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Succinate dehydrogenase-deficient GISTs are characterized by IGF1R overexpression

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Succinate dehydrogenase-deficient gastrointestinal stromal tumors (GISTs) demonstrate unique pathological and clinical features, including the absence of activating mutations of *KIT* and *PDGFRA*, and primary resistance to imatinib. They arise exclusively in the stomach and account for 5–7.5% of all adult stomach GISTs and the great majority of these tumors in childhood. Insulin-like growth factor 1 receptor (IGF1R) overexpression has been associated with wild-type and pediatric GISTs. We propose that IGF1R overexpression is a feature of succinate dehydrogenase-deficient GISTs as a group. We assessed succinate dehydrogenase complex subunit B (SDHB) and IGF1R expression by immunohistochemistry in eight known succinate dehydrogenase-deficient GISTs, three GISTs arising in the setting of neurofibromatosis type 1 syndrome and 40 unselected GISTs. Selected *KIT* and *PDGFRA* exons were amplified and sequenced from formalin-fixed paraffin-embedded tumor samples. All eight succinate dehydrogenase-deficient tumors were wild-type for *KIT* and *PDGFRA*, succinate dehydrogenase B negative and demonstrated IGF1R overexpression. The three neurofibromatosis-related tumors were succinate dehydrogenase B positive and IGF1R negative. Of the 40 unselected upper GISTs, five were wild-type for *KIT* and *PDGFRA* in the selected exons. Two of the wild-type GISTs were succinate dehydrogenase B negative and showed IGF1R overexpression and three were succinate dehydrogenase B positive and IGF1R negative. We conclude that IGF1R overexpression is a feature of succinate dehydrogenase deficient GIST as a group, rather than pediatric or wild-type GIST *per se*. Therefore, IGF1R inhibition represents a potential rational therapeutic approach in this recently recognized subgroup of GIST.
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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. In all, 85–90% of GISTs are driven by activating mutations of the *KIT* or *PDGFRA* genes.[1] The remainder are termed wild-type GISTs. As a group, wild-type GISTs are less likely to respond to imatinib and pose a major therapeutic problem.[2]

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It has recently been demonstrated that negative immunohistochemical staining for succinate dehydrogenase complex subunit B protein (SDHB) identifies a unique subgroup of wild-type GIST with different pathological and clinical features from other GISTs, including other wild-type GISTs.[3] These GISTs are now known as succinate dehydrogenase-deficient GISTs (SDH deficient GISTs).[4] The combined results of several studies have demonstrated that SDH-deficient GISTs are characterized by an exclusively gastric location, female preponderance, young age of onset and unique histological features, including a predominantly epithelioid morphology often with a plexiform growth pattern, a tendency to be associated with multifocal or

metachronous disease and consistent primary resistance to imatinib therapy.[3–9] Unlike other GISTs, the prognosis of SDH-deficient GISTs cannot be predicted by size or mitotic rate, but when metastases do occur, they may be strikingly indolent, often with relatively stable disease over years to decades.[3,4,6,7,9]

The great majority of pediatric GISTs and all the GISTs that occur in Carney Triad (the non-familial association of gastric GIST, pulmonary chondroma and extra-adrenal paraganglioma) and Carney–Stratakis Syndrome (the familial association of GIST and paraganglioma) represent specific examples of SDH-deficient GISTs.[3–5,7,8] Importantly SDH-deficient GISTs also account for between 5 (ref. 3) and 7.5% (ref. 4) of all unselected apparently sporadic gastric GISTs and in this setting their unique properties have generally not been recognized until now.

It has been consistently demonstrated that insulin-like growth factor 1 receptor protein (IGF1R) is highly overexpressed in certain subsets of GISTs, which are generally described as being either 'pediatric' or 'wild type' or both.[10–13] We hypothesized that IGF1R overexpression would be a defining feature of SDH-deficient GISTs as a group, rather than being limited to pediatric GISTs or being a feature of all wild-type GISTs or all pediatric GISTs. Therefore, IGF1R inhibition may be a rational therapeutic approach for the 5−7.5% of all gastric GISTs that are SDH deficient.

Materials and methods

Patients

The study included tumors from 51 patients for which formalin-fixed paraffin-embedded tumor blocks were available. There were eight known SDH-deficient GISTs and three GISTs arising in individuals known to have neurofibromatosis type 1 syndrome selected from the pathology consultation files of one of the authors (AG), and 40 control patients. The clinical and pathological features of six of these SDH-deficient GISTs and the three neurofibromatosis 1 syndrome-related GISTs have been previously published.[3,14] The control group comprised 40 consecutive and unselected upper gastrointestinal (gastric and duodenal) GISTs diagnosed at Royal North Shore Hospital from January 1999 to June 2004. This control group included selected patients (upper gastrointestinal GISTs cases) from a larger control cohort on which the SDHB staining has been previously described.[3]

Histology

All cases were reviewed by an experienced gastrointestinal anatomical pathologist to confirm the diagnosis of GIST (AG). The review included assessment of at least one hematoxylin and eosin-stained slide of a representative tumor section from each case and a panel of immunohistochemical stains, most of which were already performed at the time of diagnosis. The panel of immunohistochemical stains included CD117, DOG1, CD34, S100, SMA and desmin. All the GISTs were positive for either CD117 or DOG1.

Genetic Analysis

Sequencing for activating *KIT* and *PDGFR* mutations spanning the five most commonly mutated exons (*KIT* exons 9,11,13,17 and *PDGFRA* exon 18) was performed on formalin-fixed paraffin-embedded tumor blocks using standard methods as previously described.[15]

Immunohistochemistry

SDHB immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections using a commercially available mouse monoclonal antibody (ABCAM ab14714, clone 21A11) using previously described methods.[3,14,16,17] Briefly, in order for immunohistochemistry for SDHB to be considered negative (a significant result) we required the entire tumor to demonstrate absent granular cytoplasmic staining (that is, absent mitochondrial staining) and for there to be readily identifiable internal positive controls in non-neoplastic cells (endothelial cells). Negative staining of tumor cells in the absence of internal positive controls was considered an indeterminate result and immunohistochemistry was repeated.

Immunohistochemistry for IGF1R was performed on all tumors on formalin-fixed paraffin-embedded tumor block sections (cut at 4 μm), using an experimental predilute rabbit monoclonal antibody (Clone G11, cat. no. 790-4346, Ventana-Roche, Tuscon, AZ, USA), which reacts with IGF1R but does not cross-react with the insulin receptor. Staining was performed using an automated staining system, the Leica Microsystems Bond-III (Leica Microsystems; Mount Waverley, VIC, Australia), used according to the manufacturer's protocol and with the manufacturer's retrieval solutions. The primary predilute antibody was used after heat-induced epitope retrieval in acidic epitope retrieval solution (ER1 VBS part no. AR9961) for 30 min. A biotin-free detection system was used (VBS part no. DS 9713). IGF1R staining was considered positive if there was diffuse strong cytoplasmic and cytoplasmic membrane staining (arbitrarily defined as positive staining in more than 50% of the tumor cells).

SDHB and IGF1R immunohistochemistry was interpreted by a single pathologist (AG) in conjunction with a hematoxylin and eosin-stained slide. At the time of interpretation the pathologist was blinded to clinical, pathological and genetic data, as well as to other immunohistochemical data. That is, at the time of interpretation of the IGF1R staining,

Table 1 Summary of mutation analysis and SDHB staining in 40 unselected GISTs

Mutation	*Mutation site*	*SDHB immunostaining result*
Kit mutations ($n=23$)	*KIT*11 Pro551_Glu554delinsGln;	SDHB positive ($n=23$)
	*KIT*11 Val555_Gln556del; *KIT*11 Gln556_Val560del; *KIT*11 Trp557Arg ($n=2$); *KIT*11 Trp557Ser; *KIT*11 Trp557del; *KIT*11 Trp557_Lys558del ($n=2$); *KIT*11 Val559Asp; *KIT*11 Val559Gly; *KIT* 11 Val559_Glu562del; *KIT*11 Val560Asp; *KIT*11 Val560Glu; *KIT*11 Val560del; *KIT*11 Asn564_Leu576del; *KIT*11Tyr568_Leu576delinsCysCysVal; *KIT*11 Val569_Leu576del and *KIT*17 Asp820Gly; *KIT*11 Asp572_Asp579dup; *KIT*11 Tyr578_Phe591dup and *KIT* 17 Asn822Tyr; *KIT*11 Asp579del; *KIT*11 Phe584_Pro585ins13; *KIT*11 Leu589_Ser590ins14;	
PDGFRA mutations ($n=12$)	*PDGFRA*18 Arg841Lys and Asp842Val;	SDHB positive ($n=12$)
	*PDGFRA*18 Asp842Val ($n=8$); *PDGFRA*18 Asp842_Met844del; *PDGFRA*18 Asp842_Ser847delinsGluAla; *PDGFRA*18 Ile843_Asp846del	
Wild type ($n=5$)		SDHB positive ($n=3$) SDHB negative ($n=2$)

the pathologist was blinded to the SDHB staining result and vice versa.

In-Situ Hybridization

Chromogenic *in-situ* hybridization was performed on 4 μm formalin-fixed paraffin-embedded tissue sections from the eight known SDH-deficient GISTs, using a commercially available dual color probe directed against the *IGF1R* gene (chromosome 15q26.3) and the chromosome 15 centromere (INFORM *IGF1R* DNP Probe, cat. no. 800-4458 and INFORM Chromosome 15 DIG Probe, cat. no. 800-4459; Ventana-Roche). *In-situ* hybridization was performed on the BenchMark XT automated staining platform (Ventana-Roche) using the manufacturer's recommended solutions and procedures. For each tumor sample, *IGF1R* to chromosome 15 centromere ratio was determined in 30 tumor nuclei and scored by a single observer (AG).

This project met institutional and statutory guidelines and was approved by the Northern Sydney Central Coast Health Human Research Ethics Committee.

Results

Patient Characteristics

Of the eight known SDH-deficient GISTs from the study group, five occurred in females and three in males. The mean age was 35 years (range 13–63 years). All these known SDH-deficient GISTs arose in the stomach and two individuals were known to have Carney Triad. Of the three GISTs occurring in the setting of neurofibromatosis type 1 syndrome, two were females (age 22 and 28 years) and one was male (age 84 years). One arose in the stomach and two in the small intestine. Of the 40 unselected GISTs (24 proximal stomach, 14 distal stomach, 1 gastro-esophageal junction, 1 duodenum), 18 were females and 22 were males, and the mean age was 63 years (range 40−92 years).

Histology

Of the 40 unselected patients, 7 were epithelioid, 25 spindled and 8 mixed epithelioid-spindled in morphology. Of the eight known SDH-deficient GISTs, seven had pure epithelioid morphology and one had mixed epithelioid and spindled morphology. All three neurofibromatosis-associated GIST were spindled in morphology.

Genetic Analysis

All eight known SDH-deficient GISTs from the study group were wild-type for *KIT* and *PDGFRA* at the five exons examined. Of the 40 unselected control patients, five were wild-type for *KIT* and *PDGFRA* at the examined exons (Table 1).

Immunohistochemistry

All eight known SDH-deficient GISTs from the study group showed negative SDHB staining and diffuse strong positive staining for IGF1R (Figure 1). The three neurofibromatosis-related GISTs showed positive staining for SDHB and negative staining for IGF1R. Of the 40 unselected upper gastrointestinal GISTs from the control group, all 35 *KIT*- or *PDGFRA*-mutated GISTs showed positive staining for SDHB and negative staining for IGF1R. Of the five unselected GISTs that were shown to be wild type for *KIT* and *PDGFRA* at the sequenced exons, two demonstrated negative staining for SDHB (indicating that they were unsuspected SDH-deficient GISTs) and this was accompanied by strong positive staining for IGF1R. Both of these cases arose in the stomach in females (aged 41 and 45) and demonstrated epithelioid morphology.

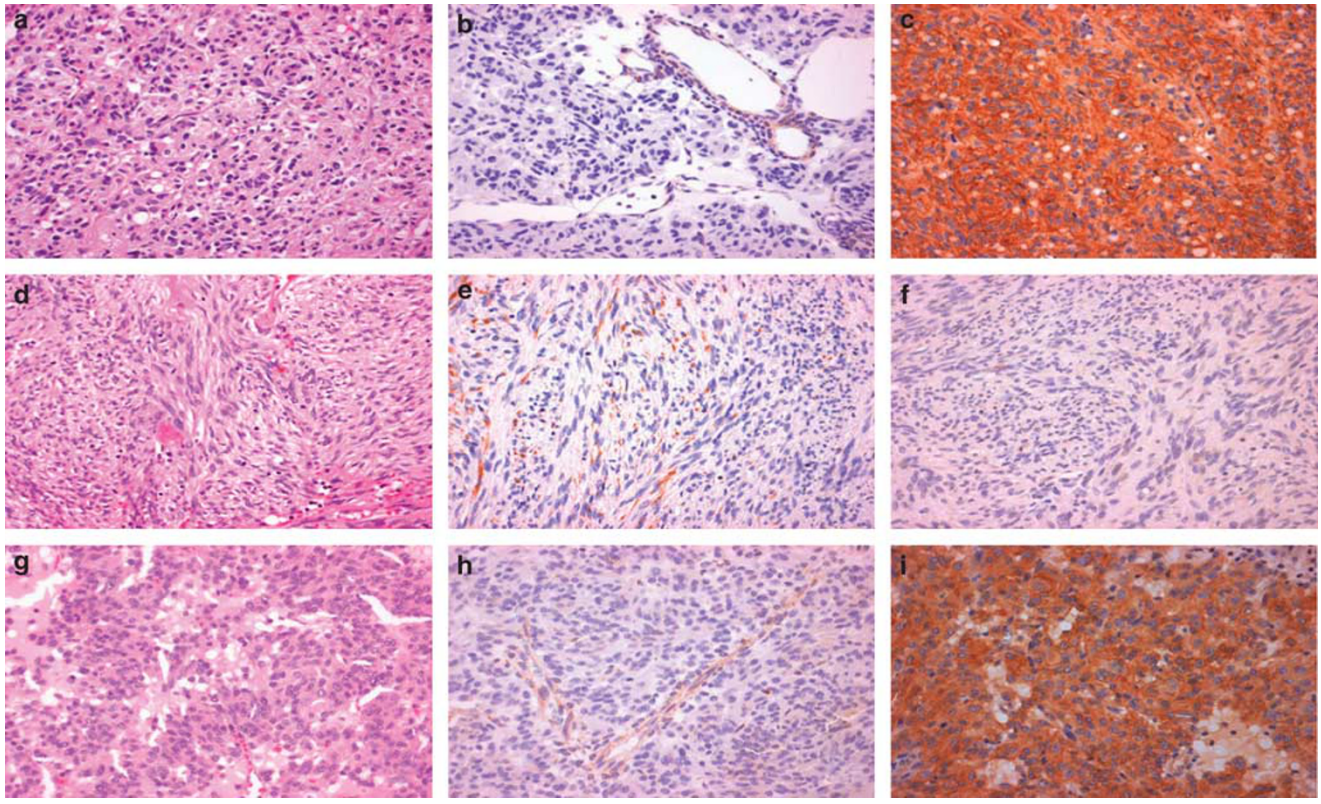

Figure 1 (**a–c**) Sporadic SDH-deficient GIST with typical epithelioid morphology (**a**), SDHB immunostaining is completely negative in tumor cells but positive in endothelial cells (positive internal control). (**b**), IGF1R immunostaining shows diffuse strong membranous and cytoplasmic staining (**c**). (**d–f**) Non SDHB-deficient GIST with usual spindle cell morphology (**d**), SDHB immunostaining shows positive granular cytoplasmic staining (**e**), IGF1R immunostaining is negative (**f**). (**g–i**) Carney Triad GIST is a specific example of SDH-deficient GIST. It shows similar morphology and immunostaining pattern as the sporadic SDH-deficient GIST shown in panels **a–c**. (**a**, **d**, **g** stained with hematoxylin and eosin; **b**, **e**, **h** SDHB immunohistochemistry; **c**, **f**, **i** IGF1R immunohistochemistry; original magnification of all images × 200).

The remaining three unselected wild-type GIST showed positive staining for SDHB and negative staining for IGF1R, and comprised one male and two females.

In-Situ Hybridization

Of the 10 SDH-deficient GISTs, *IGF1R* chromogenic *in-situ* hybridization was performed on eight tumors. There was no amplification of *IGF1R* detected in any of the eight samples (equal ratio between *IGF1R* and chromosome 15 centromere signals; Figure 2).

Discussion

It is now accepted that SDH-deficient GISTs demonstrate unique clinical and pathological features, including an exclusively gastric location, absence of *KIT* or *PDGFRA* mutations, primary resistance to imatinib and a tendency to develop multifocality and metachronous tumorigenesis—features that were previously only recognized in Carney Triad-related GISTs or pediatric wild-type GISTs, which we now consider to be specific examples of SDH-deficient GISTs.[3,4,6,7,9]

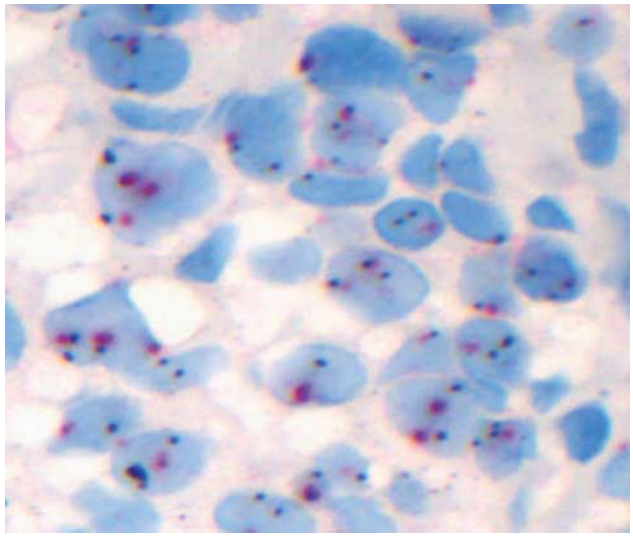

Figure 2 IGF1R chromogenic *in-situ* hybridization performed on eight SDH-deficient GISTs demonstrated equal ratio of signals for C15 centromere (red) and IGF1R (silver). This result indicates the absence of IGF1R gene amplification. (original magnification × 600).

In this study we demonstrate that IGF1R is overexpressed in 100% of SDH-deficient GISTs (10 of 10 patients) but never in non-SDH deficient GISTs

(0 of 41 patients). That is, it appears that IGF1R overexpression is yet another of the characteristic features of SDH-deficient GISTs. The fact that there was no increase in IGF1R expression in three wild-type but SDHB-positive sporadic GISTs and three GISTs occurring in individuals with neurofibromatosis (two of whom were young females) indicates that IGF1R overexpression is not a feature of wild-type GISTs or pediatric GISTs *per se*, but rather a feature of the specific subgroup of wild-type GIST recognized as SDH-deficient GISTs. We emphasize that exon 12 and exon 14 of *PDGFRA* were not sequenced. Therefore, it is possible that a GIST harboring an exon 12 or 14 *PDGFRA* mutation would be incorrectly classified as wild type in our study. However, mutations in these exons are very rare. For example, when Corless *et al*[1] sequenced 1105 GISTs, only 11 (1%) were found to harbor *PDGFRA* exon 12 mutations and only 3 (0.3%) were found to harbor exon 14 mutations. In contrast, 66 (6%) were shown to harbor exon 18 mutations.[1] Therefore, the statistical possibility that one of the GISTs that we classified as wild type may actually harbor a *PDGFRA* exon 12 or 14 mutation is unlikely to affect our basic conclusion that insulin-like growth factor 1 overexpression is a feature of SDH-deficient GISTs rather than wild-type GISTs *per se*.

When it is recognized that SDH-deficient GISTs are confined to the stomach and account for the vast majority of pediatric wild-type GISTs, our findings are in agreement with most previous studies. For example, Tarn *et al*[12] examined IGF1R protein by western blotting and immunohistochemistry and found that IGF1R overexpression was associated with wild-type GISTs and pediatric GISTs compared with mutant GISTs. Agaram *et al*[10] used differential gene expression to demonstrate that IGF1R protein was highly overexpressed in pediatric wild-type GISTs compared with adult GISTs but not overexpressed in the rare subgroup of pediatric GIST associated with *KIT* mutation. Pantaelo *et al*[13] studied eight gastric GISTs comprising two wild-type GISTs and six *KIT* mutant GISTs. Only the two wild-type gastric GISTs (both of which occurred in young patients who had metastases at presentation) demonstrated upregulation of *IGF1R* gene expression and increased protein production. Similarly, Janeway *et al*[11] used western blotting to demonstrate high IGF1R protein expression in eight of nine pediatric wild-type GISTs, and low IGF1R protein expression in five *KIT* mutant GISTs. Interestingly, in agreement with our *in-situ* hybridization study, neither Pantaelo nor Janeway's groups were able to demonstrate *IGF1R* genomic amplification by either SNP array or *in-situ* hybridization studies. Although it appears to be a constant finding, the mechanism of IGF1R overexpression in SDH-deficient GISTs is therefore currently unknown. IGF1R has been shown to be upregulated in a variety of cell types in response to mitochondrial stress induced by mitochondrial DNA depletion or by the mitochondrial toxin CCCP.[18] The mechanism for this effect appears to be mediated via elevated cytosolic calcium that activates calcineurin, which in turn leads to diverse downstream nuclear gene reprogramming.[19,20] Moreover, IGF1R links to upregulated glucose transporters in these circumstances, which is also a cardinal finding of SDHB-associated phaeochromocytoma-paragangliomas.[21] We therefore speculate that SDHB deficiency, either due to germline mutation (as in phaeochromocytoma and paraganglioma) or due to some yet-to-be-identified acquired mechanism, may induce *IGF1R* expression via the same downstream consequences of mitochondrial dysfunction.

Two immunohistochemical studies by Braconi *et al*[2] and Rios-Moreno and Jaramillo[22] are discordant with our findings and those discussed above. In Braconi's study IGF1R immunohistochemistry was performed on 13 wild-type GISTs and 81 mutant GISTs. IGF1R was strongly expressed in the cytoplasm of all GISTs. In Rios-Moreno's study 82% (18/22) of IGF1R-positive samples (cytoplasmic staining) had *KIT* mutation, 14% (3/22) *PDGFRA* mutation, and 4% (1/22) wild-type *KIT/PDGFRA*. Importantly, both these studies used a polyclonal anti-IGF1R antibody for immunohistochemistry. This polyclonal anti-IGF1R antibody has now been shown to lack specificity for the insulin-like growth factor 1 receptor—for example, by producing multiple non-specific bands on western blot and by staining positively in cell lines derived from IGF1R null mice.[23] It is therefore likely that this apparent discrepancy is accounted for by non-specific (false-positive) staining of the polyclonal anti-IGF1R antibody used in these previous two studies.

GISTs associated with neurofibromatosis type 1 syndrome are usually wild-type for *KIT* and *PDGFRA*, but have been consistently found to demonstrate positive immunostaining for SDHB.[3,8,24] The fact that we have demonstrated that our three neurofibromatosis cases show positive staining for SDHB and negative staining for IGF1R further supports our hypothesis that IGF1R overexpression is a property of SDH-deficient GISTs (which are always wild type for *KIT* and *PDGFRA*) rather than being a feature of wild-type GISTs. This is in keeping with a recent case report where a neurofibromatosis 1 syndrome-related GIST failed to respond to treatment with anti-IGF1R monoclonal antibody.[25]

Our results also support the data previously presented by Corless *et al*[26] in abstract form. They demonstrated that wild-type GISTS can be divided into two subtypes based on low or high IGF1R expression. Using quantitative RQ-PCR assay, they demonstrated that 21 of 35 wild-type GISTs had high IGF1R expression, whereas the remaining 14 showed low IGF1R expression, similar to mutant GISTs ($n = 39$). It is likely that the group of IGF1R high-expressing GISTs are SDH-deficient GISTs,

whereas the low IGF1R-expressing GISTs are likely to be wild-type but not SDH-deficient GISTs.

In summary, we have demonstrated that overexpression of IGF1R protein is a specific and consistent finding in succinate dehydrogenase-deficient GIST. IGF1R inhibition therefore represents a rational therapeutic target for this class of tumor, which accounts for between 5 and 7.5% of all gastric GISTs in adults[3,4] and perhaps also in the other tumors associated with mitochondrial complex 2 dysfunction, such as *SDH* mutated paraganglioma or the specific type of renal carcinoma associated with germline *SDHB* mutation.[14,16,17]

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Author contributions

Conception and design: Anthony J Gill. Provision of study materials and patients: Anthony J Gill, Jaswinder S Samra, Thomas J Hugh. Collection and assembly of data: Jason Chen, Adele Clarkson. Data analysis and interpretation: Angela Chou, Anthony J Gill. Manuscript writing and Final approval of manuscript: All authors.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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