Mitochondrial function and content in pheochromocytoma/paraganglioma of succinate dehydrogenase mutation carriers

E Rapizzi¹, T Ercolino², L Canu¹, V Giaché¹, M Francalanci¹, C Pratesi³, A Valeri⁴ and M Mannelli^{1,5}

¹Endocrinology Unit, Department of Clinical Pathophysiology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy

²Endocrinology Unit, Careggi Hospital, Azienda Ospedaliera Universitaria Careggi, Florence, Italy

³Vascular Surgery Unit, Department of Heart and Vessels, University of Florence, Florence, Italy

⁴Chirurgia Generale, d'Urgenza e Mini-invasiva, Careggi Hospital, Azienda Ospedaliera Universitaria Careggi, Florence, Italy

⁵Istituto Toscano Tumori, Florence, Italy

(Correspondence should be addressed to M Mannelli at Endocrinology Unit, Department of Clinical Pathophysiology, University of Florence; Email: m.mannelli@dfc.unifi.it)

Abstract

To date, the consequences of succinate dehydrogenase (SDH) impairment on overall mitochondrial functions are still obscure. In this study, we evaluated SDH activity and expression and mitochondrial homeostasis in 57 tissue samples of pheochromocytoma (PHEO)/paraganglioma (PGL) obtained from patients genotyped for PHEO/PGL susceptibility genes. The resulted SDH activity and content always decreased in *SDH*-mutated tumors, in one out of two *MAX*-mutated patients and in four patients resulted wild type (wt) at genetic screening. All these four wt patients were further screened for large deletions in *SDH* genes, *TMEM127* and *MAX* and resulted wt but two had somatic *SDHD* mutations. The RT-PCR in the *MAX*-mutated sample suggests that the decrease in *SDH* depends on complex instability and not on a reduced *SDHB* expression. *SDH* mutations neither alter citrate synthase (CS) activity nor the content of voltage-dependent anion channel (VDAC) while the expression of the mitochondrial complex IV (cytochrome *c* oxidase (COX)) was found extremely variable in all (mutated and wt) samples suggesting an impairment of mitochondrial cristae in these tumors. In conclusion, tumors from patients with germ line *SDH* mutations invariably show decreased enzymatic activity and content, but an *SDH* impairment may also depend on *SDH* somatic mutations or, seemingly, on *MAX* mutations. The impaired SDH activity in the two wt tissues suggests mutations in other still unknown susceptibility genes. Finally, the extreme variability in COX expression levels is yet to be explained and this strongly suggests to evaluate other mitochondrial features to better understand the mitochondrial role in the pathogenesis of these tumors.

Endocrine-Related Cancer (2012) 19 261–269

Introduction

Pheochromocytoma (PHEO)/paraganglioma (PGL) are neural crest-derived tumors. According to their differentiation, they can be classified into two types: PGLs that are sympathetic in origin, catecholamine secreting, and mostly located in the abdomen (mainly the adrenals) and in the thorax (sPGL) and PGLs that are parasympathetic in origin, nonsecreting or poorly secreting, and mostly located in the head and neck region (HN-PGL). In the last years, it has been demonstrated that at least 30% of all the PGLs are

indeed due to a germ line mutation in one of the susceptibility genes (Neumann *et al.* 2002).

In addition to the tumor suppressor gene *VHL* (Latif *et al.* 1993, Walther *et al.* 1999), the proto-oncogene *RET* (Neumann *et al.* 1993, Eng *et al.* 1994, Eng 1996), the tumor suppressor gene *NF1* (White & O'Connell 1991), the newly discovered *TMEM127* (Qin *et al.* 2010), and the lately found *MAX* (Comino-Méndez *et al.* 2011), this group of genes also includes the genes encoding the four subunits of the succinate dehydrogenase (SDH), enzyme involved in the tricarboxylic

acid cycle (Baysal *et al.* 2000, Niemann & Müller 2000, Astuti *et al.* 2001, Burnichon *et al.* 2010) and in the mitochondrial electron transport chain, and the recently identified gene *SDHAF2* (*SDH5*) that is responsible for the flavination of the SDHA subunit (Hao *et al.* 2009).

Mitochondrial SDH is formed by four subunits: SDHA, a flavoprotein, and SDHB, an iron–sulfur protein, are the two catalytic subunits that are anchored to the inner mitochondrial membrane by two smaller structural subunits (SDHC and SDHD), containing cytochrome *b* and ubiquinone binding sites. These subunits are entirely encoded by nuclear genes located on chromosomes 5p15 for *SDHA* (with a pseudogene on 3q23), 1p36.13 for *SDHB*, 1q21.23 for *SDHC*, and 11q23 for *SDHD*.

It has also been described in another gene, named *SDHAF1*, which encodes a protein responsible for the SDH assembling (Ghezzi *et al.* 2009), but the correlation between this gene and the onset of PGL is yet to be established.

Germ line mutations in *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes are responsible for the occurrence of syndromes named PGL4, PGL3, PGL1, and PGL2 respectively. PGL1 presents mostly benign HN-PGL possibly associated with sPGL (Neumann *et al.* 2004, Simi *et al.* 2005). PGL2 is rare, only found in two families, and exclusively presents HN-PGL (Hao *et al.* 2009). PGL3 is mainly characterized by single HN-PGL (Schiavi *et al.* 2005) but the occurrence of abdominal sPGL has also been reported (Mannelli *et al.* 2007). PGL4 presents sPGL, which, at variance with tumors developed in PGL1 and PGL3, is malignant in more than 30% of cases, leading to metastatic disease (Linnoila *et al.* 1990, Amar *et al.* 2005, Pham *et al.* 2006, Timmers *et al.* 2007).

The two most common PGL syndromes, namely PGL1 and PGL4, also differ for penetrance (higher in PGL1) and mode of genetic transmission (maternal genomic imprinting in PGL1).

Heterozygous mutations in *SDHA* have been shown to cause the occurrence of abdominal sPGL (Burnichon *et al.* 2010).

At present, very few information is available on the biochemical/functional modifications induced by gene mutations in these tumors. Indeed, to date, the correlation between *SDH* mutations and SDH activity has been studied only in a French family mutated for *SDHD* and in two case reports mutated for *SDHB* and *SDHA* respectively (Gimenez-Roqueplo *et al.* 2001, 2002, Burnichon *et al.* 2010) or in 22 cases of HN-PGL (Douwes Dekker *et al.* 2003).

In the last few years, we have diagnosed and treated many patients affected by sporadic as well as familial

PHEO/PGL. Some of these patients were found to be *SDHB*, *SDHC*, or *SDHD* mutation carriers. The type of mutations ranged from nonsense to missense, frame-shift, splicing alteration. In addition, some patients presented a clinical picture suggesting a PGL syndromic disease (i.e. multiple or recurrent PGLs) but, unexpectedly, resulted wild type (wt) at genetic analysis. Here, we discuss the results obtained on mitochondrial function in 57 cases of sPGL/HN-PGL, in which SDH activity and assembling have been evaluated.

Materials and methods

Tissue specimens

According to a protocol approved by our Local Ethics Committee, we have collected many tissue specimens as well as blood samples for DNA and mRNA analyses and biochemical and morphological assays. All patients provided informed consent. In the present work, we evaluated 57 cases of sPGL/HN-PGL (Table 1). After surgery, tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

As control, we measured SDH enzymatic activity in four different adrenal glands obtained at surgery from donors for kidney transplantation and in the normal part of one kidney surgically removed for a tumor of the upper pole.

Table 1 Schematic representation of the 57 patients analyzed

	No. of cases	Genetic analysis	Mutations
sPGL wt	32	22 wt 10 (SDHB ^a and SDHD) polymorphisms	
sPGL mut	10	3 SDHD 1 SDHC 1 SDHB 2 RET 1 VHL 2 MAX	p.Gln109X (2) p.Pro81Leu p.Trp42X p.Thr193His Fs28X p.Cys634Tyr p.Leu198Val p.Leu94Pro p.Arg33X
HN-PGL wt	4	4 wt	
HN-PGL somatic mut	2	2 SDHD	p.Asp113MetfsX21 p.Leu7CysfsX7
HN-PGL mut	9	7 SDHD 2 SDHB	p.Pro81Leu (3) p.Gln109X p.Tyr114Cys p.IVS2-1G>T Large deletion p.Cys191Tyr p.Met11le
Total	57		

^aOne patient is polymorphic for both SDHB and MAX.

Mutation analysis

DNA was extracted from the peripheral blood leukocytes of each patient with the NucleoSpin Blood L kit (Macherey-Nagel, Düren, Germany) and analyzed for germ line mutations of *SDHA* (all exons), *SDHB* (all exons), *SDHC* (all exons), *SDHD* (all exons), *SDHAF1* (all exons), *SDHAF2* (all exons), *MAX* (all exons), *TMEM127* (all exons), *VHL* (all exons), and *RET* (exons 5, 8, 10, 11, 13, 14, 15, and 16). For each gene, coding regions and exon–intron boundaries were amplified by PCR as described previously (Astuti *et al.* 2001). PCR products, purified with a commercial kit (PCR purification kit, Qiagen), were subjected to 2% agarose gel electrophoresis with ethidium bromide staining and subsequently sequenced with a genetic analyzer (ABI PRISM 310; Applied Biosystems, Milan, Italy). In all the germ line-mutated patients, loss of heterozygosity (LOH) analysis was investigated. Moreover, all the wt patients showing a decreased SDH activity and expression were also screened for somatic mutation for *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF1*, *SDHAF2*, *VHL*, *TMEM127*, and *MAX* and for large deletion for *SDHB*, *SDHC*, *SDHD*, *SDHAF1*, *SDHAF2*, and *VHL*.

Multiplex ligation-dependent probe amplification reactions

For genomic rearrangements analysis, we used commercial kits for multiplex ligation-dependent probe amplification (MLPA)-based assays (SALSA MLPA P016B *VHL* and SALSA MLPA P226 *SDHD* from MRC-Holland, Amsterdam, The Netherlands) following manufacturers' instructions.

Amplification products were diluted in HiDi formamide containing 500TAMRA internal size standards (MRC-Holland) and then separated by size with an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Electropherograms were analyzed using Coffalyser MLPA DAT Software (MRC-Holland).

Tissue homogenates and lysates

Unless specified, all reagents were purchased from Sigma–Aldrich. Homogenates were prepared as already described (Kirby *et al.* 2007) with minor modifications. Briefly, dissected tissues (50–150 mg) were finely chopped; resuspended in a solution containing 120 mM KCl, 20 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and 5 mg/ml BSA; and homogenized in a hand-held glass/glass homogenizer. The homogenate was centrifuged at 800 *g* for 10 min at 4 °C and the enzyme assays were performed on the

supernatant. For western blot analysis, chopped tissues were lysed in lysis buffer (containing 50 mM Tris–HCl, pH=7.5, 120 mM NaCl, 1 mM EGTA, 6 mM EDTA, 15 mM Na₄P₂O₇, 20 mM NaF, 1% Triton X-100, and protease inhibitor cocktail) homogenized in a hand-held glass/glass homogenizer and incubated for 30 min on ice. Lysates were clarified by centrifugation at 10 000 *g* for 15 min at 4 °C. Supernatants were quantified for protein content (Coomassie Blue reagent was from Bio-Rad; Rapizzi *et al.* 2009). All procedures were carried out on ice or at 4 °C.

SDH and citrate synthase activity

Tissue homogenates (50 µg) were incubated in a phosphate buffer containing sodium azide, 2,6-dichlorophenolindophenol (DCPIP), sodium succinate, and phenazine methosulfate. Complex II-specific activity was evaluated by measuring the decrease in absorbance due to the oxidation of DCPIP at 600 nm (Kirby *et al.* 2007). Citrate synthase (CS) activity was measured by incubating the homogenates (25 µg) in a Tris buffer containing acetyl coenzyme A, 5,5'-dithiobis(2-nitrobenzoic acid), and oxaloacetate and measuring the increase in absorbance at 412 nm (Kirby *et al.* 2007). The enzymatic activities were evaluated by photometry using the Victor3 1420 Multilabel Counter (Packard Instruments, Perkin-Elmer, Waltham, MA, USA).

In all the experiments, homogenates and lysates extracted from different healthy tissues, such as liver, kidney, and adrenal gland, were used as controls. As no relevant differences were observed among these tissues, we decided to show in the figures only the results obtained from the adrenal gland (considered to be 100%).

Western blot

Samples of clarified lysates (30 µg proteins) were separated by SDS/PAGE and transferred onto PVDF (Immobilon, Millipore, MA, USA) as described previously (Rapizzi *et al.* 2009). Bound antibodies were detected using ECL reagents (Immobilon) and analyzed using a Bio-Rad ChemiDoc Imaging System for dedicated chemiluminescent image acquisition (Imaging and Analysis Software by Bio-Rad, Quantity-One). The polyclonal anti-SDHB was from Atlas Antibodies (Stockholm, Sweden) and the monoclonal anti-subunit IV of complex IV was from Invitrogen (Molecular Probes, Inc., Eugene, OR, USA). The polyclonal anti-VDAC1, the polyclonal anti-actin, and all the secondary antibodies, such as the anti-rabbit, the anti-mouse, and the anti-goat

IgG conjugated to HRP, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from one normal adrenal gland and from the *MAX*-mutated (p.Leu94Pro) patient using RNeasy Mini Kit (Qiagen). Total RNA was reverse transcribed using TaqMan RT PCR kit (Applied Biosystems). Gene expression measurement was performed by TaqMan using the 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) as detailed elsewhere (Lombardi et al. 2008). Primers/probes for *SDHA* (Hs00417200_m1), *SDHB* (Hs01042482_m1), *SDHC* (Hs01698067_s1), and *SDHD* (Hs01098144_g1; TaqMan Gene Expression Assays; Applied Biosystems, Warrington, UK) were used. The amount of target, normalized to the human endogenous reference GAPDH (Pre-Developed TaqMan Assay Reagents, Applied Biosystems) and relative to a calibrator (quantitative PCR human reference total RNA; Stratagene, Santa Clara, CA, USA), was given by $2^{-\Delta\Delta C_t}$ calculation (Livak & Schmittgen 2001).

Statistical analysis

Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered significant. Data are reported as mean $\pm$ s.d. of at least three experiments with similar results.

Results

In this study, 42 tissue samples of sPGL and 15 samples of HN-PGL (Table 1) were evaluated. At genetic analysis, 32 sPGLs resulted wt (22 without any germ line mutations and ten with polymorphic variants) and ten resulted with germ line mutations (three *SDHD*, one *SDHC*, one *SDHB*, two *RET*, one *VHL*, and two *MAX*) while among the 15 HN-PGLs, four resulted wt, nine resulted with germ line mutations (seven *SDHD* and two *SDHB*), and two with *SDHD* somatic mutations. A significant decrease in SDH activity, ranging from 60 to 80%, was found in all the *SDH*-mutated sPGLs, and as shown in Fig. 1A, a decrease in SDH activity, ranging from 40 to 80%, was also found in two out of 32 wt sPGLs and one *MAX*-mutated sPGL. Tumors mutated for *RET* and *VHL* presented a SDH activity comparable with that of controls.

Similar results were obtained in HN-PGL tissues (Fig. 2A). In fact, all the *SDH*-mutated tumors showed a significant decrease in SDH activity, ranging from 50 to 90%. The two tissue samples showing a decreased

Figure 1 SDH activity and expression in sPGL. (A) The histogram represents the SDH activities expressed as the percentage of the activity measured in tissue homogenates. (B) Blot is representative of three independent experiments, and SDHB protein expression values were normalized on actin (blot not showed). Bars are the mean $\pm$ s.d. of three independent experiments, $*P < 0.05$ compared with control. Two out of 32 wt patient tumors (dark gray bars), one *MAX*-mutated out of five tumors mutated for other susceptibility genes (black and white bars), and all *SDH*-mutated tissues (white bars) show a significantly decreased SDH activity (A) and *SDHB* expression (B) compared with control (black bar). Bar (a) is the mean $\pm$ s.d. of SDH activity and expression of the 30 wt sPGL showing no significant differences compared with control. Bars (b and c) represent the highest and lowest level of SDH activity (A) and expression (B) measured in wt sPGL.

SDH activity and classified as wt at genetic analysis were found to have *SDHD* somatic mutations. One of these mutations (p.Asp113MetfsX21) has been previously described by Cascon et al. (2002), while the second is a novel mutation (p.Leu7CysfsX7; Fig. 3).

The *SDH* expression in tumor samples, as evaluated by western blot analysis, closely correlated with the results of enzymatic assay, either in sPGL or in HN-PGL samples (Figs 1B and 2B respectively). LOH was found only in 70% of the patients with a decreased SDH activity and expression, while the remaining 30% showed a loss of SDH protein and function even in the absence of LOH.

Figure 2 SDH activity and expression in HN-PGL. (A) The histogram represents the SDH activities expressed as the percentage of the activity measured in tissue homogenates. (B) Blot is representative of three independent experiments, and SDHB protein expression values were normalized on actin (blot not showed). Bars are the mean $\pm$ s.d. of three independent experiments, * $P < 0.05$ compared with control. The two patients with the somatic mutations (light gray bars) and all *SDH*-mutated tissues (white bars) show a significantly decreased SDH activity (A) and *SDHB* expression (B) compared with control (black bar).

To investigate the mechanism responsible for the decreased SDH activity and expression in one of the two *MAX*-mutated samples (p.Leu94Pro), we performed an RT-PCR study on the four *SDH* subunit transcript levels. While in the control normal adrenal tissue, mRNA expression of the four subunits resulted, as expected, similar, in the *MAX*-mutated tissue, the transcript levels of *SDHB*, *SDHC*, and *SDHD*, were found slightly increased compared with control (Fig. 4).

At variance with SDH activity and expression, the activity of CS, which is an enzyme present in the mitochondrial matrix and is involved in the TCA cycle (Fig. 5), and the expression of voltage-dependent anion channel (VDAC), a protein located on mitochondria outer membrane (Fig. 6), were found normal and comparable among all the tissues both in sPGL and in HN-PGL while the expression of cytochrome *c* oxidase

(COX), which is an enzyme of the respiratory chain located in the inner mitochondrial membrane as mitochondrial complex IV, has been found extremely variable among the different tissue samples (Fig. 7).

Discussion

To the best of our knowledge, here for the first time SDH activity and expression have been systematically studied in sPGLs and HN-PGLs. Indeed, until now, only two research groups measured SDH activity in *SDH*-mutated tumors. In particular, [Gimenez-Roqueplo *et al.* \(2001\)](#) reported that in a French family, a mutation in *SDHD* results in the lack of SDH activity, and they also found a loss of SDH activity in two patients with *SDHB* and *SDHA* mutations ([Gimenez-Roqueplo *et al.* 2002](#), [Burnichon *et al.* 2010](#)). [Douwes Dekker *et al.* \(2003\)](#) also investigated SDH expression beside the SDH activity, but only in HN-PGLs.

As expected, we also observed that in *SDH*-mutated patients, SDH enzymatic activity is always decreased and that this loss of activity closely correlates with a decrease in protein expression.

This observation is in accordance with that previously reported by Dahia *et al.* (2005) and by Favier *et al.* (2009). Indeed, as already suggested for the not flavinated subunit A (Hao *et al.* 2009), in presence of mutations in one of the SDH subunits, the nonfunctional assembled SDH complex might be unstable and, as a consequence, more susceptible to degradation.

Interestingly, in addition to the *SDH*-mutated samples, a decrease in SDH activity and expression was also found in three sPGLs, two classified as wt and one as *MAX*-mutated, and in two HN-PGLs classified as wt at peripheral DNA genetic screening.

Figure 3 Electrophoresis patterns of the two somatic *SDHD* mutations. The DNA mutations are indicated by the small arrow.

Figure 4 mRNA expression levels of SDH subunits. Real-time PCR analysis in a normal adrenal gland and in the *MAX*-mutated (p.Leu94Pro) tissues. Quantitative mRNA analysis was performed by simultaneous amplification of the target *SDHA*, *SDHB*, *SDHC*, and *SDHD* transcripts. Results are expressed as fold changes according to the $2^{-\Delta\Delta C_T}$ method, using *SDHA* of the normal control as calibrator. The experiment was performed in triplicate.

Concerning the *MAX*-mutated tissue, some hypotheses can be assumed to explain the decreased complex II activity and expression. Indeed, we cannot exclude that this finding might be due to a mutation in another still unknown gene involved in the correct SDH assembling/function. Protein *MAX* is a member of the basic helix–loop–helix leucine zipper family of transcription factors and, depending on its association with *MYC* or *Mad*, the formed complex behaves as a transcriptional activator or a transcriptional repressor respectively. Accordingly, *MAX* might regulate the expression of some genes implicated in SDH formation, but this hypothesis is not confirmed by our RT-PCR experiments. Indeed, in the *MAX*-mutated tissue, the *SDHB*, *SDHC*, and *SDHD* transcripts were slightly increased not only in comparison with the *SDHA* transcript but also in comparison with the *SDH* transcripts of the healthy adrenal gland, thus suggesting an SDH complex instability and disassembling more than a negative transcriptional regulation in the *MAX*-mutated tissue.

As the other *MAX*-mutated tissue showed a normal SDH activity, a study on a larger number of *MAX*-mutated tissues is necessary to understand the relationship, if any, between SDH activity and *MAX* mutations.

The presence of *SDHD* somatic mutations explains the reduced SDH activity and content in the two HN-PGLs resulted wt at peripheral DNA genetic screening. Such somatic mutations as well as large deletions were not found in the two sPGLs resulted with wt at genetic screening. Therefore, a possible explanation for such a finding might be a mutation in one so far undiscovered susceptibility gene involved in SDH assembling and function.

It is well known that mitochondria are dynamic organelles that can change in number and morphology. Mitochondrial integrity is maintained through a continuous process by which mitochondria divide and fuse forming a dynamic interconnecting network, which undergoes remarkable structural changes both during apoptosis and under oxidative stress.

Figure 5 Citrate synthase (CS) activity in sPGL (A) and HN-PGL (B). The histogram represents the CS activities expressed as the percentage of the activity measured in tissue homogenates. Bars are the mean $\pm$ s.d. of three independent experiments (each of them conducted in duplicate samples). In both sPGL and HN-PGL, all wt (dark gray bars), *RET*-, *VHL*-, *MAX*- (black and white bars), and *SDH*-mutated (light gray or white bars) patient tumors show a CS activity comparable with control (black bar). In A, bar (a) is the mean $\pm$ s.d. of CS activity of the 32 wt sPGL, bars (b and c) are the highest and lowest CS activity, respectively, measured in wt tumors.

Figure 6 Study of voltage-dependent anion channel (VDAC) expression by western blot analysis in sPGL and HN-PGL. Blot is representative of three independent experiments, and the bar graphs are the mean $\pm$ s.d. of VDAC protein expression values normalized on actin (blot not showed). In both sPGL and HN-PGL, all wt (dark gray bars), *SDH*-polymorphic (light gray bars), *RET*-, *VHL*-, *MAX*- (black and white bars), and *SDH*-mutated (light gray or white bars) tumors show VDAC expression levels comparable with control (black bar). In A, bar (a) is the mean $\pm$ s.d. of VDAC expression of the 32 wt sPGL, bars (b and c) are the highest and lowest VDAC levels, respectively, measured in wt tumors.

To investigate whether our patients were also showing other alterations in other mitochondrial enzyme activities, we studied the activity of the CS. The CS is an enzyme present in the mitochondrial matrix and belongs to the TCA. Interestingly, CS activity was comparable among all the tissues, thus suggesting that enzyme function within the mitochondrial matrix was not impaired by the presence of *SDH* mutations.

As *SDH* is a complex present in the inner mitochondrial membrane and personal preliminary data obtained by electron microscopy on *SDH*-mutated sPGL and HN-PGL seem to suggest a structural alteration of mitochondrial cristae, we also investigated the expression levels of VDAC, a channel present on mitochondrial outer membrane and of COX, an enzyme of the respiratory chain present in the inner mitochondrial membranes. Interestingly, the expression of COX has been found extremely variable

in *SDH*-mutated as well as in *SDH* wt tumoral tissues, while VDAC content was comparable among all the samples. These observations suggest that in these tumors, only the mitochondrial cristae are altered, while the matrix and the outer membrane do not seem to be consistently modified.

In conclusion, an impairment of *SDH* activity can not only be due to germ line mutations in *SDH*-related genes but also due to somatic *SDH* mutations in tumor

Figure 7 Study of cytochrome *c* oxidase (COX) expression by western blot analysis in sPGL and HN-PGL. Blot is representative of three independent experiments, and the bar graphs are the mean $\pm$ s.d. of COX protein expression values normalized on actin (blot not showed). * $P < 0.05$ vs control. In sPGL, eight out of 32 wt tumors (dark gray bars), the two *MAX*-mutated tissues (black and white bars), and one *SDH*-mutated patient (white bars) show a significantly decreased COX expression compared with control (black bar), while three wt tumoral tissues (dark gray bars) and four out of five *SDH*-mutated tissues (white bars) show a significantly increased COX expression. In the HN-PGL, one out of four wt tumors (dark gray bars), one *SDH* somatic-mutated tumor (light gray bars), and four *SDH*-mutated patients (white bars) show a significantly decreased COX expression, while one *SDH* somatic-mutated tumor (light gray bars) and one *SDH*-mutated tumoral tissue (white bars) show a significantly increased COX expression compared with control (black bar). In A, bar (a) is the mean $\pm$ s.d. of COX expression of the 21 wt sPGLs not showing significant differences compared with control. Among these patients, bars (b and c) represent the wt patients with highest and lowest COX levels respectively.

tissue and possibly due to germ line mutations in other known (i.e. *MAX*) or unknown susceptibility genes. Moreover, SDH impairment seems to alter neither the outer mitochondrial membrane, as shown by the normal *VDAC* expression, nor the mitochondrial matrix, as shown by the normal *CS* activity. At variance, the wide difference in *COX* activity, either in *SDH*-mutated or wt tissues, suggests an alteration in mitochondrial cristae in PHEO/PGL, but additional studies are necessary to explain such high variability and to fully understand the mitochondrial role in the pathogenesis of these tumors.

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.

Funding

This work has been supported by Fondazione Cassa di Risparmio di Pistoia e Pescia (Prot. 2010.0278), Istituto Toscano Tumori (ITT, Prot. AOOGR/325462/Q.80.110) to M Mannelli and E Rapizzi is recipient of ITT fellowship.

Acknowledgements

E Rapizzi, T Ercolino, L Canu, and M Mannelli are members of the ENS@T (European Network for the Study of Adrenal Tumors).

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Received in final form 3 February 2012

Accepted 8 February 2012

Made available online as an Accepted Preprint
9 February 2012