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) that cause 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 that carry SDH defects, but they have not been studied in CTr. For the present study, we examined mitochondrial structure in human tumors and GI tissue (GIT) of mice with SDH deficiency. Tissues from 16 CTr tumors ($n = 12$), those with isolated GIST ($n = 1$), and those with CSS caused by *SDHC* ($n = 1$) and *SDHD* ($n = 2$) mutations were studied by electron microscopy (EM). Samples of 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 GISTs of patients with SDH. In tumor cells from patients, regardless of diagnosis or tumor type, cytoplasm contained an increased number of mitochondria with a ‘hypoxic’ phenotype: mitochondria were devoid of cristae, exhibited structural abnormalities, and were of variable size. Occasionally, mitochondria were small and round; rarely, they were thin and elongated with tubular cristae. Many mitochondria exhibited amorphous fluffy material with membranous whorls or cystic structures. A similar mitochondrial hypoxic phenotype was seen in *Sdhb*^{+/-} mice. We concluded that tissues from SDH-deficient tumors, those from mouse GIT, and those from CTr tumors shared identical

Key Words

- GIST
- mitochondria
- Carney triad
- succinate dehydrogenase

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 does.

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Introduction

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

GISTs are the most common mesenchymal neoplasm of the GI tract, and they mainly occur in the stomach (60–70%) (El-Rifai *et al.* 2000, Corless & Heinrich 2008) and small intestine (25–35%) (El-Rifai *et al.* 2000, Miettinen *et al.* 2006, Corless & Heinrich 2008); they rarely appear in the large intestine or colon (5–10%) (Huang *et al.* 2006) and esophagus (Gouveia *et al.* 2005). GISTs typically occur later in life and they are rare in children and young adults (Kaemmer *et al.* 2009, Janeway & Pappo 2012). GISTs are believed to originate from the interstitial cells of Cajal, the pacemaker cells that regulate peristalsis in the digestive tract (Parkin & Chugh 2011). Worldwide, GISTs occur at an incidence of around 11–19.6 per million (Goettsch *et al.* 2005, Nilsson *et al.* 2005, Chan *et al.* 2006, Bulbul Dogusoy 2012, Vukobrat-Bijedic *et al.* 2012), which equates to 3300–6000 new cases being reported annually in the USA.

Molecularly, most GISTs are driven by gain-of-function mutations in *KIT* or platelet-derived growth factor receptor- α (*PDGFRA*) (Lasota & Miettinen 2008); however, a small subset of GISTs lack such mutations and are termed ‘wild-type’ (WT) GISTs. The latter constitute about 15% of the GISTs that are identified in adult patients and more than half of the tumors seen in pediatric patients (Hirota *et al.* 1998, Heinrich *et al.* 2003, Lasota & Miettinen 2008, Doyle *et al.* 2012). Recently, we identified succinate dehydrogenase (SDH) deficiency, which activates oncogenesis by inhibiting hypoxia-induced factor (HIF)- α prolyl hydroxylase (Lancaster 2002) as a cause of

WT-GISTs. (Stratakis & Carney 2009, Gaal *et al.* 2011, Janeway *et al.* 2011, Celestino *et al.* 2012). SDH consists of four subunits that are encoded by the *SDHA*, *SDHB*, *SDHC*, and *SDHD* genes, which are collectively known as *SDHx*. Mutations in these genes that were known to predispose individuals to hereditary PGLs and pheochromocytomas (Astuti *et al.* 2001, Velasco *et al.* 2005) were additionally found to be responsible for 10–15% of WT-GISTs (Celestino *et al.* 2012). A loss of SDHB immunostaining has been seen in the majority of the WT-GISTs that have been studied to date (Gaal *et al.* 2011, Janeway *et al.* 2011, Celestino *et al.* 2012, Killian *et al.* 2013), which indicates that SDH deficiency is present even in those WT-GISTs that do not harbor *SDHx* DNA defects, possibly because of *SDHx* epigenetic down-regulation. SDH is involved in catalyzing the oxidation of succinate to fumarate in the Krebs cycle, and it participates in oxidative phosphorylation (Lancaster 2002). All SDH subunits are encoded by nuclear genes, and *SDHx*-deficient tumors bear inactivating germline mutations as well as a loss of the corresponding normal allele (Baysal *et al.* 2000, Niemann & Muller 2000, Astuti *et al.* 2001, Velasco *et al.* 2005, Burnichon *et al.* 2010, Gill *et al.* 2011, Celestino *et al.* 2012).

Histologically, GISTs consist of spindle cells, epithelioid cells, or a mixture of both, and they typically express the KIT (c-KIT) protein (Miettinen & Lasota 2006a). At the ultrastructural level, GISTs from different anatomical locations have been examined, with particular focus on the variability of tumor cells (ranging from non-specialized spindle cells that have similarities to fibroblasts to smooth muscle cells that exhibit neuronal features) (Segal *et al.* 1994, Yantiss *et al.* 2002, Min & Leabu 2006). These studies have helped to better classify this heterogeneous group of neoplasms. However, the ultrastructural features of the mitochondria (whose integral role resides in cellular metabolism) in these tumors have not yet been examined. In the present paper, we present ultrastructural evidence for significant abnormalities in the appearance of mitochondria in tumors from patients with CTr that are similar to those seen in *SDHB*-deficient tumors; interestingly, tissues from mice with a heterozygous deletion in

Sdhb also showed mitochondrial structural abnormalities, and this last point has, to our knowledge, never before been demonstrated.

Materials and methods

Patient case evaluations

Sixteen cases were identified, including six from the Mayo Clinic, nine from the National Institutes of Health, and one from la Timone University Hospital, Marseille, 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 three male and 13 female patients (but only one male patient had 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 an isolated GIST ($n=1$); and tumors from patients with the dyad (CSS), including a GIST caused by *SDHC* ($n=1$) and a GIST ($n=1$) and a PGL ($n=1$) caused by an *SDHD* mutation, each of which were from a sibling with CSS from the same family. Their mutations and pathology (with regard to SDHB

immunohistochemistry) have been described by our group previously (Janeway et al. 2011, Matayakhina et al. 2007); all of the tumors demonstrated negative SDHB immunohistochemistry (data not shown but previously published (Janeway et al. 2011, Matayakhina et al. 2007)).

Electron microscopy

For nine cases specimens were obtained from fresh tissue samples and were diced into 1 mm³ cubes, fixed in 2.5% glutaraldehyde, post-fixed in osmium, embedded in Epon epoxy resin, and routinely processed for transmission electron microscopy (EM). Specimens for another seven cases were retrieved from formalin-fixed paraffin-embedded tissue; although these showed suboptimal preservation, we were able to make observations pertaining to mitochondrial ultrastructure. A total of five to 15 sections were analyzed for each patient sample.

Mouse studies

Sdhb^{+/-} mice (obtained from Dr Maher) (Maher et al. 2011) were maintained on a C57BL/6 genetic background.

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
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 ^a	None
14	9	M	GSS m + PGL nf	Dyad ^a	<i>SDHC</i> c.405 + 1G > A (IVS5 + 1G > C)/p.Met136Leufs*3
15	20	F	PGL m, nf	Isolated PGL	<i>SDHD</i> c.388insG/p.Ala130Glyfs*61 ^b
16	16	M	GSS multiobulated + PGL m, nf + pigmented penile spots	Dyad ^a	<i>SDHD</i> c.388insG/p.Ala130Glyfs*61 ^b

m, multiple; nf, nonfunctioning; f, functioning; met, metastatic; PGL, paraganglioma; GIST, gastrointestinal stromal tumor; GSS, gastric stromal sarcom; IVS5, intervening sequence intron5.

^aDyad, the dyad of PGLs and GISTs or Carney–Stratakis syndrome (CSS).

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

Mice were killed (by CO₂ inhalation) at 12 months of age ($n=4$, all female), and their GI tissue (GIT) was dissected. Small intestines (duodena) were isolated and fixed in 2.5% glutaraldehyde, post-fixed in osmium, embedded in EPON, and routinely processed for transmission EM (Laboratory of Pathology, National Cancer Institute (NCI), NIH, Bethesda, MD, USA). All of the 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 with normal control tissues. The descriptions highlight the salient abnormal features of the various sets of our tumor specimens. The two patients with GISTs that

harbored *SDHB* or *SDHC* mutations had a similar mitochondrial morphology to that observed in the CTr samples. A summary of the mitochondrial ultrastructural features is presented in the following sections.

Gastrointestinal stromal tumors

GISTs were characterized by plump, epithelioid cells that contained a centrally located, large, round nucleus with a prominent nucleolus and diffuse chromatin (Fig. 1A) as well as an increased number of morphologically abnormal mitochondria (Fig. 1A, B and C). Most of the mitochondria contained remnants of cristae and hyaline aqueous solution. Throughout the cytoplasm, glycogen granules were evident, which is a sign of hypoxic conditions and is simultaneously present in cases of dysfunctional mitochondria. Cytoplasmic membranes were well defined and had frequent microvillus-like filopodia (Fig. 1A, B and D). Some oval and spindle cells with indented nuclei,

Figure 1

(A, B, C and D) Gastric stromal sarcoma (case no. 1). Luxuriant filopodal cell borders (F), numerous glycogen granules (arrow), and prominent nucleoli (B). Mitochondria showed abnormal cristae structure (*) (B). Moderately increased numbers of mitochondria (C and D), all showing abnormal morphology; no cristae (*). (E and F) Gastric stromal sarcoma (case no. 2, specimen 6). There were fewer numbers of oval and spindle cells with heavily lobulated nuclei (E, arrows), intracytoplasmic filamentous aggregates, and slender-surface filopodia (F). A few short intercellular attachments were also present. Polygonal and spindle cells were packed with cystic-looking mitochondria (*) without cristae and with

intra-mitochondrial membranous inclusions (H, *). (G, H and I) CTr gastrointestinal stromal tumors (case no. 2, specimen 6). Oval and spindle cells were in close apposition. Slender filopodia (F) were evident with few short intercellular attachments. The cytoplasm contained strands of rough endoplasmic reticulum (RER), branching ER, intermediate filaments, and increased numbers of mitochondria (*). Mitochondria exhibited variable sizes and structural abnormalities – some were small and round, whereas others were thin and elongated with tubular cristae. Many mitochondria had partial to complete loss of cristae and exhibited amorphous material that contained membranous whorls or cystic structures.

Figure 2

(A and B) Ultrastructural features of a paraganglioma (PGL) that exhibited many abnormal mitochondria (*) and dense core granules (arrows) (case no. 1).

intracytoplasmic filamentous aggregates, slender-surface filopodia, and a few short, intercellular attachments were present as well (Fig. 1E and F). These polygonal and spindle cells were packed with abnormal cystic-looking mitochondria that were devoid of cristae and had intra-mitochondrial membranous inclusions. Some of the mitochondria were small and round or thin and elongated, but many were enlarged and exhibited partial or complete loss of cristae and amorphous fluffy material with membranous whorls or cystic structures (Fig. 1G, H and I). No autophagy or mitophagy processing was seen. In general, the morphology of the mitochondria in the GIST cells can be described as being very close to those of a primitive neoplasm.

Paragangliomas

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

CTr lung chondroma

The only evaluated case of lung chondroma showed fibroblastic-looking cells with dilated endoplasmic reticulum. The mitochondria were morphologically abnormal, with degenerated inner membranes and absent cristae, or only their remnants were present (Fig. 3E and F). Interestingly, we also detected the presence of leaky extranuclear chromatin (Fig. 3F).

Figure 3

(A, B, C and D) Ultrastructural features of a paraganglioma (PGL) that exhibited polygonal and spindle cells packed with cystic-looking mitochondria (*) without cristae and with intra-mitochondrial membranous inclusions (case no. 2). The cell surfaces were smooth. (E and F) Lung chondroma; the majority of the mitochondria were devoid of cristae (*). Leaky chromatin was present in the chondroma (E, arrow).

Figure 4

Ultrastructural features of a WT (A) and *Sdhb*^{+/-} (B) 12-month-old mouse duodenum. Mitochondria (*) in the *Sdhb*^{+/-} duodenum have partial to complete loss of cristae as compared with the WT duodenum.

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

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

Discussion

GISTs are the most frequent spindle cell tumor of the GI tract, and they are thought to arise from the interstitial cells of Cajal (Min & Leabu 2006). GISTs occur most 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, they are found in the esophagus and rectum. GISTs exhibit heterogeneous ultrastructural features (Matsumoto et al. 1997, Kindblom et al. 1998, Yantiss et al. 2002, Park et al. 2004). In clinical studies to date the histopathology, immunohistochemical, and genetic characteristics of GISTs have been examined (Hirota & Isozaki 2006, Miettinen & Lasota 2006a,b, Paral et al. 2010); however, there have been no reported studies in which the ultrastructure of the mitochondria in GISTs and their morphology in CTr have been examined.

In the present study, we provide insight into the mitochondrial ultrastructure in CTr and in GISTs and

PGLs caused by SDH deficiency. We determined that in all of the clinical cases examined, mitochondria had a strikingly similar morphology – a hypoxic phenotype, a general lack of cristae, vacuoles, and varied sizes. The prominence of the features reflected the severity/aggressiveness of the tumor. Patients with the dyad of PGLs and GISTs (CSS) without other tumors harbor loss-of-function mutations in *SDHx* subunit genes. The results of the present study indicate that the mitochondrial ultrastructure in the tumors of these patients is almost identical to that in tumors from patients with CTr. Interestingly, we recently found high succinate levels, as assessed by ¹H high-resolution magic angle spinning nuclear magnetic resonance spectroscopy, in two CTr-associated PGLs (Imperiale et al. 2015). The CTr-related PGL's metabolomic profile was indeed consistent with SDH deficiency.

We also utilized a mouse model with an *Sdhb* heterozygous deletion (Maher et al. 2011). Unlike in humans (where there is a high penetrance of PGLs in individuals with *SDHB* mutations), no PGLs, GISTs, or any other tumors have been detected in *Sdhb*^{+/-} mice (Maher et al. 2011), 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 in the present study, there are mitochondrial defects in the GI cells of these mice. It is unclear how these defects influence disease progression; mitochondria play a central role in orchestrating many apoptotic processes (Newmeyer & Ferguson-Miller 2003), but it is possible that mice require more genetic hits in order for GISTs and PGLs to develop. SDH deficiency is apparently enough to produce a phenotype in the mouse pituitary gland, although tumors also fail to develop there, which is unlike the situation in humans (Xekouki & Stratakis 2012, Xekouki et al. 2015).

The results of the present ultrastructural study indicate that mitochondria from both *SDH*-mutant tumors and those associated with CTr have considerable similarity, particularly with respect to their increase in number, size, and loss or complete absence of cristae. Tumor cells have the ability to successfully escape hypoxia-mediated death as a result of the reduced expression or mutation of p53 (Moll & 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 up-regulation is facilitated by the induction of hypoxia-inducible factor-1 (HIF-1) (Wang & Semenza 1993). Tumors from patients with *SDHx* mutations indeed have higher levels of HIF-1 (Hagg & Wennstrom 2005). It has been previously 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 & Stratakis 2012, Xekouki *et al.* 2012).

Although further studies on live cells are needed to identify the contribution of the abnormal mitochondria to CTr pathogenesis, the present study constitutes yet another observation in the endeavor to better describe the molecular etiology of CTr. It is now tempting to speculate that the still-elusive gene(s) may be involved somehow in mitochondrial function along with the down-regulation of *SDHB* (Gaal *et al.* 2011, Janeway *et al.* 2011) and *SDHC* (Haller *et al.* 2014).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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