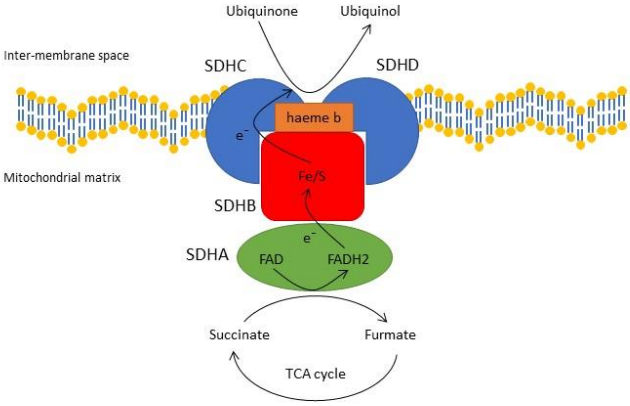


Figure 1. Structure and function of the succinate dehydrogenase complex – included with the authors permission[23]

Currently the PGL syndromes associated with SDH-mutations are grouped into five types according to the subunit which is mutated *(see table 1*). Each of these groups, numbered PGL1-5, are associated with a different spectrum of SDH-related tumours including PC, TAPGLs, HNPGLs, GIST, RCC and PA.

Table 1. SDH associated familial paraganglioma syndromes

Syndrome	**PGL1**	**PGL2**	**PGL3**	**PGL4**	**PGL5**
SDH subunit	SDHD	SDHAF2	SDHC	SDHB	SDHA
Tumour most commonly found	HNPGL	HNPGL	HNPGL SDH-deficient RCC	TAPGL SDH-deficient RCC	SDH-deficient GIST

The reported rate of SDH mutations in PGLs varies significantly between series, between 15% and 54%.[17,25] Mutations in SDHB and D are the most common of the four subunits and correspond to syndromes PGL4 and PGL1 respectively.[2] SDHB mutated tumours (PGL4) are usually abdominal and have the highest risk of metastases. Up to 71% of paragangliomas with SDHB mutations have been shown to metastasize compared to only 3% of non-SDH mutated cases.[10,17] Furthermore, SDHB mutations, which show incomplete penetrance, result in tumours at younger ages.[26] In contrast, tumours with SDHD mutations are typically found in the head and neck region, are multiple and recurrent with a very low rate of metastases.[2]

According to the SDH mutation database (an online resource capturing all reported SDH sequence variants involved in PC/PGL) up to 289 sequence variants of SDHB have currently been described with the majority of these mutations being missense mutations.[27]

2.5. Mechanisms of tumorigenesis in SDH deficient tumours

As mentioned above, mutations in SDH result in failure to inactivate HIFα.[28] Although the mechanisms of tumorigenesis in SDH deficient tumours are still not

entirely clear, activation of the hypoxia response pathway is believed to play a role. Accumulation of succinate results in triggering of downstream signals that simulate hypoxia resulting in expression of genes involved in angiogenesis.[28–31]

2.6. Head and neck PGLs

Head and neck PGLs account for the majority of PGLs and have exceptionally high rates of SDH mutations with Mannelli reporting a rate of 31%.[32] SDHD mutations are the most common of the SDH mutations in HNPGLs.[32–34] Tumours with SDHD mutations have higher rates of multifocality than tumours due to other SDH mutations.[33,34] SDHD mutations also occur in apparently sporadic HNPGLs.[34] SDHD is maternally imprinted and therefore tumours only develop when the gene is paternally inherited (parent of origin mode of inheritance).[10,32,34] This pattern of inheritance is not seen with SDHA and SDHB.

2.7. Thoraco-abdominal PGLs

TAPGLs are not as common as PCs and HNPGLs however their rate of malignancy is high.[32] TAPGL were found to be associated with SDH mutations in 33%[32] and 41%[35] of cases in two studies. The majority of these mutations were of SDHB.[32,35] As stated previously SDHB mutation are associated with high rates of metastases ranging from 38-71%.[10,17,35,36]

2.8. Spinal paragangliomas

Central nervous system PGLs are rare tumours that most often occur in the cauda equina region of the spinal canal. Little information is available regarding the association of spinal PGLs with SDH mutations or with other PGL associated syndromes. A study that investigated 22 spinal PGLs for SDHD mutations showed only one case to be associated with germline SDHD mutations.[37]

2.9. Pheochromocytoma

The rate of SDH mutations in PCs ranges from 2.8%[32] to 4.5%[35] with equal numbers attributable to SDHB and SDHD. These patients develop tumours at a younger age and have higher rates of bilateral disease.[10]

2.10. Immunohistochemistry (IHC) for SDHB

Despite algorithmic approaches to better target patients for specific genetic testing and thereby reduce costs, the burden of genetic screening is still high.[19] In 2009 it was reported that IHC for SDHB could be used to identify cases with underlying SDH germline mutations.[38] Destabilisation of any of the four subunits of SDH can be detected by immunohistochemical analysis for SDHB.[38] SDHB immunohistochemistry has therefore emerged as a more cost effective method to 'triage' genetic testing of SDH genes as it selects out patients who can then undergo further confirmation of the presence of SDH gene mutation.[19,38]

Inactivation of any of the SDH genes (A, B, C or D) result in loss of enzymatic activity and therefore loss of staining of SDHB. Negative staining indicates that any of the four subunits of SDH may be mutated and then allows for further more targeted genetic testing.[19] Several studies have demonstrated that SDH IHC has a high diagnostic accuracy (sensitivity and specificity of up to 100% reported in some studies)with low inter-observer variability and with a good negative predictive value.[19,38] A large study using web based virtual microscopy showed substantial inter-observer agreement in interpretation of SDHB immunohistochemistry with kappa values of 0.7338.[39]

Mutations of one of the SDH subunits are almost always due to a germline mutation and are very rarely somatic.[39–42] The detection of these cases is therefore of paramount importance as loss of immunohistochemical staining in these cases therefore signifies likely syndromic disease due to germline SDH mutations or more rarely, hypermethylation of SDHC.[43]

2.11. Interpretation and reporting of SDHB IHC

Correct interpretation of SDHB IHC is crucial if it is to be a useful diagnostic tool. There are potential pitfalls in interpretation and strict guidelines for correct interpretation have been advised.[10,44] SDHB IHC should be interpreted as positive/retained if the staining is granular cytoplasmic within the tumour cells (as it is a mitochondrial enzyme), even if this staining is patchy. Negative staining or loss of staining is demonstrated by loss of cytoplasmic staining in the tumour cells with retention of staining in sustentacular cells and endothelium (positive internal controls). The entire tumour must lack staining for a result to be interpreted as

negative/lost. Staining is said to be equivocal if there is a cytoplasmic blush or only focal positivity. False negatives can be avoided by well-defined internal controls and following clear and strict protocols.

Conventional “positive” and “negative” descriptors used when reporting the results of immunohistochemical staining can be confusing when applied to stains where positive staining is a normal result and negative staining is abnormal. It is therefore advised that these results should be clearly reported in the pathology report as normal intact staining or abnormal loss of staining.[45]

2.12. SDH deficiency in South Africa

Currently the percentage of PC/PGLs with mutations of SDH in our setting is unknown. To the authors’ knowledge no published studies have been conducted in South Africa to determine if the prevalence rates locally are comparable to that reported in international literature.

2.13. Hypothesis

We hypothesize that the prevalence of SDH deficiency in PC and PGLs at Tygerberg Hospital in South Africa is similar to international figures (between 15 and 54%).

3. AIMS

3.1. Primary aim

The main aim of this study was to determine the prevalence of succinate dehydrogenase deficiency based on loss of immunohistochemical staining for SDHB in biopsy and resection specimens of PGL and PC between 2005 and 2015 at Tygerberg Hospital in Cape Town.

3.2. Secondary aims

The secondary aims were to stratify paragangliomas by their location/site into HNPGL, TAPGL and other and to compare the prevalence of loss of staining of SDHB by site.

Other aims were to compare SDH status with clinical parameters including age, sex and clinical behaviour and to assess the inter-observer agreement in interpretation of the immunohistochemical stain.

3.3. Motivation for this study

Currently the percentage of PC/PGL with mutations of SDH in our setting is unknown. If the prevalence is found to be equivalent to studies conducted elsewhere, then genetic and IHC testing for the mutation on a routine basis might prove valuable. As discussed above the implications of loss of SDH staining are of prognostic value and allow for recognition of syndromic cases. Early detection of syndromic disease in individual patients and their family members with screening for development of metastases and/or other SDH related tumours may have lifesaving

implications. Immunohistochemistry is widely available in South Africa, relatively easy to perform and interpret and significantly more cost effective than genetic testing. Development of this test in our setting, once the prevalence is known, is therefore potentially beneficial to patients, their families and the clinical team.

4. METHODS

4.1. Methodology

4.1.1. Study design

This was a retrospective descriptive laboratory-based study.

4.1.2. Inclusion criteria

Biopsy and resection specimens from patients diagnosed with PGL and/or PC between 2005 and 2015 at Tygerberg Hospital were included.

4.1.3. Exclusion criteria

Cases of PGL and PC where the tissue wax blocks could not be retrieved from the archive were excluded from this study. Cases in which there was disagreement about the diagnosis of PC/PGL upon review were also excluded.

4.1.4. Sample size

A total of 65 cases of PC/PGL between 2005 and 2015 were identified. Four patients had multiple specimens of PC/PGL, either recurrences or metastases (three patients had three cases each, one patient had two cases). Only one case per patient was included as SDH mutations are almost exclusively germline and the presence of an SDH mutation would therefore be present in all PC/PGLs from the same patient. A total of 58 patients were therefore identified. A further six cases were excluded – one in which the preferred diagnosis was a neuroendocrine tumour and five for which the wax blocks could not be retrieved. A total of 52 cases were therefore included in the final sample (*see Figure 2*).

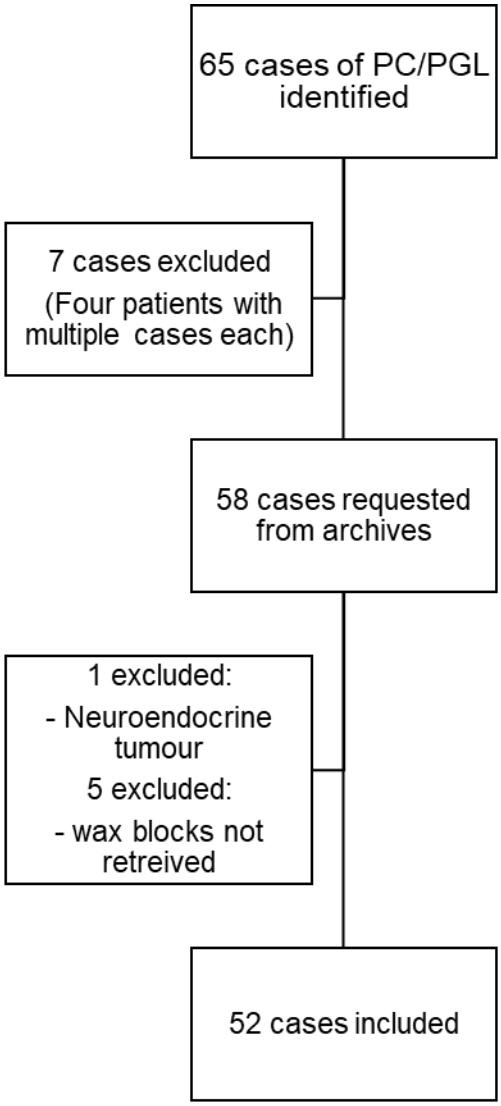


Figure 2. Flow diagram of the case selection process.

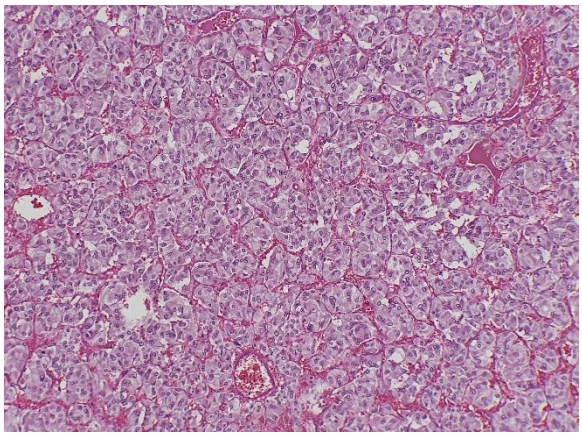
4.2. Detailed methods

4.2.1. Specimen retrieval

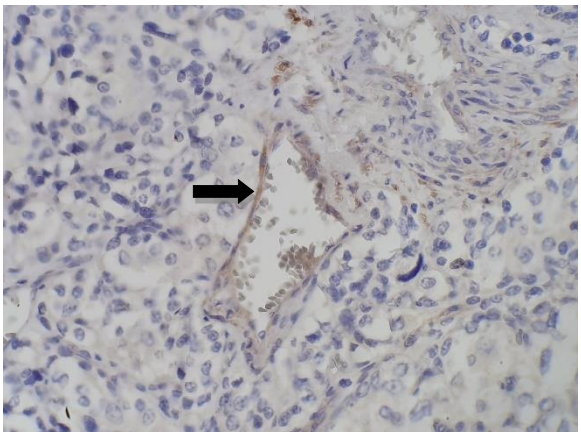
Specimens were identified on the DISA laboratory system with key word search and/or Snomed codes for PC and PGL. The slides and tissue blocks were retrieved from the archive at the Division of Anatomical Pathology, National Health Laboratory Service (NHLS), Tygerberg Hospital.

4.2.2. Tissue wax block selection and histopathological evaluation

All the available histopathology slides of each case included in the study were reviewed by the PI (CBB) together with a consultant anatomical pathologist (AvW). If the diagnosis was agreed upon, a tissue block was selected for immunohistochemical staining. If tumour was present in more than one block the most appropriate block in terms of quality and quantity of tumour was selected for further immunohistochemical staining. Where possible, blocks were chosen that also included some normal background tissue to assist with inclusion of internal controls. Cases for which tissue wax blocks could not be retrieved (five cases) or in which there was uncertainty about the diagnosis (one case) were excluded.



a) Haematoxylin and eosin stained section, 100x magnification: paraganglioma in a patient with confirmed SDH mutation.



b) SDHB immunohistochemistry showing loss of staining in the tumour cells with retained granular cytoplasmic staining in the endothelial cells (internal control), see arrow.

Figure 3. Paraganglioma in a patient with confirmed SDH mutation.

4.2.3. Immunohistochemical staining for SDHB

SDHB immunohistochemical staining was performed on an automated immunohistochemical stainer (Bond III, Leica Biosystems) according to standard

operating procedures (SOP) and the manufacturer's instructions. Two SDHB antibodies were acquired for optimisation in our laboratory as this stain was not yet available to the NHLS. The initial antibody acquired was from ABCAM (rabbit polyclonal IgG, ab151684). Numerous attempts to optimize this stain were performed but our laboratory was unable to achieve a consistent result with appropriate positive internal controls using this antibody. A second antibody was then acquired from Sigma Aldrich (rabbit polyclonal IgG, HPA002868 antibody) which was successfully optimised at dilutions of 1:400 with antigen retrieval using heat. The commercially available ER2 heat induced epitope retrieval was used which has a pH of 8.9-9.1 and is EDTA based. The epitope retrieval was applied for 20 minutes at 100 degrees Celsius. This stain was validated using two cases of PGLs in which the SDH mutation status of the patients was known (germline testing had been performed). In the case in which the patient was known to have an SDH mutation the immunohistochemistry showed loss of staining as expected (*Figure 3*). In the case in which the patient had no SDH mutation by germline testing the immunohistochemistry showed retention of staining (*Figure 4*).

The following steps were followed in our laboratory according to our SOP. Tissue sections were cut at 3-5µm and placed onto super frost plus slides. The slides were then baked for 30 minutes at 70°C. Slides were then placed into the BOND automated staining machine which was run according to manufacturer's instructions (*see table 2*). Bond wash was prepared by adding 100 ml of BOND Wash concentration to 900ml of deionised water. The slides were placed in a DAB enhancer for 4 minutes. The slides were then dehydrated in a series of graded alcohol and cleared in xylene before mounting.

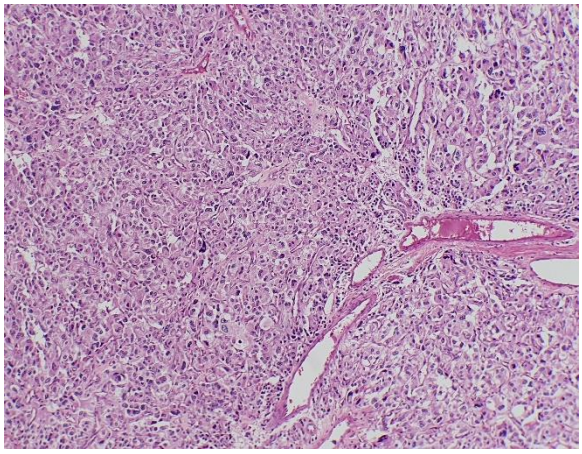
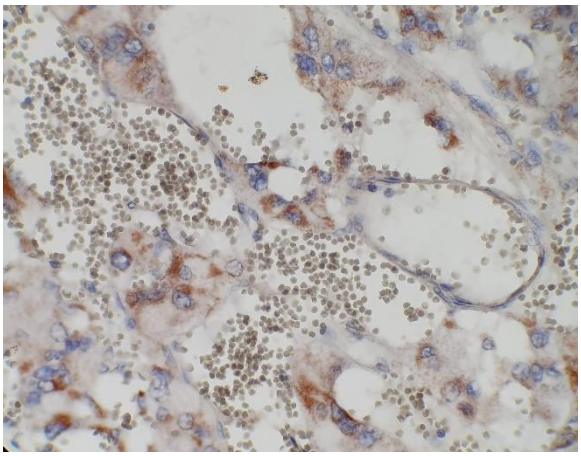
	
a) Haematoxylin and eosin stained section, 40x magnification: pheochromocytoma in a patient with no SDH mutation on germline testing.	*b) SDH immunohistochemistry showing retained granular cytoplasmic staining in the tumour cells.*

Figure 4. Pheochromocytoma in a patient with no SDH mutation on germline testing.

4.2.4. Interpretation of the IHC stain

Positive staining/normal retained staining was interpreted as granular cytoplasmic staining in the tumour cells. Any amount of positive staining was interpreted as retained staining. Negative staining/loss of staining was interpreted as complete absence of cytoplasmic staining in the tumour cells with positive staining of the external and internal controls. Internal controls included sustentacular cells and endothelial cells. The IHC stains were interpreted by the PI and a consultant anatomical pathologist (AvW) independently and the results were then compared.

Table 2. Leica Bond III staining protocol.

	Repeats/cycles	Time	Temperature
Dewax	X3		72˚C
100% Alcohol	X3		
Bond wash	X3		
Retrieval SDHB	X2	20 min	100˚C
Bond wash	X4		
Bond wash	X1	3 min	
Optimally diluted antibody	X1	15 min	
Bond wash	X1	2 min	
Bond wash	X2	1 min	
Post primary	X1	8 min	
Wash	X3		
Polymer	X1	8 min	
Wash	X2	2 min	
Deionized water	X1		
Peroxide block	X1	5 min	
Wash	X1	1 min	
Wash	X2		
Distilled water	X1		
Mixed DAB refine	X1		
Mixed DAB refine	X1	10 min	
Deionized water	X3		
Haematoxylin	X1	5 min	
Deionized water	X1		
Wash	X1		
Deionized water	X1		

4.2.5. Data management

Electronic capturing of de-identified data was performed on a password protected Excel spreadsheet which only the PI and supervisor had access to. Cases were stratified based on the patient age, site of the lesion, and presence or absence of

metastatic disease. The information required to perform this stratification was obtained from the pathology laboratory information system. A unique random study number was assigned to each pathology specimen. This study number as well as age, site of tumour, presence of metastases and loss or retention of SHDB staining was captured on the Excel spreadsheet.

4.2.6. Security and backup

The Excel spreadsheet was password protected and only the PI and supervisor had access to this password. The document was saved on the PI's computer which was locked in an access-controlled office. On-line backup of the spreadsheet was performed using Google Drive (also password protected).

4.2.7. Statistical considerations

Data was analysed using SPSS (Version 25) with the threshold for significance set at $p = 0.05$. Data was presented as means and standard deviations for continuous variables, and proportions for categorical data. A Mann-Whitney U test was used to compare the median age of patients who had retention and loss of staining. Chi-square tests were used to determine if there was an association between retention of staining and (a) sex, and (b) site of tumour. Inter-observer reliability for coding of retained or lost staining was assessed using Cohen's kappa.

4.3. Ethical considerations

4.3.1. Waiver of consent

A waiver of consent was requested to review the PGL and PC biopsy/resection specimens as well as to perform additional immunohistochemical stains (SDHB) on the wax tissue blocks of these cases. The specimens were de-identified and the

result of the immunohistochemically staining would in no way influence the current clinical management/care of the patient and therefore involved minimal risk. The SDH immunohistochemistry was performed on the tumour only with no germline testing performed in this study. The two cases used for validation of the immunohistochemical staining had given informed consent to the clinical team for performance of germline testing which was not performed as part of this study but as part of the patients' clinical management. The practicality of attempting to obtain consent from patients/relatives of the cases sampled over a ten-year period would make this study impossible to perform. Procedures to protect confidentiality were maintained throughout the study.

4.3.2. Confidentiality

Strict patient confidentiality was maintained throughout this study. Specimens were de-identified and assigned unique study numbers. Only the PI and supervisor had access to the cases and to the data which was password protected and kept in an access-controlled office at all times.

4.3.3. Ethics approval

This study received ethical approval from the Stellenbosch University Health Research Ethics Committee (HREC) on 14 March 2017 (reference number: S17/02/041). An annual renewal of ethics approval was obtained from the HREC following submission of annual progress reports.

4.4. Funding

Funding for this study was obtained from the NHLS Research Trust Development Grant (Grant number: 00494643).

5. RESULTS

A total of 65 cases of PC/PGL between 2005 and 2015 were identified. Four patients had multiple specimens of PC/PGL, either recurrences or metastases. Only one case per patient was included. A total of 58 patients were therefore identified. A further six cases were excluded – one in which the diagnosis was disputed and five for which the wax blocks could not be retrieved. A total of 52 cases were therefore included in the final sample (*figure 2*).

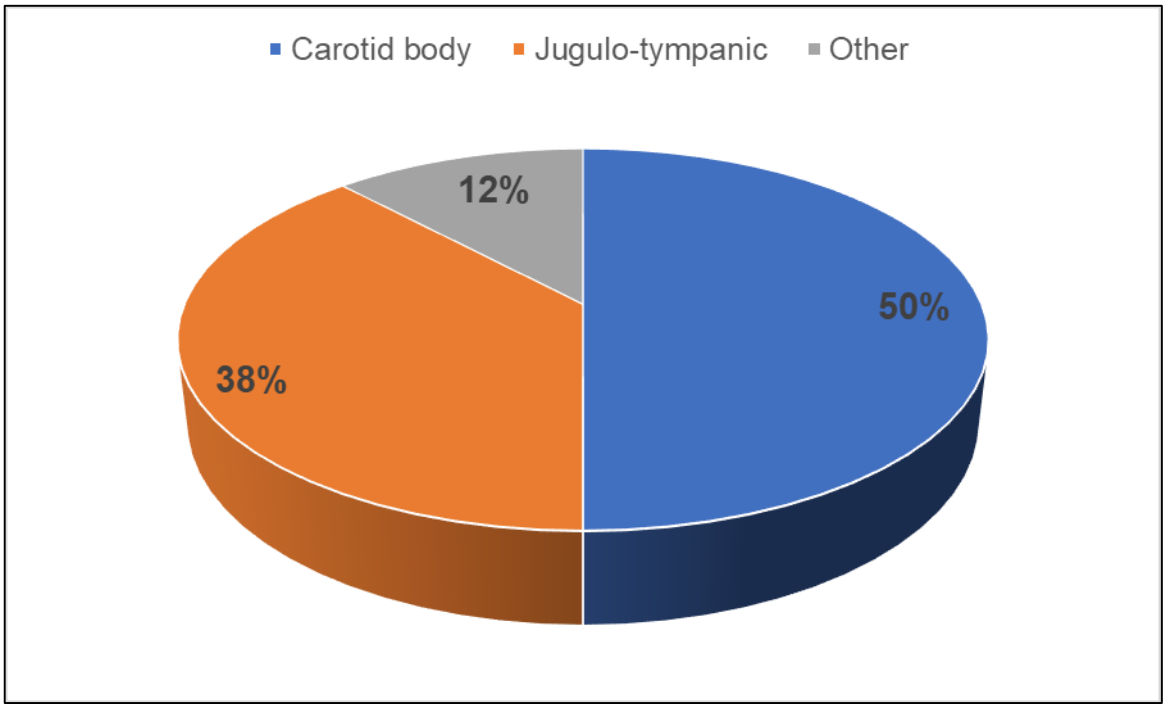


Figure 5. Diagrammatic representation of head and neck tumours by specific site.

The mean age of the patients included was 41.8 years (*SD* =16.4 years; range: 7 – 71 years). Females were more strongly represented with thirty-four females (65%) and eighteen males. Tumours located in the head and neck region made up 50% of the sample (*n*=26). The majority of HNPGLs were carotid body tumours (50%)

followed by jugulo-tympanic tumours (38%) (*See figure 5*). Other head and neck sites included neck (not further specified), laryngeal and skull (not further specified). Thoraco-abdominal cases made up 46% of the sample (n=24) with the majority occurring in the adrenal gland (58%) and para-aortic sites (25%) (*See figure 6*). Other thoraco-abdominal sites included liver, pelvic and retroperitoneal (not further specified). The remainder of the cases were spinal (4%, *n*=2, *see figure 7*). A total of three patients (6%) had metastatic disease.

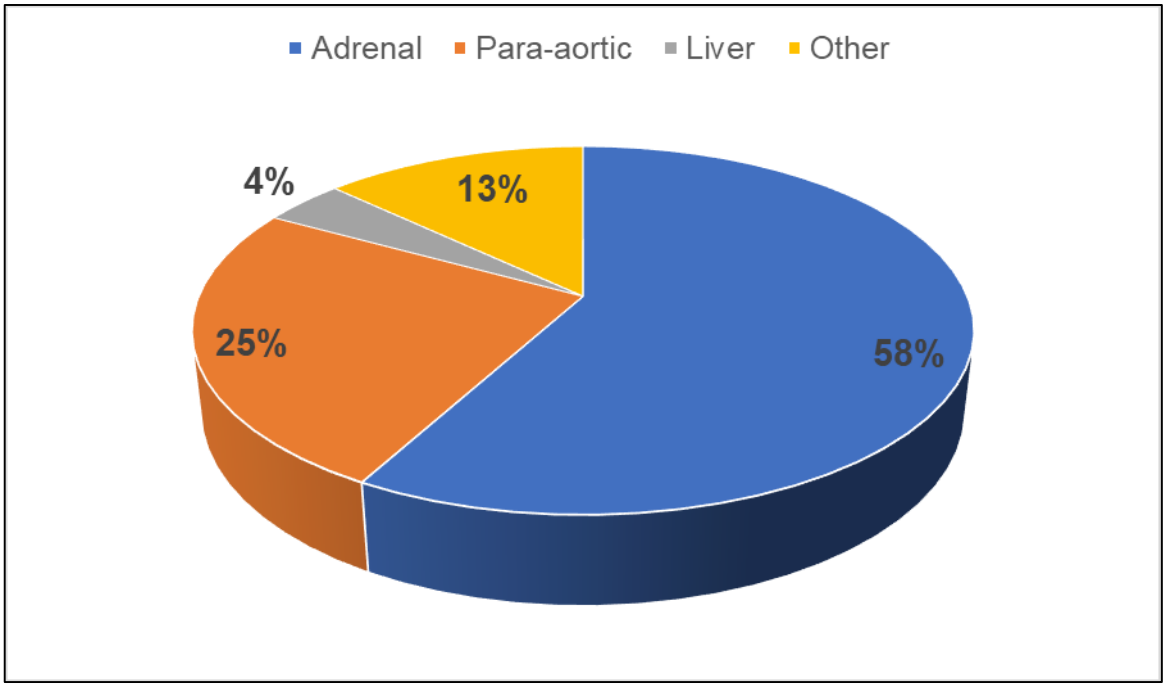


Figure 6. Diagrammatic representation of thoraco-abdominal tumours by specific site.

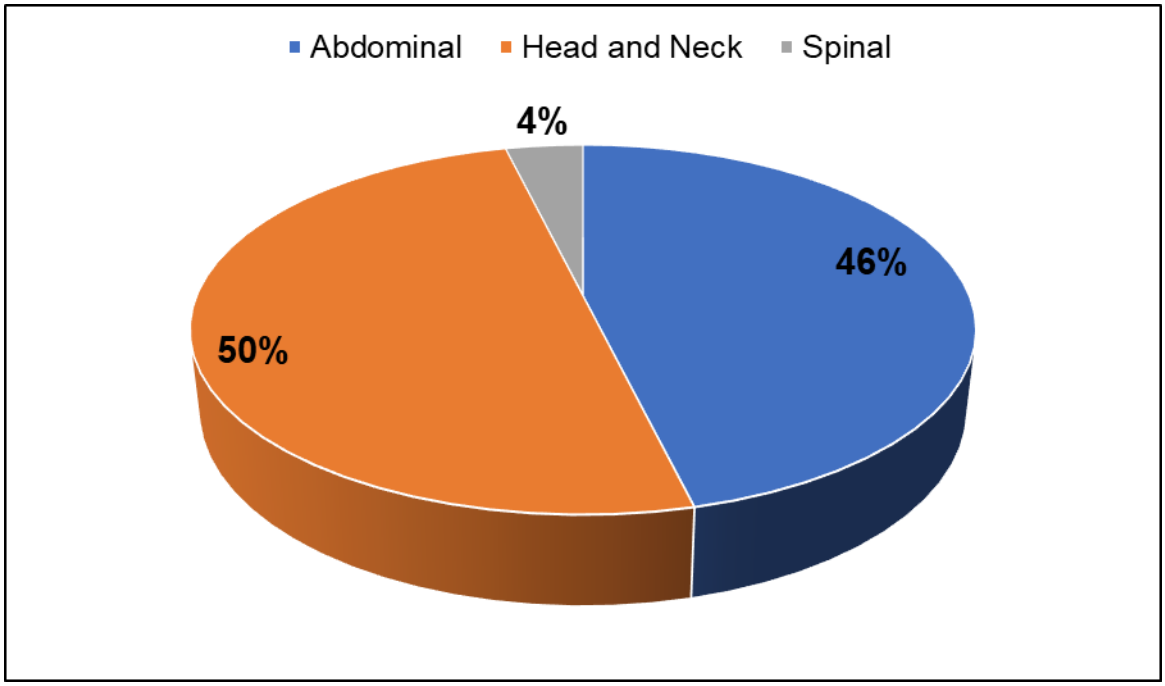
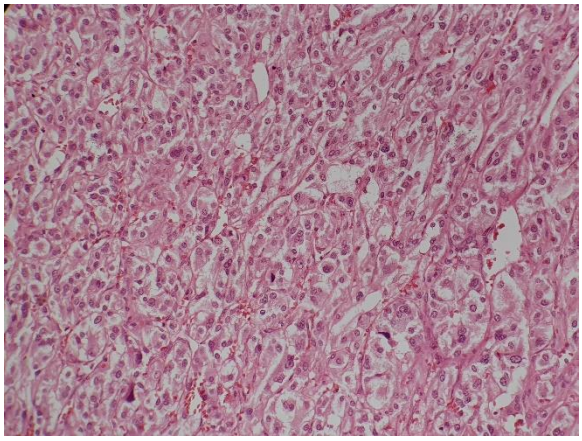
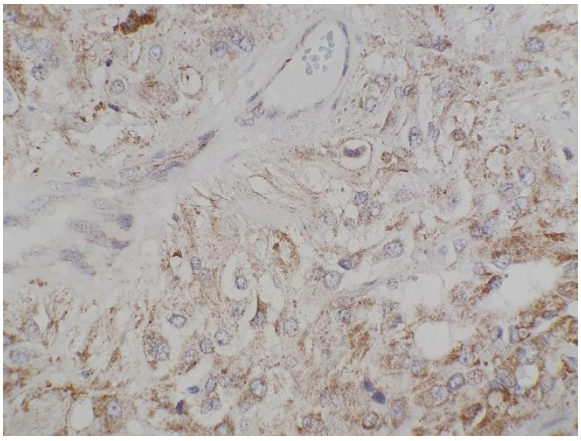


Figure 7. Diagrammatic representation of tumours by site.

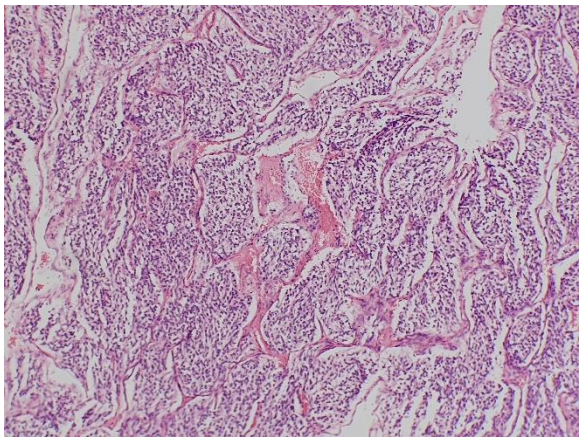
Loss of SDHB staining was present in 36% of cases (*n*=19). Retained staining was therefore seen in 64% (*n* = 33) and no cases were excluded for equivocal staining (*See figure 8 and 9*). Patients who had loss of staining were significantly younger than those who had retained staining (z = -3.59, p < .001). The median age of those who showed loss of staining was 26 years (IQR: 21 – 41), compared to 50.5 years (IQR: 36 – 61) for those who showed retained staining (*see table 3*). Sex was not associated with loss of staining ($\chi 2$ = 2.15, p = .142), with 9 of the 18 males (50%) compared to 10 of the 34 females (29.4%) showing loss of staining. Site of tumour was also not associated with loss of staining ($\chi 2$ = 0.94, p = .333), with 7 of the 24 TAPGLs (29.2%) compared to 11 of the 26 HNPGLs (42.3%) showing loss of staining (*see table 4*). A summary of these findings can be seen in Table 5.



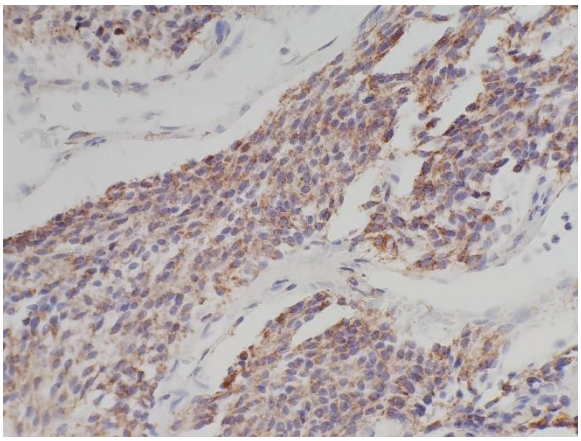
a) Haematoxylin and eosin stained section, 100x magnification: adrenal pheochromocytoma.



b) SDHB immunohistochemical stain of (a) showing retained staining with granular cytoplasmic staining.

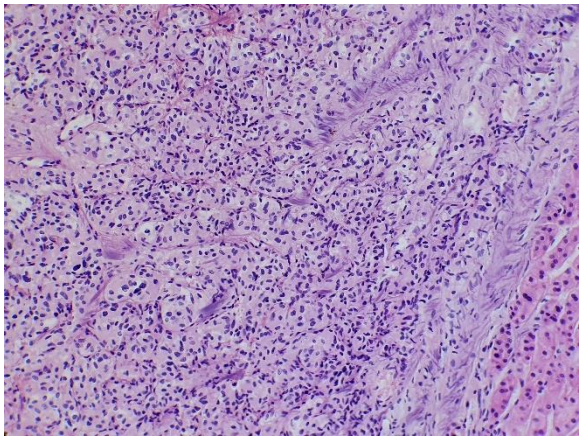


c) Haematoxylin and eosin stained section, 40x magnification: jugulo-tympanic paraganglioma.

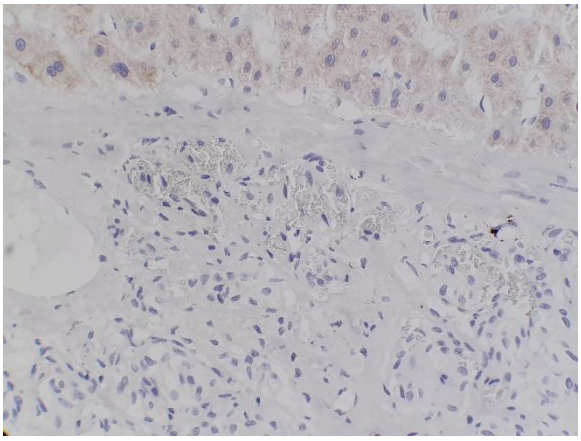


d) SDHB immunohistochemical stain of (c) showing retained staining with granular cytoplasmic staining.

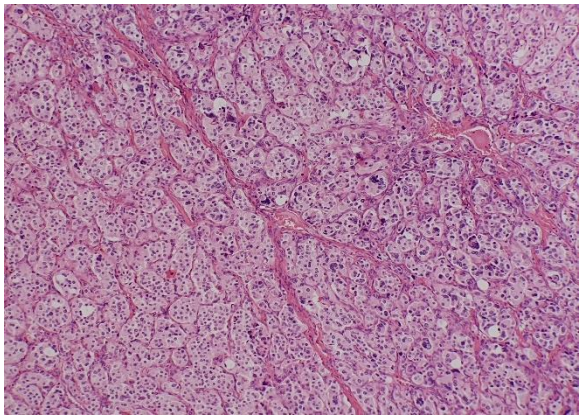
Figure 8. Pheochromocytoma and paraganglioma with retained SDHB immunohistochemical staining.



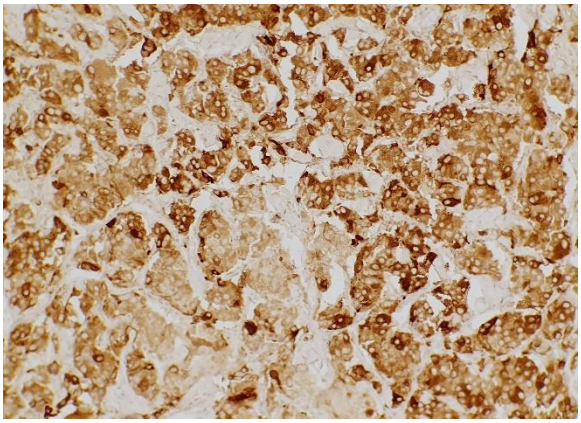
a) Haematoxylin and eosin stained section, 40x magnification: metastatic paraganglioma in the liver, note the tumour (left) and the background liver parenchyma (bottom right).



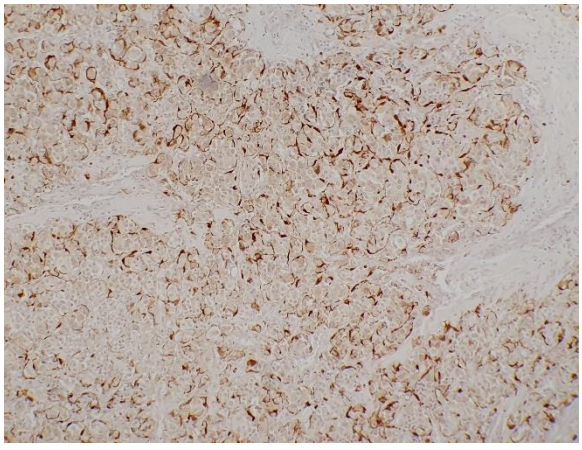
b) SDHB immunohistochemical stain showing loss of staining in the tumour in (a) (bottom) with granular cytoplasmic staining in the adjacent hepatic parenchyma (top).

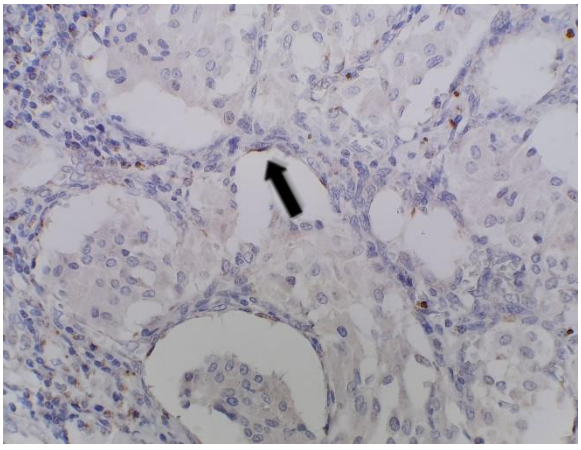


c) Haematoxylin and eosin stained section, 40x magnification: carotid body paraganglioma.



d) Chromogranin-A, granular cytoplasmic staining in the carotid body paraganglioma seen in (c).





e) S100 immunohistochemistry showing sustentacular cells around nests of tumour cells in the paraganglioma seen in (c).	*f) SDHB immunohistochemical stain showing loss of staining in the tumour with retained granular cytoplasmic staining in sustentacular and endothelial cells (arrow).*

Figure 9. Paragangliomas with loss of SDHB immunohistochemical staining.

Interpretation of the IHC stain was performed by the PI and a consultant anatomical pathologist independently. The inter-observer agreement between the two interpreters was excellent (*Cohen's kappa* = 0.917, $p < .001$).

Table 3. Loss of immunohistochemical staining by age.

Staining	**Loss of staining**	**Retention of staining**
Mean age	26 years (IQR: 21 – 41)	50.5 years (IQR: 36 – 61)
Mann-Whitney U test	$z = -3.59$, $p < .001$	

Table 4. Cases with loss of immunohistochemical staining by site.

Tumour site	**HNPGL**	**TAPGL**	**Spinal PGL**
Number	26	24	2
Number with loss of staining	11 (42.3%)	7 (29.2%)	1 (50%)
Chi square test	$\chi 2 = 0.94$, $p = .333$		

Table 5. Summary of 52 PC/PGL cases

	Adrenal	Para-aortic	Other TAPGL	Carotid body	Jugulo-tympanic	Other HNPGL	Spinal
All tumours	14	6	4	13	10	3	2
SDHB lost	1 (7%)	3 (50%)	3 (75%)	7 (54%)	4 (40%)	0	1 (50%)
Age (mean)	23	16-26 (20)	7-53 (33)	24-55 (36)	21-47 (29)	-	40
Sex (M/F)	1/0	1/2	2/1	2/5	2/2	-	1/0
SDHB retained	13 (93%)	3 (50%)	1 (25%)	6 (46%)	6 (60%)	3 (100%)	1 (50%)
Age (mean)	19-67 (45)	44-49 (47)	43	22-71 (49)	27-68 (49)	36-65 (54)	57
Sex (M/F)	5/8	0/3	1/0	0/6	1/5	2/1	0/1

6. DISCUSSION

6.1. Study design

This was a retrospective descriptive laboratory-based study. Cases were identified from the NHLS archives at Tygerberg Hospital using the laboratory electronic information system (DISA). Cases diagnosed as PC or PGL between 2005 and 2015 were selected using key word searching. The cases identified were assigned unique study numbers and captured on a password protected Excel spreadsheet. Cases for which there was disagreement about the diagnosis (1 case), or for which the wax blocks could not be retrieved (5 cases), were excluded. Four patients had multiple specimens and only one case per patient was selected for inclusion. A total of 52 cases meeting inclusion criteria were identified.

The histology (haematoxylin and eosin stained slides) of each case was reviewed by the PI and a consultant anatomical pathologist (AvW). Any additional immunohistochemical or histochemical stains that were performed at the time of diagnosis and that were available were reviewed. Wax blocks were selected during this review process on which to perform immunohistochemistry. An attempt was made in all included cases to utilise the most appropriate wax block based on quality and amount of tumour present as well as the presence of adjacent normal tissue to act as positive internal controls.

Immunohistochemistry was performed on the selected wax blocks according to the laboratory SOP using the Leica BOND III as described above (methods). The initial antibody acquired (ABCAM) could not be successfully optimised in our laboratory. A

consistent result with appropriate positive and negative controls was not achieved despite multiple attempts over a period of a few months. A second antibody was then acquired from Sigma Aldrich and successfully optimised and validated using two cases with known positive and negative germline testing for SDH mutations.

The immunohistochemistry was interpreted independently by the PI and AvW with cases blinded by their unique study number. The interpretation of the staining was then compared between the two interpreters. Interpreters followed the guidelines for interpretation of retention/loss of staining as outlined in more detail above (see methods). In only two of the fifty-two cases was there initial disagreement in interpretation. These two cases were then reviewed again by the two interpreters together and a consensus was reached. In both of these cases, upon review of the interpretation criteria and re-screening of the slide the interpreters agreed on retained/lost staining.

Germline testing for SDH mutation or sequencing of the tumours was not performed in this study and this data was not available for any of the included cases. Two cases used to validate the immunohistochemical staining had this information available (one with a known germline SDH mutation and one with no SDH mutation). These cases were diagnosed outside of the time frame (2005-2015) used for this study and were therefore not included.

6.2. Demographics

The age range of patients included in this study was broad. The youngest patient was 7 years old and the eldest 71 years. The mean age was 41.8±16.4 years. This

is similar to what is reported in the literature.[46–49] The WHO Classification of tumours of Endocrine organs reports a mean age of 41-47 years.[1]

Females made up the majority of patients included in this study. Thirty-four of the fifty-two patients were female (65%) compared to only eighteen males. Of the head and neck PGLs, 73% occurred in females. This is reflected in the literature with a reported female to male ratio of 8:1 for HNPGL, particularly at high altitudes.[50,51] Information regarding the altitude at which patients lived was not available, however our setting is coastal with a low altitude.

6.3. Tumour site

Fifty percent of cases included in this study were paragangliomas occurring within the head and neck region. The most common site within the head and neck was the carotid body (50% of cases), followed by jugulo-tympanic tumours (38%). This is comparable to the literature which reports carotid body PGLs as the most common site in the head and neck (57%).[47,49] Only one laryngeal tumour was identified. Laryngeal PGLs are known to be exceptionally rare.[47,49] In the remainder of the HNPGLs the site listed was not specific enough to classify further such as ‘neck’ and ‘skull’.

Forty-six percent of the included cases were TAPGLs with the majority, 58%, represented by PC (adrenal tumours). The mean age of patients with adrenal tumours was 44 years and the male to female ratio was roughly equal, similar to what is reported in the WHO Classification of Endocrine tumours. Other thoraco-abdominal sites included para-aortic, liver, pelvic and retroperitoneal.

6.4. Presence of metastatic disease

Information regarding the presence of metastatic disease was only available for three patients. This did not allow for any statistically significant correlations to be drawn. It is however noted that all three of these patients did have loss of staining of SDHB, i.e. likely had SDH mutations.

6.5. Immunohistochemistry

Loss of SDH staining was present in 36% of cases. This falls within the range reported in the literature of 15-54%.[17,25] Loss of staining was significantly correlated with a younger age at presentation (z = -3.59, p < .001). The median age of those who had retained staining was 50.5 years (IQR: 36 – 61), compared to 26 years (IQR: 21 – 41) for those who showed loss of staining. This is supported by the literature with patients known to have SDH mutations developing disease at significantly younger ages than those in whom disease is sporadic.[26]

Sex was not associated with loss of staining ($\chi 2 = 2.15$, $p = .142$). Familial PGL/PC syndromes caused by SDH mutations have an equal sex distribution and therefore this association would not be expected.

The site of tumour was also not associated with loss of staining ($\chi 2 = 0.94$, $p = .333$). Since loss of staining for SDHB will be present if there is mutation of any of the SDH subunits, this association would also not be expected. On germline testing tumour site should correlate with specific SDH subunit mutation (with SDHB most common in thoraco-abdominal cases and SDHD most common in head and neck cases).

6.6. Inter-observer variability

Interpretation of the IHC stain was performed by the PI and a consultant anatomical pathologist independently. Once interpretation was completed independently the results were compared and any cases that were initially disagreed upon were reviewed again together to come to a consensus. Only two cases were interpreted differently on initial review. Following review of these two cases together, a consensus was easily reached in both cases. The inter-observer agreement between the two interpreters was excellent (*Cohen's kappa* = 0.917, $p < .001$). This inter-observer agreement is similar to what has been reported in the literature. Of note the reported excellent inter-observer agreement in the literature was in a setting with sub-specialist endocrine pathologists.[39] The two interpreters in this study had no prior experience with this stain and are general pathologists. We acknowledge that interpretation of this stain can be difficult as it requires identification of loss of a granular cytoplasmic stain. However, our excellent inter-observer variability demonstrates that following strict and clear guidelines should allow accurate interpretation of this stain by other general pathologists in our setting.

The prevalence of SDH loss in our setting is comparable to the literature and highlights the need for performance of this stain in our setting. While multigene panel germline testing will probably become more accessible and cost-effective and may eventually obviate the need for immunohistochemical staining in PGL/PC, many patients in South Africa currently do not have access to genetic testing upon diagnosis of a PC or PGL as this is still costly and not widely available. Immunohistochemistry is widely available in South African anatomical pathology

laboratories, is relatively affordable, and can be used to assess the need for further targeted germline testing. Interpretation of immunohistochemistry is part of routine training as a pathologist in South Africa and based on our reported inter-observer variability we expect that general pathologists in our setting would be capable of interpreting this stain if following the necessary guidelines.

6.7. Limitations

The limitations of this study are the small sample size and the lack of confirmatory testing of cases which showed loss of staining on immunohistochemistry by sequencing or germline testing. Based on the published literature, the use of the immunohistochemical stain is an excellent surrogate marker for SDH mutation however this has not been proven in our setting.

Future studies using a larger sample size, perhaps with multicentre data from various centres in South Africa, may help to generate statistically significant results. Although sequencing or germline testing is costly, a study which correlates SDH mutation status with SDHB immunohistochemical staining will be of value in validating the use of IHC instead of genetic testing in our setting.

7. CONCLUSION

7.1. Summary of findings

Fifty-two cases of PC and PGL were identified at Tygerberg Hospital NHLS between 2005 and 2015. The prevalence of SDH deficiency in these cases based on immunohistochemical staining for SDHB is 36%. Head and neck paragangliomas made up 50% of the sample with 46% occurring at thoraco-abdominal sites and 4% were spinal. There was no statistically significant correlation between loss of staining and tumour site. Patients who had loss of staining were significantly younger than those who had retained staining (z = -3.59, p < .001). There was no association between sex and loss of staining. Sixty-five percent of cases occurred in females with the majority of head and neck cases (73%) being female. The number of cases with metastatic disease was too few to generate statistically significant results. All three cases that had metastatic disease had loss of SDHB staining. The inter-observer agreement in interpretation of the SDHB immunohistochemical stain was excellent (*Cohen's kappa* = 0.917, p < .001).

7.2. Conclusions

Our findings largely correlate with the literature with our prevalence rate of 36% falling within the reported ranges (15-54%).[17,25] The correlation between age and retention of staining is also in keeping with what would be expected as familial PGL/PC occur at a younger age. The inter-observer agreement in IHC interpretation was excellent in this study, similar to other studies performed in more specialised centres.

7.3. Summary of contributions

To the authors' knowledge this is the first study that has assessed the prevalence of SDH deficiency in PGL/PC in South Africa. Based on our findings of a similar rate of SDH deficiency in these tumours as in the published literature, we can recommend that this stain is useful to perform in our setting. Access to genetic testing is limited in South Africa while IHC is widely available, cost effective and relatively easy to interpret. Our reported inter-observer variability, which mirrors that of rates published by highly specialised centres, highlights that although sometimes challenging to interpret, with the correct guidelines, other generalist pathologists in our setting should be able to achieve similar results. This test will therefore be a useful surrogate marker of SDH deficiency and should be made available to practicing pathologists in our setting to perform routinely on all PC/PGL cases.

7.4. Future research

There is a need for a larger study with a larger sample size in order to generate more statistically significant results. A multi-institutional study pooling cases from centres across the country would be ideal. Although access to genetic testing is limited, a study correlating loss of SDHB staining with germline SDH mutation status should ideally be performed in our setting to validate the correlation of the stain with the mutation status.

7.5. Recommendations

- Succinate dehydrogenase immunohistochemistry should be performed routinely on all PC/PGL cases in our setting in order to screen for syndromic associations and for prognostic information.
- The SDH immunohistochemical stain should be made available to general pathologists practicing in the NHLS.
- Interpretation of this stain should follow strict guidelines as published in the literature.

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