SDHB loss predicts malignancy in pheochromocytomas/sympathetic paragangliomas, but not through hypoxia signalling

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Abstract

Prediction of malignant behaviour of pheochromocytomas/sympathetic paragangliomas (PCCs/PGLs) is very difficult if not impossible on a histopathological basis. In a familial setting, it is well known that succinate dehydrogenase subunit B (SDHB)-associated PCC/PGL very often metastasise. Recently, absence of SDHB expression as measured through immunohistochemistry was shown to be an excellent indicator of the presence of an *SDH* germline mutation in PCC/PGL. SDHB loss is believed to lead to tumour formation by activation of hypoxia signals. To clarify the potential use of SDHB immunohistochemistry as a marker of malignancy in PCC/PGL and its association with classic hypoxia signalling we examined SDHB, hypoxia inducible factor-1 α (Hif-1 α) and its targets CA-9 and GLUT-1 expression on protein level using immunohistochemistry on a tissue micro array on a series of familial and sporadic tumours of 115 patients. Survival data was available for 66 patients. SDHB protein expression was lost in the tumour tissue of 12 of 99 patients. Of those 12 patients, 5 had an *SDHB* germline mutation, in 4 patients no germline mutation was detected and mutational status remained unknown in parts in 3 patients. Loss of SDHB expression was not associated with increased classic hypoxia signalling as detected by Hif-1 α , CA-9 or GLUT-1 staining. Loss of SDHB expression was associated with an adverse outcome. The lack of correlation of SDHB loss with classic hypoxia signals argues against the current hypoxia hypothesis in malignant PCC/PGL. We suggest SDHB protein loss as a marker of adverse outcome both in sporadic and in familial PCC/PGL.

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Introduction

Pheochromocytomas (PCCs) are rare tumours of neural crest-derived chromaffin cells. Most tumours arise in the adrenal gland, but up to 23% (Karagiannis *et al.* 2007) are localised in extra-adrenal tissue and are called sympathetic paragangliomas (PGLs). About 30% of these

tumours occur in familial tumour syndromes (Neumann *et al.* 2002, Amar *et al.* 2005, Tischler 2008, Komminoth 2009) including neurofibromatosis type 1 (NF1), von Hippel–Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN2) and succinate dehydrogenase (SDH) syndromes.

The underlying molecular mechanisms leading to PCCs/sympathetic PGLs are not fully understood. Several studies suggest that classic hypoxia signalling involving hypoxia inducible factor-1 α (Hif-1 α) may play a central role, at least in the context of VHL deficiency (Maxwell *et al.* 1999). VHL inactivation leads to classic hypoxia signalling by inhibiting the degradation of the Hif-1 α : under normoxic conditions, Hif-1 α is hydroxylated and degraded. Under hypoxia and in some types of cancer it stabilises and accumulates in the nucleus. This leads to an upregulation of the Hif target genes *CA-9* and *GLUT-1* on the one hand and to an increased expression of VEGF, resulting in an increased microvessel density, on the other hand (Couvelard *et al.* 2005). This mechanism is supported by expression analysis of PCC: on the RNA level, Eisenhofer *et al.* (2004) described the activation of hypoxia-driven angiogenic pathways in VHL syndrome PCCs. Hif-1 α was not in the list of upregulated genes, possibly because it is regulated on protein rather than on mRNA level (van Uden *et al.* 2008). Dahia *et al.* (2005) described a hypoxia-induced expression profile in both VHL- and SDH-induced PCCs/PGLs. By gain- and loss-of-function analyses they additionally suggested that the link between hypoxia signals (through pVHL) and mitochondrial signals (through SDH) is mediated by Hif-1 α . Succinate accumulating due to SDH mutations can inhibit the degradation of Hif-1 α as do VHL mutations in this *in vitro* model. This is thought to cause upregulation of Hif targets leading to tumorigenesis (Dahia *et al.* 2005, Selak *et al.* 2005, 2006) in SDH-associated PCCs/PGLs. These hypotheses have been challenged by a recent study on hypoxia signalling in hereditary PCCs/PGLs: on mRNA levels there was no difference of Hif-1 α expression between the different tumour types but Hif-2 α was over-expressed in VHL- and SDH-related tumours, a finding that was also reflected by protein expression analysis (Favier *et al.* 2009). Hif-2 α is reported to be associated with a poor patient outcome in several tumour types (Qing & Simon 2009). Other results also point towards alternative mechanisms than classic hypoxia signalling: VHL mutants leading to the VHL2c phenotype consisting exclusively of PCCs retain their ability to downregulate Hif (Hoffman *et al.* 2001). In *Caenorhabditis elegans*, a subset of genes dysregulated in vhl mutants is not normalised in vhl/hif-1 double mutants (Bishop *et al.* 2004). Failure of developmental apoptosis may be the hypoxia-independent mechanism of PCC pathogenesis (Lee *et al.* 2005), possibly with a regulatory loop including Hif (Maxwell 2005).

In the familial setting, it is well known that SDH subunit B (SDHB)-associated PCCs/PGLs very often lead to metastases (Timmers *et al.* 2007), sometimes many years after resection (Maier-Woelfle *et al.* 2004). Prediction of malignancy in sporadic tumours is an unsolved problem; the only definite evidence of malignancy is the detection of metastases. The 'PCC of the adrenal gland scaled score (PASS)', a morphological scoring system to identify more aggressive tumours (Thompson 2002), has not proved to be useful due to great interobserver variability in a recent study (Wu *et al.* 2009). An increased risk of malignancy seems to be indicated by Ki-67 proliferation indices >2 or 3%, but this is of limited clinical use (van der Harst *et al.* 2000, August *et al.* 2004, Kimura *et al.* 2005, Strong *et al.* 2008). Recently, loss of SDHB immunohistochemistry was shown to be an excellent indicator of the presence of an SDH germline mutation in PCCs and PGLs (van Nederveen *et al.* 2009). Its potential use for predicting biological behaviour is unknown.

To clarify the potential use of SDHB immunohistochemistry as a marker of malignancy and its association with classic hypoxia signalling, we decided to examine SDHB, Hif-1 α and its targets CA-9 and GLUT-1 expression on protein level on a series of familial and sporadic PCCs and sympathetic PGLs and to correlate the results with survival data.

Materials and methods

Patients and tumour specimens

All PCCs and sympathetic PGLs analysed at the Institute of Surgical Pathology, University Hospital Zurich in the years from 1975 to 2006 were included.

Clinical data and follow-up information were extracted retrospectively from patient charts. A questionnaire enquiring about tumour relapse or progression was sent to family doctors.

Tumour specimens

We analysed PCC/PGL specimens from 115 patients (62 males, 46 females and sex could not be evaluated in 7 patients). The available paraffin specimens comprised 112 primary tumours and 3 metastases. Of the 112 primary tumours, 26 were of extra-adrenal abdominal localisation (19 males and 8 females), 76 originated from the adrenal medulla (37 males, 32 females and 7 not specified) and 8 originated extra-abdominally. Information about the localisation was not available in two tumours. Of the three metastases one was of an extra-adrenal abdominal primary

(patient with known *SDHB* germline mutation), one of an extra-abdominal primary (patient without *SDHB* germline mutation) and the one metastasis was of an unknown primary (mutational status of the patient unknown). A tissue micro array (TMA) comprising these 115 tissues was constructed as described previously (Bubendorf *et al.* 2001).

Syndromic patients

One male patient suffered from NF1 disease clinically, five patients from VHL disease (four with proven *VHL* mutation, one clinical VHL disease with multiple bilateral clear cell renal cell carcinomas in addition to the PCC (one male, three females and one not specified)) and six patients from MEN2 (proven *RET* mutation (three males and three females)) (Table 1). An overview of patients and follow-up data is given in Table 2.

Immunohistochemistry

The analysis was performed on 4 µm sections from the TMA, which was stained with antibodies against CA-9, Hif-1α, CD34, GLUT-1 and SDHB. The immunohistochemical staining for the antigens was performed on automated staining systems (CA-9 on Bond Refine, Vision BioSystems Ltd, Newcastle upon Tyne, UK; Hif-1α on Bond Refine, Vision BioSystems Ltd; CD34 on Ventana BenchMark, Ventana Medical Systems, Tucson, AZ, USA; GLUT-1 on Ventana BenchMark). For SDHB staining, the slides were pretreated by microwave heating in Tris/EDTA buffer, pH 9.0 at 100 °C for 40 min or citrate buffer, pH 6.0 for 15 min.

After rinsing in tap water followed by incubation in 3% H₂O₂ in PBS for 15 min the SDHB antibody was incubated overnight at 4 °C. The presence of tumour tissue was verified by synaptophysin and H&E stainings in all punch cylinders.

The following antibodies were used: CA-9 polyclonal antibody ab15086 (Abcam, Cambridge, UK), dilution 1:200; Hif-1α monoclonal antibody ab16066 (Abcam), dilution 1:500; GLUT-1 polyclonal antibody AB1341 (Chemicon International, Temecula, CA, USA), dilution 1:1000 and SDHB rabbit polyclonal HPA002868 (Sigma-Aldrich Corp.), dilution 1:250; CD34 clone QBEND/10 (MCAP 547, Serotec, MorphoSys, Oxford, UK), dilution 1:800.

Visualisation was accomplished using the avidin–biotin complex method leading to a brown staining signal. As controls, for SDHB and CD34 endothelial cells served as internal positive control, for CA-9 and GLUT-1 normal liver tissue was used and for Hif-1α glioblastoma tissue was used.

For CA-9 cytoplasmic and/or membranous staining was scored positive. For Hif-1α nuclear and cytoplasmic stainings were evaluated separately. For GLUT-1 cytoplasmic staining was scored positive. For Hif-1α and GLUT-1, depending on the intensity of staining, a semiquantitative scoring system was used, comprising strongly positive, weakly positive and negative immunoreactivities. For CA-9 stainings were scored as either negative or positive. In all stainings tumours with <5% positive tumour cells were scored as negative.

SDHB was scored as positive if the cytoplasm showed a strong dot-like positivity. We categorised

Table 1 Results of immunohistochemical hypoxia stainings in familial tumours

Patient no.	Syndrome	Sex	Organ	SDHB	Hif-1α	CA-9
40	NF1	M	Adrenal gland	+	+	–
28	RET(MEN2)	M	Adrenal gland	+	–	–
39	RET(MEN2)	M	Adrenal gland	+	–	–
60	RET(MEN2)	M	Adrenal gland	+	–	–
61	RET(MEN2)	F	Adrenal gland	+	–	–
71	RET(MEN2)	F	Adrenal gland	+	–	–
117	RET(MEN2)	F	Adrenal gland	+	–	–
5	SDHB	M	Abdominal ea	NA	–	–
11	SDHB	M	Abdominal ea	–	+	+
33	SDHB	F	Abdominal ea	–	–	–
77	SDHB	M	Abdominal ea	–	–	–
78	SDHB	M	Abdominal ea	–	–	–
21	VHL	M	Adrenal gland	NA	–	NA
54	VHL	F	Adrenal gland	+	+	+
115	VHL	F	Adrenal gland	+	+	–
126	VHL	UK	Adrenal gland	+	–	–
42	VHL	F	Adrenal gland	+	–	–

–, no staining; +, positive staining; NA, not assessable; ea, extra-adrenal; UK, unknown.

Table 2 Clinical characteristics of 115 pheochromocytomas (PCC) patients

	Total	Men	Women	Unknown
Patients	115	62 (53.9%)	46 (40.0%)	7 (6.1%)
Multifocal	19 (16.5%)	11 (9.6%)	7 (6.1%)	1 (0.9%)
Follow-up available	66 (57.4%)	35 (30.4%)	31 (27.0%)	0 (0%)
Tumour-related death	15 (13.0%)	5 (4.3%)	3 (2.6%)	7 (6.1%)
No tumour-related death/alive	59 (51.3%)	30 (26.1%)	29 (25.2%)	0 (0%)
Follow-up				
Range (month)	2–291	5–291	2–188	–
Mean (month)	78.39	77.4	79.52	–
Median (month)	55.5	52	71	–
Syndromic patients				
NF1	1 (0.9%)	1 (0.9%)	0 (0%)	0 (0%)
VHL	5 (4.3%)	1 (0.9%)	3 (2.6%)	1 (0.9%)
MEN2	6 (5.2%)	3 (2.6%)	3 (2.6%)	0 (0%)
SDHB	5 (4.3%)	4 (3.5%)	1 (0.9%)	0 (0%)
Localisation (primary tumour)				
Unknown	3 (2.6%)	2 (1.7%)	1 (0.9%)	0 (0%)
Adrenal	76 (66.1%)	37 (32.2%)	32 (27.8%)	7 (6.1%)
Extra-adrenal	33 (28.7%)	22 (19.1%)	11 (9.6%)	0 (0%)

the tumour as negative if the cytoplasm was negative in the presence of internal positive control in endothelial cells. Tumours with homogeneous faint cytoplasmic staining were scored negative (van Nederveen *et al.* 2009).

Microvessel density was calculated by counting all vessel sections in tumour tissue of each TMA cylinder with the help of a grid and a counting device in relation to the area consisting of tumour tissue. The number of vessel sections per square millimetre was calculated. Examples of immunohistochemical stainings are given in Fig. 1.

All immunohistochemical stainings were evaluated in a blinded manner by at least two pathologists separately (A B, A P and F v N).

SDHB mutation analysis

In patients with negative *SDHB* immunohistochemistry germline mutation analysis of the *SDH* genes was performed on peripheral blood or non-neoplastic paraffin tissue after obtaining informed consent. Where no non-neoplastic tissue was available, mutation analysis was performed in tumour tissue. All mutation analyses were performed at least twice in two laboratories (Bern or Zurich and Rotterdam).

DNA was extracted from peripheral blood using the Puregene kit (GentraSystems, Minneapolis, MN, USA) according to the manufacturer's instructions. DNA was extracted from paraffin tissue as described previously (Maier-Woelfle *et al.* 2004). Mutation analysis was performed by denaturing gradient gel electrophoresis-based mutation analysis as described previously

(Maier-Woelfle *et al.* 2004). PCRs were repeated for all samples with abnormal banding patterns followed by cycle sequencing. Mutation analysis was repeated in a second laboratory by cycle sequencing in all patients where no *SDHB*-mutation was detected.

SDHB deletion analysis

In all *SDHB* immunonegative cases where no *SDHB* mutation was detected fluorescence *in situ* hybridisation (FISH) analysis was performed to detect copy number losses. Deletion analysis was performed by double-target FISH as described before (Schmitt *et al.* 2009) using a chromosome 1-specific centromere probe in combination with a 1p36.1–1p35-specific probe containing the *SDHB* gene. Images were recorded with Analysis software (Olympus Biosystems, Hamburg, Germany).

Statistical analysis

The statistical analysis was performed with SPSS version 16.0.1 (SPSS Software, Chicago, IL, USA). We used two-sided Pearson's χ^2 -test and Spearman correlation coefficient to analyse dependence of the data. Kaplan–Meier curves were used for demonstration of survival. *P* values <0.05 were considered to indicate statistical significance.

Ethics

The study was approved by the local ethical committee (Kantonale Ethikkommission, StV 37-2006).

Figure 1 Immunohistochemistry of PCC/PGL. (A) Negative for CA-9; (B) positive for CA-9; (C) nuclear negativity for HIF-1 α ; (D) nuclear positivity for HIF-1 α ; (E) negative for SDHB (positive signal in endothelial cells); (F) positive for SDHB (note granular cytoplasmic staining); (G) first quartile microvessel density (MVD); (H) second quartile MVD; (I) third quartile MVD; (J) fourth quartile MVD.

Results

Follow-up

Follow-up data were available for 66 patients (57.4%). The survival data ranged from 2 to 291 months (mean: 78.39 months; median: 55.5 months). In 15 patients (13.0%), death was caused by tumour (5 males, 3 females and 7 not specified) (Table 2).

CA-9 immunohistochemistry

Tumours of 111 patients (96.5%) could be evaluated for CA-9 protein expression by immunohistochemistry. In total, 15 (13.5%) of these were scored positive (11 males and 4 females), 96 (86.5%) were negative (48 males, 41 females and 7 without details about sex).

Hif-1 α immunohistochemistry

Hif-1 α could be evaluated in 114 patients (99.1%). Nuclear staining was negative in 110 of 114 (96.5%) patients (60 males, 43 females and sex of 7 patients unknown), only 4 of 114 (3.5%) patients showed nuclear staining (2 males and 2 females).

Regarding cytoplasmic staining, 6 of 114 (5.3%) showed a strong positive staining (4 males, 1 female and sex of 1 patient unknown) and 14 of 114 (12.3%) patients were weakly positive (6 males, 4 females and sex of 4 patients unknown) whereas the majority of patients (94 of 114, 82.5%) were negative (52 males, 40 females and sex of 2 patients unknown).

GLUT-1 immunohistochemistry

GLUT-1 could be evaluated in 112 patients (97.5%), 6 of them showed a strong positivity (3 males, 2 females and sex of 1 patient unknown), 2 showed a strong positivity (1 male and 1 female) and 104 of 112 patients (56 males, 42 females and sex of 6 patients unknown) were negative.

SDHB immunohistochemistry

SDHB immunohistochemistry could be evaluated in 99 patients (86.1%). In 16 patients SDHB immunohistochemistry could not be evaluated due to lack of a positive internal control in the endothelium of the intratumoural vessels. In all, 12 of 99 (12%) patients were SDHB negative, including all the 5 patients, in whom an *SDHB* germline mutation was detected (10 males and 2 females); 87 of 99 (87%) were weakly or strongly positive (41 males, 39 females and 7 unknown). Follow-up was available for 60 of these 99 patients including 9 of the 12 SDHB immunonegative patients.

Eleven of the 12 (92%) SDHB-negative tumours were localised outside the adrenal gland (i.e. extra-adrenal sympathetic PGLs).

Three of the 12 (25%) SDHB-negative tumours showed a strong positivity in the CA-9 staining, and 9 (75%) were negative (not significant in cross tabulation).

None of the SDHB-negative patients showed a strong nuclear positivity for Hif-1 α , all 12 were negative for Hif-1 α in the nucleus. Two (17%) SDHB-negative patients showed a strong cytoplasmic positivity for Hif-1 α , 1 (8%) was weakly positive and 9 (75%) were negative for Hif-1 α .

Mutation status in SDHB-negative tumours

In two brothers an *SDHB* germline mutation was already known. Mutation analysis of the *SDHB*, *SDHC* and *SDHD* genes was performed in all the remaining 10 patients. *SDHB* germline mutation was excluded in

one patient by analysing non-neoplastic tissue. In three additional patients SDHB mutations were excluded in tumour tissues as no non-neoplastic tissue was available. In the remaining three patients SDHB mutations could be excluded in parts of the *SDHB* gene in tumour tissue, the remaining exons were not amplifiable (Table 3). All patients without an *SDHB* germline mutation were tested for *SDHC* and *SDHD* mutations. *SDHC* mutations were absent in all three informative patients and *SDHD* mutations were absent in all five informative patients.

Microvessel density

In 109 evaluable cases the number of vessel sections in the area of the cylinder (0.28 mm²), corrected for non-neoplastic portions if present, ranged from 3.75 to 1144 (mean 229 and median 122). Tumours were subdivided into quartiles according to the number of vessel sections per mm². The first quartile ranged from 13 to 535 vessel sections per mm², the second from 548 to 1083 vessel sections per mm², the third from 1268 to 1530 vessel sections per mm² and the fourth from 1641 to 4046 vessel sections per mm².

SDHB deletion analysis

In seven SDHB immunonegative cases without a detectable *SDH* mutation SDHB deletion analysis was performed by FISH. One SDHB immunonegative case with an *SDHB* germline mutation and a known deletion served as a positive control. In five of these seven SDHB immunonegative cases no SDHB deletion was detected. Two cases were not evaluable in repeated analyses.

Statistical analysis

SDHB immunoreactivity did neither correlate with cytoplasmic CA-9 staining ($P=0.150$; $r_s=0.000$) nor with nuclear or cytoplasmic Hif-1 α staining ($P=0.555$; $r_s=0.109$ and $P=0.399$; $r_s=-0.010$ respectively) nor with GLUT-1 ($P=0.780$; $r_s=0.083$).

Significant correlation was observed between both nuclear and cytoplasmic Hif-1 α immunoreactivity and CA-9 staining ($P=0.000$; $r_s=0.348$ and $P<0.000$; $r_s=0.123$ respectively).

Survival analysis was performed for all immuno-histochemical markers. The survival of patients with SDHB immunonegative tumours was significantly adverse compared with SDHB-positive tumours

Table 3 Clinical characteristics of SDHB immunonegative tumours

PGL-no.	Sex	Syndrome	SDHB mutation	Tumour	Localisation of primary tumour	Localisation of metastasis	Multi-focal	Follow-up (age at time of death)
aPGL001	M	No	All ex neg (tu)	Primary tumour	Abdominal ea/paraaortal	No	No	AWD
aPGL007	M	SDHB	Del 632G in Exon 5	Metastasis	Abdominal ea/pararenal	Spine	No	DOD (37)
aPGL030	M	No	All ex neg. (tu + nnt)	Primary tumour	Abdominal ea/pararenal	Liver, bones, lymph nodes	No	DOD (62)
aPGL031	M	SDHB	C. 307insC/p.103 Trp fs	Primary tumour	Abdominal ea/paraaortal		No	NA
aPGL047	M	No	Ex 4/6 neg. remaining NA (tu)	Primary tumour	Adrenal gland	No	No	NA
aPGL058	M	No	Ex 1/2/3/4/5/6/7 neg. 8 NA (tu)	Primary tumour	Abdominal ea/paraaortal	Aorta, small bowel, bones	Yes	AWD
aPGL091	F	SDHB	Del 632 G in Exon 5	Primary tumour	Abdominal ea/pararenal	No	No	AWD
aPGL204	M	SDHB	H132P	Primary tumour	Abdominal ea/paraaortal	Lymph nodes	No	DOD (43)
aPGL205	M	SDHB	H132P	Metastasis	Abdominal ea/paraaortal	Bones	Yes	DOD (60)
aPGL243	F	No	All ex neg. (tu)	Metastasis	Mediastinal	Brain	Yes	DOD (56)
aPGL320	M	No	Ex 1/2/4/5/6 neg (tu)	Primary tumour	Abdominal ea/paraaortal	No	No	AWD
aPGL347	M	No	Ex 6 neg. (tu)	Primary tumour	Abdominal ea/paraaortal	No	Yes	NA

SDHB, succinate dehydrogenase subunit B; Del, deletion; ins, insertion; ea, extra-adrenal; NA, not assessable; tu, tumour; nnt, non-neoplastic tissue; AWD, alive with disease; DOD, death of disease.

Figure 2 Kaplan–Meier curve depicting percentage of tumour-specific survival for PCC/PGL with (upper curve) and without (lower curve) SDHB immunoreactivity.

($P<0.0001$; Fig. 2). No survival difference between a weak and strong SDHB positivity was found. An extra-adrenal localisation of the primary tumour was also associated with a shortened tumour-specific survival ($P=0.005$). The survival depending on hypoxia markers CA-9, Hif-1 α or GLUT-1 offered no significant trend.

Increased microvessel density correlated significantly with loss of SDHB ($P=0.017$; $r_s=-0.289$) but not with Hif-1 α in the nucleus ($P=0.192$; $r_s=0.171$), Hif-1 α in the cytoplasm ($P=0.496$; $r_s=0.048$), GLUT-1 ($P=0.990$; $r_s=-0.040$) and CA-9 ($P=0.186$; $r_s=0.070$). An overview over the correlation results is given in Table 4.

Discussion

Prediction of malignant behaviour of PCCs is very difficult if not impossible on a histopathological basis. A proposed morphological scoring scheme did not prove useful in a recent analysis due to large inter-observer variability (Thompson 2002, Kimura *et al.*

2005, Wu *et al.* 2009). The present survival analysis on 66 unselected PCC/sympathetic PGL patients with follow-up revealed SDHB immunohistochemistry as a possible prognostic marker. The survival of patients with SDHB immunonegative tumours was significantly worse than of patients with SDHB-positive tumours. Five of nine patients with SDHB immunonegative tumours and available follow-up died because of the tumours; of those five patients three had an *SDHB* germline mutation. A tumour-related death did not occur in the six strongly SDHB-positive tumours and only twice in the 46 weakly SDHB-positive tumours with available follow-up. A possible explanation of these findings is that the SDHB-negative tumours arise in patients with the SDHB-associated PCC/PGL syndrome. Indeed, we could demonstrate an *SDHB* germline mutation in five of the nine immunonegative patients we were able to examine. *SDHB* mutations were excluded in all coding regions of four patients. We were only able to exclude *SDHB* mutations in the remaining three immunonegative patients in a minority of SDHB exons. In tumours with *SDHB* germline mutation inactivation of the wild-type allele occurs by loss of heterozygosity (LOH) (Gimenez-Roqueplo *et al.* 2002, Maier-Woelfle *et al.* 2004); we were able to exclude such LOH by FISH analysis in five of the seven immunonegative cases without germline mutation. *SDHB* germline deletions are described in up to 30% of SDHB kindreds (McWhinney *et al.* 2004, Tischler 2008). In these tumours LOH would also be expected. Therefore, we postulate that at least these five patients with SDHB immunonegative tumours without mutation or deletion suffer from sporadic disease. van Nederveen *et al.* (2009) described SDHB loss by immunohistochemistry in 11–16% of sporadic tumours, a similar frequency as described in this study. Complete loss of granular SDHB immunopositivity has been shown

Table 4 Correlations and correlation coefficient of immunohistochemistry and number of vessels

	Hif-1 α nucleus	Hif-1 α cytoplasm	SDHB	GLUT1	Number of vessels	Localisation
CA9	0.000	0.000	0.150	0.834	0.186	0.406
Spearman correlation	0.348	0.123	0.000	−0.006	−0.070	
Hif-1 α nucleus		0.643	0.555	0.853	0.192	0.291
Spearman correlation		−0.088	0.109	−0.053	0.171	
Hif-1 α cytoplasm			0.399	0.301	0.496	0.174
Spearman correlation			−0.010	0.148	0.048	
SDHB				0.780	0.017	0.000
Spearman correlation				0.083	−0.289	
GLUT1					0.990	0.665
Spearman correlation					−0.40	

P values bold numbers indicate statistical significance.

Figure 3 Kaplan–Meier curve depicting percentage of tumour-specific survival for PCC/PGL in dependence of microvessel density (displayed in quartiles). There is no significant correlation between microvessel density and survival.

as a predictor of *SDH* germline mutation with a positive predictive value of at least 92% and a negative predictive value of 100% (van Nederveen et al. 2009). In our series, both tumours with a known *SDH* germline mutation stained negative for SDHB immunohistochemistry, confirming the high negative predictive value of a positive SDHB staining. From the clinical point of view our data also stress the role of localisation of the tumour. In our series there was a strong correlation of extra-adrenal localisation with poor prognosis and *SDHB* germline mutation, which is in line with previous findings (Neumann et al. 2002, Maier-Woelfle et al. 2004, Amar et al. 2005, Lenders et al. 2005, Tischler 2008).

One aim of our study was to examine the possible link between SDHB immunostaining, which is indicative of enzymatic activity of the SDH complex (van Nederveen et al. 2009) with classic hypoxia signalling, as suggested by several authors (Dahia et al. 2005, Pollard et al. 2006). Classic hypoxia signalling through Hif-1 α and its target genes *CA-9* and *GLUT-1* plays a central role in the development of clear cell renal cell carcinoma and glioblastoma (Kaelin 2004, Kaur et al. 2005). Thus, we examined the expression of Hif-1 α , *CA-9* and *GLUT-1* by immunohistochemistry. Classic hypoxia signalling as characterised by an accumulation of these proteins in our study could be detected only in a small minority of tumours and did not have any influence on survival. There was no association of SDHB loss with *CA-9*, *GLUT-1* and Hif-1 α protein level. In contrast, SDHB protein loss correlated with a high microvessel density. Moreover, SDHB loss correlated with an adverse outcome, while a high microvessel density did not significantly correlate with a poor survival (Fig. 3). The two markers of classical

hypoxia, Hif-1 α and *CA-9*, showed a strong correlation with each other, arguing against technical problems in identifying classic hypoxia signals. These findings argue against an important role of classic hypoxia signalling in the development of PCCs/PGLs. However, pseudohypoxia, which is mediated by Hif-2 α , might play a role as suggested by Favier et al. (2009) who found a high Hif-2 α expression in both VHL- and SDH-associated PCCs. However, while the same authors described an upregulation of glucose transporters solely in VHL-associated PCC we cannot confirm these findings: in our study all VHL-associated cases were immunonegative for GLUT-1.

Alternatively, other mechanisms than pseudohypoxia signals might be involved in the genesis of SDHB-associated and most sporadic PCCs even in the setting of the VHL syndrome, such as apoptosis and inhibition of microtubule stabilisation as suggested by other authors (Hergovich et al. 2003, Lee et al. 2005). Further studies will be needed to elucidate the mechanisms of tumourigenesis in PCCs/PGLs.

In summary, we suggest SDHB protein loss as a marker of adverse outcome both in sporadic and in familial PCCs/PGLs. Inclusion of this marker in the assessment of PCCs/PGLs might be mandatory for two reasons: first, for direction of molecular genetic testing towards the *SDH* genes in the case of absent staining, and second as a prognostic marker. We suggest to include patients with sporadic SDHB-negative tumours in more stringent follow-up protocols.

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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