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Carney Triad, SDH-deficient tumors, and *Sdhb*$^{+/-}$ mice share abnormal mitochondria

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Abstract

Carney Triad(CTr) describes the association of paragangliomas(PGL), pulmonary chondromas, and gastrointestinal(GI) stromal tumors(GISTs) with a variety of other lesions including pheochromocytomas and adrenocortical tumors. The gene(s) causing CTr remain(s) unknown. PGL and GISTs may be caused by loss-of-function mutations in succinate dehydrogenase (SDH) (a condition known as Carney-Stratakis syndrome (CSS)). Mitochondrial structure and function are abnormal in tissues carrying SDH defects but they have not been studied in CTr. For this study, we examined mitochondrial structure in human tumors and GI tissue(GIT) of mice with SDH deficiency. Tissues from 16 CTr tumors (n=12), and those with isolated GIST(n=1), with CSS caused by SDHC(n=1), SDHD(n=2) mutations were studied by electron microscopy (EM).

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GIT from mice with a heterozygous deletion in Sdhb (Sdhb+/−, n=4) were also studied by EM. CTr patients presented with mostly epithelioid GISTs that were characterized by plump cells containing a centrally located round nucleus and prominent nucleoli; these changes were almost identical to those seen in the GIST of patients with SDH. In tumor cells from patients, regardless of diagnosis or tumor type, cytoplasm contained increased mitochondria with “hypoxic” phenotype: mitochondria were devoid of cristae, exhibited structural abnormalities and of variable size. Occasionally, mitochondria were small/round, rarely thin, elongated with tubular cristae. Many mitochondria exhibited amorphous fluffy material with membranous whorls or cystic structures. Similar mitochondrial hypoxic phenotype was seen in Sdhb+/− mice. We conclude tissues from SDH-deficient tumors and mouse GIT and from CTr tumors shared identical abnormalities in mitochondrial structure and other features. Thus, the still elusive CTr defect(s) is(are) likely to affect mitochondrial function just like germline SDH-deficiency.

Keywords

GIST; mitochondria; Carney Triad; Succinate dehydrogenase

Introduction

Carney triad (CTr) is a syndrome that describes the association of paragangliomas (PGLs) with gastrointestinal stromal tumors (GISTs) and pulmonary chondromas (PCH); other lesions, including pheochromocytomas, esophageal leiomyomas and adrenocortical adenomas, have also been described (Carney, 1999, 2009; Stratakis and Carney, 2009). CTr is a novel form of multiple endocrine neoplasia (MEN) predominantly affecting females; it is caused by a yet unknown genetic defect (Matyakhina et al., 2007). The dyad of PGLs and GISTs (Carney–Stratakis syndrome, CSS) is inherited in an autosomal dominant manner relating to germline mutations in *SDHB*, *SDHC* and *SDHD* genes (but not *KIT* or *PDFGRA*) (Stratakis and Carney, 2009).

GISTs are the most common mesenchymal neoplasm of the gastrointestinal tract occurring in the stomach (60–70%) (Corless and Heinrich, 2008; El-Rifai et al., 2000) and small intestine (25–35%) (Corless and Heinrich, 2008; El-Rifai et al., 2000; Miettinen et al., 2006); they occur rarely in the large intestine or colon (5–10%) (Huang et al., 2006) and esophagus (Gouveia et al., 2005), typically later in life with only a few cases in the pediatric population and young adults (Janeway and Pappo, 2012; Kaemmer et al., 2009). GISTs are considered to originate from the interstitial cells of *Cajal*, the pacemaker cells that regulate peristalsis in the digestive tract (Parkin and Chugh, 2011). Worldwide, GISTs occur at an incidence of around 11 to 19.6 per million (Bulbul Dogusoy, 2012; Chan et al., 2006; Goettsch et al., 2005; Nilsson et al., 2005; Vukobrat-Bijedic et al., 2012), equating to 3,300 to 6,000 new cases reported annually in the United States.

Molecularly, most GISTs are driven by gain-of-function mutations in *KIT* or platelet-derived growth factor receptor-α (*PDGFRA*) (Lasota and Miettinen, 2008), however, a small subset of GISTs lack such mutations and are termed ‘wild-type’ (WT) GISTs. The latter constitute about 15% of GISTs that are identified in adult patients and more than half of the tumors

seen in pediatric patients (Doyle et al., 2012; Heinrich et al., 2003; Hirota et al., 1998; Lasota and Miettinen, 2008). Recently, we identified succinate dehydrogenase (SDH) deficiency, which activates oncogenesis by inhibiting hypoxia-induced factor (HIF)-alpha prolyl hydroxylase (Lancaster, 2002) to be associated with most WT-GISTs at the protein level (Celestino et al., 2012; Gaal et al., 2011; Janeway et al., 2011; Stratakis and Carney, 2009). SDH consists of four subunits, encoded for by the *SDHA*, *SDHB*, *SDHC*, and *SDHD*, genes. Mutations in these genes, collectively known as *SDHx*, which were known to predispose individuals to hereditary PGL and pheochromocytomas, were additionally found to be responsible for 10 to 15% of WT-GISTs (Celestino et al., 2012): loss of SDHB immunostaining was seen in the majority of WT-GISTs studied to date (Celestino et al., 2012; Gaal et al., 2011; Janeway et al., 2011; Killian et al., 2013), suggesting SDH deficiency is present even in these WT-GISTs that do not harbor *SDHx* DNA defects, possibly due to *SDHx* epigenetic down regulation (Astuti et al., 2001; Velasco et al., 2005). SDH is involved in catalyzing the oxidation of succinate to fumarate in the Krebs cycle, and participates in oxidative phosphorylation (Lancaster, 2002). All SDH subunits are encoded for by nuclear genes and *SDHx*-deficient tumors bear inactivating germline mutations as well as loss of the corresponding normal allele (Astuti et al., 2001; Baysal et al., 2000; Burnichon et al., 2010; Celestino et al., 2012; Gill et al., 2011; Niemann and Muller, 2000; Velasco et al., 2005).

Histologically, GISTs consist of spindle cells, epithelioid cells, or a mixture of both, and typically express the KIT (c-KIT) protein (Miettinen and Lasota, 2006a). GISTs with mutations in *PDGFRA* are reported to be of gastric origin, are of epithelioid cell type, and have the KIT-positive phenotype (Lasota and Miettinen, 2008). At the ultrastructural level, examination of GISTs from different anatomic locations have been examined, with particular focus on the variability of tumor cells (ranging from non-specialized spindle cells with similarities to fibroblasts to smooth muscle cells exhibiting neuronal features) (Min and Leabu, 2006; Segal et al., 1994; Yantiss et al., 2002). These studies have helped to identify the many overlapping ultrastructural characteristics and have contributed to the better classification of this heterogeneous group of neoplasms. However, these studies have not examined the ultrastructural features of the mitochondria (whose integral role resides in cellular metabolism) in these tumors. Here we present ultrastructural evidence for significant abnormalities in the appearance of mitochondria in tumors from patients with CTr, similar to those seen in *SDHB*-deficient tumors; interestingly, tissue from mice with a heterozygous deletion in *Sdhb* also showed mitochondrial structural abnormalities, which has never been shown before.

Materials and Methods

Patient Case Evaluations

Sixteen cases were identified. Six (6) from Mayo Clinic, nine (9) from National Institutes of Health, and one (1) from la Timone University Hospital, Marseille, France, France (Taieb et al., 2012).

Tumors from a total of 16 patients were studied; the patient's clinical data are presented in Table 1. There were 3 male and 13 female patients (with only one male patient with CTr). A

total of 19 tumors were investigated: CTr-associated GISTs (n=13), PGL (n=1), and chondroma (n=1); a tumor from a patient with isolated GIST (n=1), and tumors from patients with the dyad (CSS), a GIST caused by *SDHC* (n=1), and a GIST (n=1) and a PGL (n=1) caused by an *SDHD* mutation, each from a sibling with CSS from the same family. Their mutations and pathology (with regards to SDHB immunohistochemistry) have been described by our laboratory previously (23, 24); all tumors demonstrated negative SDHB immunohistochemistry (data not shown and previously published (23, 24)).

Electron Microscopy

Nine cases were processed from fresh tissue samples and were diced into $1mm^3$ cubes, fixed in 2.5% glutaraldehyde, post-fixed in osmium, embedded in EPON and routinely processed for transmission electron microscopy. Another 7 cases were retrieved from formalin-fixed paraffin-embedded tissue; although these showed suboptimal preservation we could successfully make observations pertaining to mitochondrial ultrastructure. A total of 5–15 sections were analyzed per each patient sample.

Mouse Studies

Sdhb$^{+/-}$ mice (obtained from Dr. Maher) (Louis J. Maher III, 2011) were maintained on a C57BL/6 genetic background. Mice were sacrificed (CO_2 inhalation) at 12 months of age (n=4; all female) and gastrointestinal tissue (GIT) was dissected. The small intestine (duodenum) was isolated and fixed in 2.5% glutaraldehyde, post-fixed in osmium, embedded in EPON and routinely processed for transmission electron microscopy (Laboratory of Pathology, National Cancer Institute (NCI), NIH, Bethesda, MD 20892, USA). All animal experiments were approved by the National Institute of Health Animal Ethics Committee (06-033).

Results

Ultrastructural findings in human tumors with SDH deficiency

The abnormalities of the tumor cells are described in comparison to normal controls. Our descriptions highlight the salient abnormal features of the various sets of our tumor specimens. Two patients with GISTs that harbored *SDHB* or *SDHC* mutations had similar mitochondrial morphology to that observed in CTr samples. A summary of mitochondrial ultrastructural features is presented below:

GISTs

GISTs were characterized by plump, epithelioid cells containing a centrally located round large nucleus with prominent nucleolus and diffused chromatin (Figure 1A) and a good number of morphologically abnormal mitochondria (Figure 1A–C). Most mitochondria contained remnants of cristae and hyaline aqueous solution. Throughout the cytoplasm glycogen granules were evident, which is a sign of hypoxic conditions and simultaneously present in cases of dysfunctional mitochondria. Cytoplasmic membranes were well defined and with frequent microvillus-like filopodia (Figure 1A, B and D). Few oval and spindle cells with indented nuclei, intracytoplasmic filamentous aggregates, slender surface filopodia and a few short intercellular attachments were present as well (Figure 1E and F).

These polygonal and spindle cells were packed with abnormal cystic-looking mitochondria without cristae and with intra-mitochondrial membranous inclusions. Some mitochondria were small and round, or thin and elongated and many were enlarged with partial or complete loss of cristae exhibiting amorphous fluffy material with membranous whorls, or cystic structures (Figure 1G–I). No autophagy or mitophagy processing was seen. In general, the morphology of the mitochondria in GIST cells can be described as being very close to those of a primitive neoplasm.

PGLs

PGLs were characterized by scant cytoplasm, diffused chromatin, the presence of intracytoplasmic dense core secretory granules (Figure 2A and B) and, a large number of morphologically abnormal mitochondria. The latter was similar to the GISTs. However, unlike the GISTs, the mitochondria in the PGLs appeared more numerous and occupied most of the cytoplasm; they also exhibited larger size than those of GIST (Figure 3A–D). The cell membranes were occasionally degenerate and cells appeared as if they were multinucleated; nuclei had various shapes ranging from round to lobulated (Figure 3A). Again, no autophagy or mitophagy processing was seen. Adjacent endothelial cells did not have any of these mitochondrial abnormalities (images not shown here).

CTr Lung Chondroma

The only evaluated case of lung chondroma (Figure 3E and F) showed fibroblastic looking cells with dilated endoplasmic reticulum (ER). The mitochondria wer morphological abnormal, with degenerate inner membrane and absent cristae or only their remnants present (Figure 3E & F). Interestingly, we also detected presence of leaky extranuclear chromatin (Figure 3F).

Ultrastructural findings in mice with SDH deficiency (the $Sdhb^{+/-}$ mouse)

In mouse GIT, the duodenum in particular, Cajal cell morphology had similar characteristics to that of patients with epithelioid GISTs; cytoplasm contained numerous mitochondria that displayed highly abnormal morphology including disintegration of the inner membrane and lack of cristae, budding of mitophagic vesicles, and variability of sizes with some swelling that gave some round shape; the latter seemed to be due to accumulation of aqueous substance (Figure 4A and B). Chromatin appeared pycnotic and the nuclear membrane separating from cytoplasm or slightly degenerate (Figure 4A). Additionally, some short rough endoplasmic membrane strands present (Figure 4A & B), and lysosomes were visible (figure 4B).

Discussion

GISTs are the most frequent spindle cell tumor of the gastrointestinal tract, thought to arise from interstitial cells of Cajal (Min and Leabu, 2006). GISTs occur more frequently in the stomach (Durham et al., 2004); to a lesser extent (in approximately 30% of patients) GISTs can be found in the small bowel, and in 10% or fewer cases in the esophagus as well as rectum. GISTs exhibit heterogeneous ultrastructural features (Kindblom et al., 1998; Matsumoto et al., 1997; Park et al., 2004; Yantiss et al., 2002). Clinical studies to date have

examined the histopathology, immunohistochemical, as well as genetic characteristics (Hirota and Isozaki, 2006; Miettinen and Lasota, 2006a, b; Paral et al., 2010), however, there have been no reported studies to date examining the ultrastructure of the mitochondrion in GISTs and their morphology in CTr.

In this study, we provide insight into mitochondrial ultrastructure in CTr and in GIST and PGL caused by SDH deficiency. We have identified that, in all clinical cases examined, mitochondria have a strikingly similar morphology – that of a hypoxic phenotype; generally lacking cristae, exhibit vacuoles, and varying in size; the more striking features reflect the severity/aggressiveness of the tumor. Patients with the dyad of PGL and GISTs (Carney-Stratakis syndrome or CSS) without other tumors harbor loss-of-function mutations in *SDHx* subunit genes. The current study shows that mitochondrial ultrastructure in tumors of these patients is almost identical to tumors from patients with CTr. Interestingly, we have recently found high succinate levels assessed by ^{1}H high-resolution magic angle spinning nuclear magnetic resonance (HRMAS NMR) spectroscopy in 2 CTr-associated PGLs (Imperiale et al., 2015). CTr-related PGLs metabolomic profile was indeed consistent with a SDH deficiency.

We also utilized a mouse model with an *Sdhb* heterozygous deletion (Maher III et al., 2011). Unlike in humans (where there is high penetrance of PGL in individuals with *SDHB* mutations) no PGLs or GISTs or any other tumors have been detected in *Sdhb*$^{+/-}$ mice (42), except for the recent description by our laboratory of modest pituitary gland hyperplasia and increased growth hormone and prolactin secretion (Xekouki et al., 2015). However, as seen here, there are mitochondrial ultrastructure defects in the gastrointestinal cells of these mice. It is unclear how these defects influence disease progression; mitochondria play a central role in orchestrating many apoptotic processes (Newmeyer and Ferguson-Miller, 2003), but it is possible that mice require more genetic hits for GIST and PGLs to develop, SDH deficiency apparently is enough to produce a phenotype in the mouse pituitary gland, although tumors fail to develop there, too, unlike the situation in humans (Xekouki and Stratakis, 2012; Xekouki et al., 2015).

Our ultrastructural study indicates that mitochondria from both *SDH*-mutant tumors and those associated with CTr have considerable similarity, particularly with respect to their increase in numbers, size and loss or complete absence of cristae. Tumor cells have the ability to successfully escape hypoxia-mediated death as a result of lowered expression or mutation of p53 (Moll and Schramm, 1998). Under hypoxic conditions, mitochondria are unable to provide enough ATP for cell survival, therefore tumor cells must up-regulate the glycolytic pathway; this is facilitated by the induction of hypoxia-inducible factor 1 (HIF-1) (Wang and Semenza, 1993). Tumors from patients with *SDHx* mutations have, indeed, higher levels of HIF-1(Hagg and Wennstrom, 2005). Previous studies have proposed that enlarged mitochondria arise from HIF-1-induced fusion and that these enlarged mitochondria confer resistance to apoptosis (Chiche et al., 2010). We have seen a similar phenotype in human mutant *SDHx*-associated pituitary tumors (Xekouki et al., 2012; Xekouki and Stratakis, 2012).

Although further studies on live cells are mandated to identify the contribution of the abnormal mitochondria in CTr pathogenesis, this is yet another observation that endeavors to describe better the molecular etiology of the CTr. It is now tempting to speculate that the yet elusive gene(s) are involved somehow in mitochondrial function beyond the down-regulation of *SDHB* (Gaal et al., 2011; Janeway et al., 2011) and *SDHC* (Haller et al., 2014).

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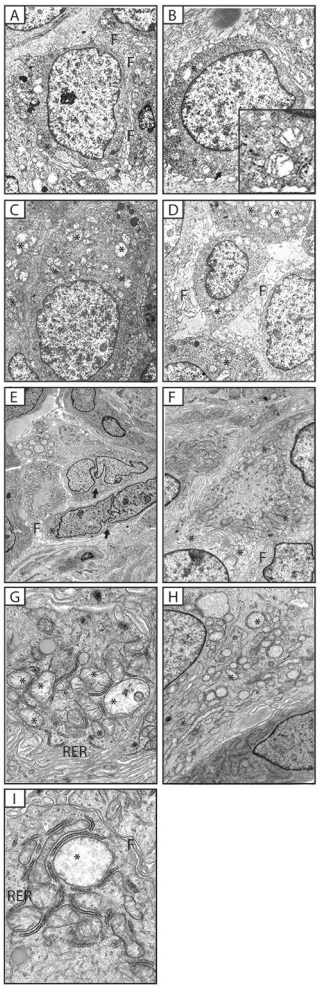

Figure 1.

(A–D) Gastric stromal sarcoma (Case No. 1). Luxuriant filopodal cell borders (marked as ***F***). Numerous glycogen granules (***arrow***) and prominent nucleoli (**B**). Mitochondria show abnormal cristae structure (*) (and **inset B**). Moderately increased numbers of mitochondria (**C – D**), all showing abnormal morphology; no cristae (*). **(E – F)** Gastric stromal sarcoma (Case No 2, specimen 6) Fewer numbers of oval and spindle cells with heavily lobulated nuclei (**E,** ***arrow***) intracytoplasmic filamentous aggregates, slender surface filopodia (***F***) and a few short intercellular attachments were present. Polygonal and spindle cells were packed with cystic-looking mitochondria (*) without cristae and with intra-mitochondrial membranous inclusions (**H,** *). **(G–I)** CTr Gastrointestinal Stromal Tumors (Case no 2, specimen 6). Oval and spindle cells are in close apposition. Slender filopodia (***F***) are evident with few short intercellular attachments. The cytoplasm contains strands of rough endoplasmic reticulum (***RER***), branching ER, intermediate filaments and increased numbers of mitochondria (*). Mitochondria exhibit variable sizes and structural abnormalities – some are small and round, others are thin and elongated with tubular cristae. Many mitochondria have partial to complete loss of cristae and exhibit amorphous amorphous material containing membranous whorls or cystic structures.

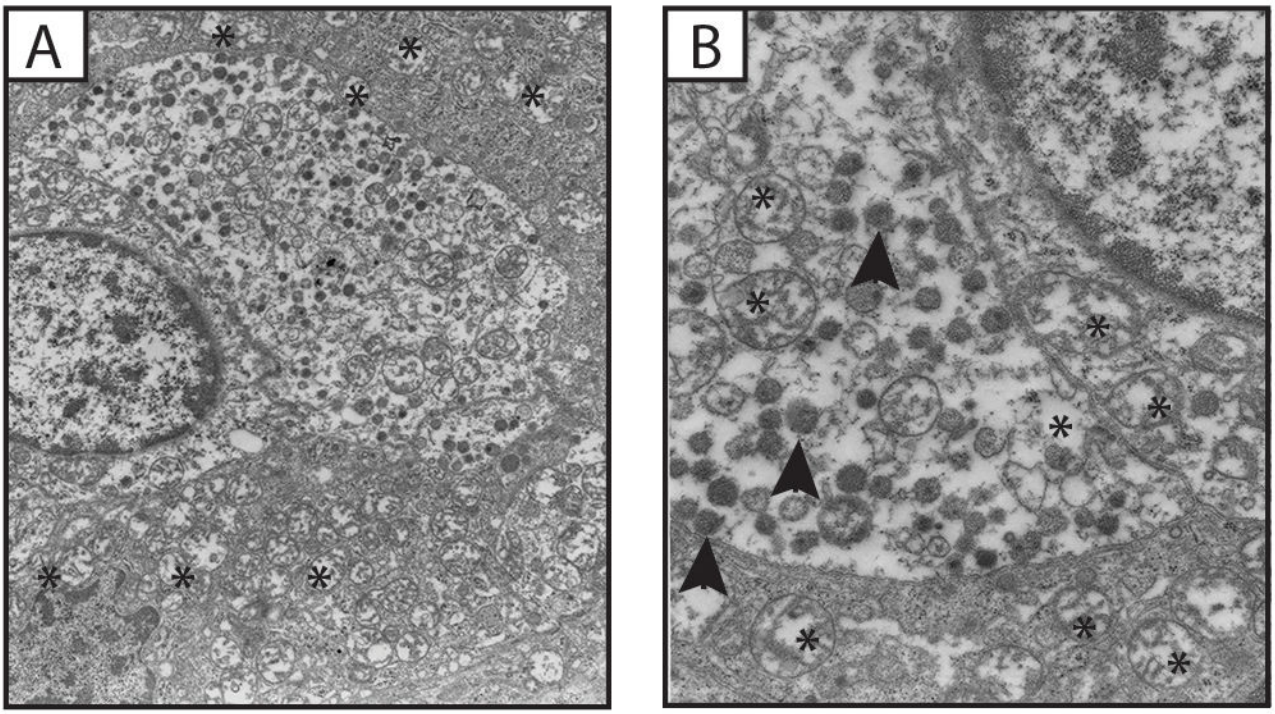

Figure 2.
Ultrastructural features of a Paraganglioma (PGL) exhibiting many abnormal mitochondria (*) and dense core granules (***arrowheads***) (Case No 1).

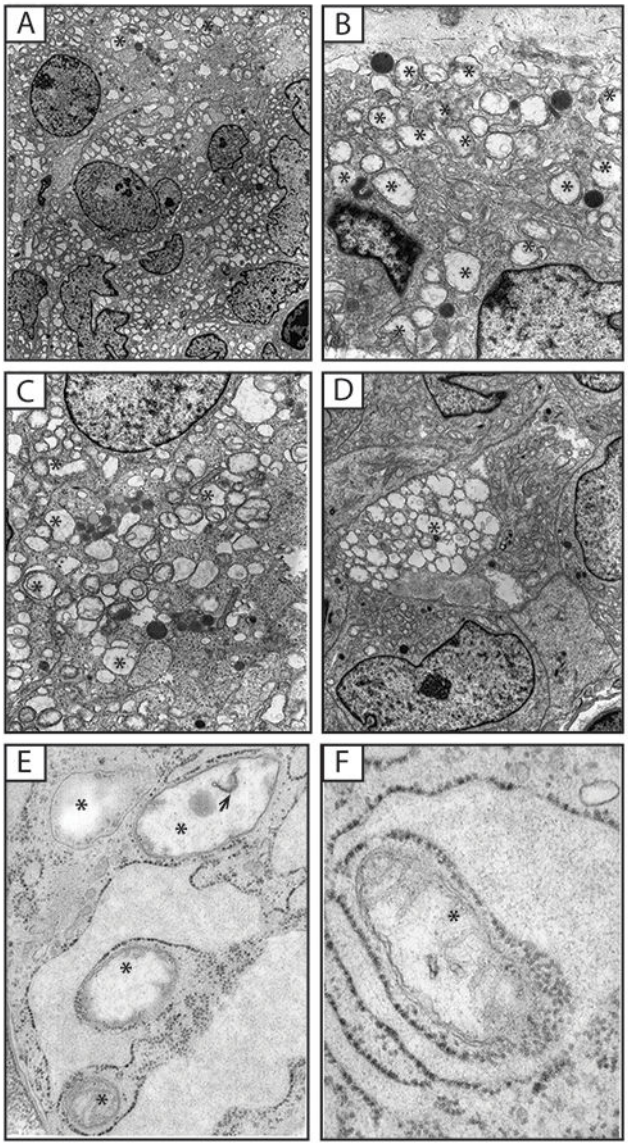

Figure 3.

(A–D) Ultrastructural features of a Paraganglioma (PGL) (Case No 2) exhibiting polygonal and spindle cells packed with cystic-looking mitochondria (*) without cristae and with intra-mitochondrial membranous inclusions. The cell surfaces are smooth. **(E–F)** Lung chondroma, majority of mitochondria are devoid of cristae (*). Presence of leaky chromatin in the chondroma (**E, *arrow***).

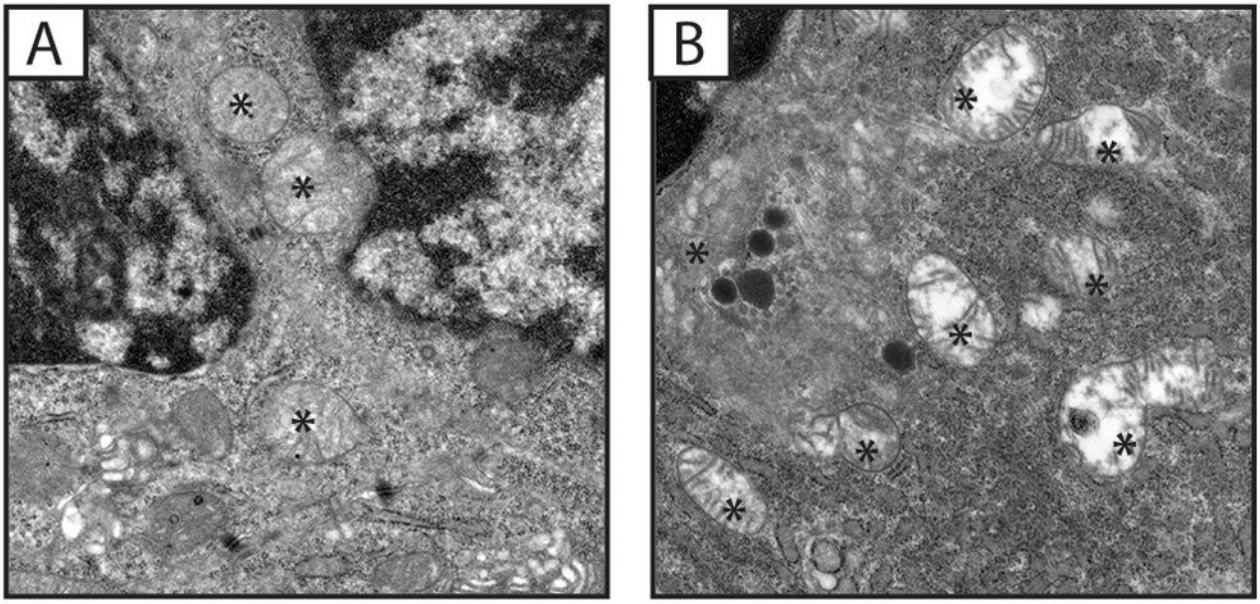

Figure 4.

Ultrastructural features of **(A)** wild type and **(B)** *Sdhb*$^{+/-}$ 12-month old mouse duodenum. Mitochondria (*) in *Sdhb*$^{+/-}$ duodenum have partial to complete loss of cristae, compared to wild type.

Table 1

Clinical and molecular findings in the patients that were included in the present investigation.

Patient/Case No.	Age at diagnosis (years)	Sex	Clinical Presentation	Dyad/Triad	SDHx Mutation
1	29	F	PGL	Triad	No
			Stomach GIST	Triad	None
2	28	F	Stomach GIST	Triad	None
			Duodenal GIST	Triad	None
			Stomach GIST	Triad	None
3	20	F	Stomach GIST	Triad	None
4	15	F	Stomach GIST	Triad	None
5	17	M	Stomach GIST	Triad	None
6	52	F	Stomach GIST	Triad	None
7	15	F	lung chondroma	Triad	None
8	20	F	Stomach + Duodenum GIST	Triad	None
9	14	F	GIST m, met + PGL m	Triad	None
10	34	F	GIST m, met	Triad	None
11	12	F	GIST + chondroma + PGL	Triad	None
12	25	F	GIST met + PGL + adrenocortical tumor	Triad	None
13	18	F	Stomach GIST	Dyad*	None
14	9	M	GSS m + PGL nf	Dyad*	*SDHC* c.405+1G>A (IVS5+1G>C)/p.Met136Leufs*3
15	20	F	PGL m, nf	Isolated PGL	*SDHD* c.388insG / p.Ala130Glyfs*61 #
16	16	M	GSS multiobulated; PGL m, nf; Pigmented penile spots	Dyad*	*SDHD* c.388insG / p.Ala130Glyfs*61 #

Abbreviations: m = multiple; nf = nonfunctioning; f = functioning; met = metastatic; PGL= paraganglioma; GIST = gastrointestinal stromal tumor; IVS5 = intervening sequence intron 5;

*Dyad = the dyad of PGLs and GISTs or Carney-Stratakis syndrome (CSS);

#These two patients also have a co-segregating variation in *SDHB* c.423+20T>A