Overexpression of miR-210 is associated with SDH-related pheochromocytomas, paragangliomas, and gastrointestinal stromal tumours

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Abstract

miR-210 is a key regulator of response to hypoxia. Pheochromocytomas (PCs) and paragangliomas (PGLs) with germline *SDHx* or *VHL* mutations have pseudohypoxic gene expression signatures. We hypothesised that PC/PGLs containing *SDHx* or *VHL* mutations, and succinate dehydrogenase (SDH)-deficient gastrointestinal stromal tumours (GISTs), would overexpress miR-210 relative to non-*SDH* or -*VHL*-mutated counterparts. miR-210 was analysed by quantitative PCR in i) 39 PC/PGLs, according to genotype (one *SDHA*, five *SDHB*, seven *VHL*, three *NF1*, seven *RET*, 15 sporadic, one unknown) and pathology (18 benign, eight atypical, 11 malignant, two unknown); ii) 18 GISTs, according to SDHB immunoreactivity (nine SDH-deficient and nine SDH-proficient) and iii) two novel *SDHB*-mutant neurosphere cell lines. miR-210 was higher in *SDHx*- or *VHL*-mutated PC/PGLs (7.6-fold) compared with tumours without *SDHx* or *VHL* mutations ($P=0.0016$). miR-210 was higher in malignant than in unequivocally benign PC/PGLs ($P=0.05$), but significance was lost when benign and atypical tumours were combined ($P=0.08$). In multivariate analysis, elevated miR-210 was significantly associated with *SDHx* or *VHL* mutation, but not with malignancy. In GISTs, miR-210 was higher in SDH-deficient (median 2.58) compared with SDH-proficient tumours (median 0.60; $P=0.0078$). miR-210 was higher in patient-derived neurosphere cell lines containing *SDHB* mutations (6.5-fold increase) compared with normal controls, in normoxic conditions ($P<0.01$). Furthermore, siRNA-knockdown of SDHB in HEK293 cells increased miR-210 by 2.7-fold ($P=0.001$) under normoxia. Overall, our results suggest that SDH deficiency in

Key Words
- pheochromocytoma
- paraganglioma
- gastrointestinal stromal tumour
- succinate dehydrogenase
- miR-210

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-13-0519

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PC, PGL and GISTs induces miR-210 expression and substantiates the role of aberrant hypoxic-type cellular responses in the development of these tumours.

Endocrine-Related Cancer (2014) **21**, 415–426

Introduction

Pheochromocytomas (PCs) are catecholamine-secreting tumours of chromaffin cells of the adrenal medulla and are closely related to paragangliomas (PGLs) which arise from sympathetic and parasympathetic chains (Favier *et al.* 2005). Patients with these tumours often present with features of hormone excess, including hypertension, cardiovascular instability or local compressive symptoms. Up to 30% of PC/PGL are associated with germline mutations, most commonly in genes encoding succinate dehydrogenase (SDH) subunits A, B, C and D (*SDHA, SDHB, SDHC, SDHD*; collectively *SDHx*), but also in *VHL, RET* (Buffet *et al.* 2012), *TMEM127* (Qin *et al.* 2010), *MAX* (Comino-Mendez *et al.* 2011), *SDHAF2* (Bayley *et al.* 2010) and *KIF1Bβ* (Yeh *et al.* 2008). The mechanistic link between mutation in these genes and PC/PGL tumourigenesis is not clear; however, at least two lines of evidence implicate oxygen tension and/or sensing. First, hypoxia has been implicated in PGL development, particularly those located in the head and neck which are typically *SDHD*-mutated (Astrom *et al.* 2003). Second, on the basis of microarray data, PC/PGLs appear to divide into two groups: those associated with a 'hypoxic' gene expression signature (including *SDHx* and *VHL* mutated tumours) and those associated with a 'kinase activation' signature (including *RET, NF1, TMEM127* and *MAX* associated tumours) (Dahia *et al.* 2005, Comino-Mendez *et al.* 2011). VHL has an established biological role in the regulation of hypoxic response via degradation of hypoxia inducible factor, *HIF1α*. The involvement of SDH with hypoxia, however, has been less obvious. One hypothesis is that *SDHx* mutation leads to intracellular succinate accumulation which in turn stabilises HIFα and therefore activation of a hypoxic gene signature (Favier & Gimenez-Roqueplo 2010).

Gastrointestinal stromal tumours (GISTs) arise from the pacemaker cells of the gastrointestinal tract, and 85–90% of GISTs are driven by activating mutations of the tyrosine kinases *KIT* or *PDGFRA* (Corless *et al.* 2005). SDH-deficient GISTs are a newly recognised subtype of GIST defined by the loss of immunohistochemical staining for SDHB (Gill *et al.* 2010*a*). In contrast to usual (or SDH proficient) GISTs, SDH-deficient GISTs arise exclusively in the stomach (where they account for 5–7.5% of GISTs) and demonstrate several other unique clinical and pathological features (Gill 2012). Of note, they are not associated with KIT or PDGFRA mutations but instead appear to be driven primarily by the dysfunction of the mitochondrial complex II similar to *SDHx* mutated PGL (Janeway *et al.* 2011). SDH-deficient GISTs could therefore be expected to demonstrate a hypoxic rather than a kinase activation phenotype.

There has been increasing interest in the role of microRNA (miRNA) in tumour development. These non-coding RNAs, 20–22 nucleotides in length, regulate gene expression via binding to 3′UTRs of their related parent mRNAs. miR-210 has become known as a key regulator of hypoxia, acting on a number of targets including, *E2F3, NPTX1, RAD52, ACVR1B, MNT, CASP8AP2, FGFRL1* and *HOXA1* and *-9* (Fasanaro *et al.* 2009, Chan *et al.* 2012). In many cell types, miR-210 expression has been shown to be increased under hypoxia, and in turn may contribute to tumourigenesis through regulation of genes that are involved in a number of cellular processes, including cell cycling, differentiation, migration and escape from apoptosis (Fasanaro *et al.* 2009). Further, miR-210 has been associated with the development and aggressiveness of a number of cancers, including head and neck squamous cell carcinoma (Gee *et al.* 2010), pancreatic cancer (Greither *et al.* 2010), renal cell cancer (Juan *et al.* 2010, Neal *et al.* 2010), breast cancer (Foekens *et al.* 2008) and lung cancer (Puissegur *et al.* 2010).

In this study, the expression of miR-210 was studied in order to determine its involvement in the pathogenesis of PC/PGLs. We hypothesised, firstly, that miR-210 would be upregulated to a greater degree in those tumours typically associated with a pseudohypoxic gene expression signature, namely those associated with *SDHx* or *VHL* mutation. Second, we questioned whether overexpression of miR-210 would be associated with malignancy. Third, to further substantiate the role of miR-210 in SDH-related tumourigenesis, we examined miR-210 expression in GISTs, by comparing SDH-deficient with SDH-proficient GISTs, as well as in two novel cell lines containing germline *SDHB* mutations.

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Subjects and methods

Patient and tissue samples

PCs and PGLs Tumour samples and paired peripheral blood samples were obtained from 39 patients, consisting of 31 PCs, seven extra adrenal PGLs and one metastasis from a malignant PC previously presenting with a PC (Table 1). Normal adrenal medulla tissue was obtained from five patients who had undergone total unilateral adrenalectomy for small (<4 cm) benign non-functioning

Table 1 Patient and tumour characteristics: pheochromocytomas and paragangliomas

ID	Age[a]	Sex	Tumour	Pathology[b]	Germline mutation[c]	Follow Up (years)	Size (mm)	SDHA IHC	SDHB IHC	VHL Somatic mutation
P1	27	F	EA PGL	Malignant	VHL (c.470C>T (p.Thr157Ile)	NA	13			
P2	41	F	PC	Malignant	SDHB (c.72+1G>T)	5	40			
P3	57	M	PC	Malignant	Neg	7	23×55		Pos	Neg
P4	11	F	PC	Atypical	VHL (p.Val81Leu)	NA				
P5	45	F	PC	Atypical	RET	NA	<30			
P6	21	F	PC	Benign	RET	NA	40			
P7	52	F	PC	Malignant	Neg	7	70	Pos	Pos	Neg
P8	64	F	PC	Benign	RET	NA	179			
P9	49	M	EA PGL	Metastasis	SDHB (c.200+3G>C)	NA	45			
P10	76	M	PC	Benign	Neg	NA	20	Pos	Pos	Neg
P11	43	F	PC	Benign	RET	NA	17	Pos	Pos	
P12	40	M	PC	Atypical	VHL (p.Arg161X)	NA	10			
P13	46	M	EA PGL	Atypical	SDHA	11	20	Negative	Negative	
P14	40	M	PC	Atypical	Neg	NA	65	NA	Pos	Neg
P15	40	F	PC	Benign	SDHB (c.268C>T (p.Arg90X))	NA	40	Pos	Negative	
P16	39	M	PC	Benign	VHL (p. Arg161X)	NA	50	Pos	Pos	
P17	34	F	PC	Atypical	Neg	2	80	Pos	Pos	Neg
P18	11	M	PC	NA	VHL	NA	NA			
P19	71	M	PC	Malignant	NF1	NA	78	Pos	Pos	
P20	76	F	PC	Benign	Neg	NA	10	NA	NA	
P21	63	M	PC	Benign	Neg	NA	92	NA	NA	
P22	69	F	PC	Benign	Neg	NA	25	Pos	Pos	Neg
P23	58	F	PC	Atypical	NF1	NA	35	Pos	Pos	
P24	37	F	PC	Benign	Neg	NA	37	Pos	Pos	
P25	62	M	Metastasis (P3)	Metastasis	Neg	NA	65	Pos	Pos	Neg
P26	NA	M	PC	NA	RET	NA	10	Pos	Pos	
P27	61	F	PC	Benign	Neg	7	70	Pos	Pos	
P28	NA	M	PC	Malignant	Neg	NA	NA		Pos	
P29	32	M	PC	Benign	VHL	NA	75			
P30	66	M	EA PGL	Benign	Neg	NA	46	Pos	Pos	NA
P31	33	M	EA PGL	Malignant	SDHB (c.268C>T (p.Arg90X))	7	105			
P32	31	F	PC	Benign	RET	NA	32			
P33	48	F	PC	Benign	Neg	6	35	Pos	Pos	Neg
P34	61	M	PC	Malignant	RET	NA	80	Pos	Pos	
P35	35	F	PC	Benign	NF1	NA	35	Pos	Pos	
P36	29	F	PC	Benign	VHL	NA	35	Pos	Pos	
P37	19	M	EA PGL	Atypical	SDHB (p.Ile127Ser)	4	20	Pos	Neg	
P38	36	M	EA PGL	Benign	Neg	1	95	Pos	Pos	Neg
P39	NA	M	PC	Malignant	NA	NA	NA		Pos	Neg

NA, information not available; F, female; M, male; PC, pheochromocytoma; EA PGL, extra-adrenal paraganglioma; SDHA, succinate dehydrogenase subunit A; SDHB, succinate dehydrogenase subunit B; VHL, von Hippel–Lindau; Neg, negative; RET, rearranged during transfection.
[a]Age at time of surgery.
[b]Tumours were classified as either benign, where there was no evidence of metastases; atypical, where there was no evidence of metastases but there was evidence of local invasion, vascular invasion, or high PASS score; or malignant, if there was clinical and radiological evidence of distant metastases either at the time of surgery or subsequently at follow up.
[c]Germline tests performed included SDHB (all exons), SDHD (all exons), RET (exon 11 only), and VHL (all exons).

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DOI: 10.1530/ERC-13-0519

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adrenal cortical adenoma or aldosterone-producing adenoma (Conn's syndrome), where the remainder of the adrenal gland was well preserved. Tumours were classified as either benign ($n=18$), where there was no evidence of metastases; atypical ($n=8$), where there was no evidence of metastases but with evidence of local invasion, vascular invasion or high PC of the adrenal gland scaled score (PASS score) ≥ 4 (Thompson 2002), or malignant ($n=11$) if there was unequivocal evidence of distant metastases either at the time of surgery or subsequently at follow-up. Two tumours did not have complete information regarding pathology. Genotyping was carried out on DNA extracted from peripheral blood leukocytes as previously described (Meyer-Rochow *et al.* 2010). In one patient, the genotype was unknown. Briefly, PCR and denaturing HPLC (dHPLC) was carried out for all coding exons of *SDHB*, *SDHD*, *VHL* and *RET* exons 11 and 13. Where a mutation was identified it was confirmed by direct sequencing. Tumours obtained from patients with clinical features of neurofibromatosis were classified as being affected by *NF1* mutations. In 11/15 tumours where no germline mutation was identified with direct sequencing ('sporadic'), immunohistochemistry (IHC) for *SDHA* and *SDHB* was also carried out to indicate absence of *SDHx* mutations (Gill *et al.* 2010*a*); residual tumour was unavailable for IHC in the three remaining samples. Tumour DNA was extracted from the 11 sporadic tumours using QIAamp DNA kit (Qiagen) and sequenced for exons 1–3 of *VHL* using previously published primers (Stolle *et al.* 1998). All patients provided written informed consent for tissue and blood banking, according to a protocol endorsed by the Northern Sydney Health Human Research and Ethics Committee. Tumour tissue was surgically removed and snap–frozen in liquid nitrogen and the samples were stored at $-80\,^{\circ}C$ in the Neuroendocrine Tumour Bank of the Kolling Institute of Medical Research. The presence of tumour was histologically confirmed by touch preparations or sections of tissue before use in this study. Additionally, normal adrenal medulla, used as the reference tissue in this study, was confirmed by a pathologist (A J G).

Gastrointestinal stromal tumours Formalin-fixed and paraffin-embedded tumour blocks were available for 18 patients with GISTs (Table 2). Tumours demonstrating loss of SDHB staining by IHC were classified as SDH deficient ($n=9$), while those positive for SDHB by IHC were classified as SDH-proficient ($n=9$). The SDH-deficient cases have been included in previously described cohorts (Gill *et al.* 2010*b*, 2011*a*,*b*, Chou *et al.* 2012, Dwight *et al.* 2013). Mutation analysis of *SDHB*, *SDHC* and *SDHD* was carried out in SDH-deficient GISTs as previously described (Gimm *et al.* 2000, Aguiar *et al.* 2001, Gimenez-Roqueplo *et al.* 2002, Benn *et al.* 2003, Schiavi *et al.* 2005). Additionally, *SDHA* mutation analysis of the SDH-deficient GISTs has previously been described (Dwight *et al.* 2013), with two associated with germline *SDHA* mutations (Table 2). The use of these samples was approved by the Northern Sydney Local Health District Human Research Ethics Committee.

RNA extraction and RT-qPCR

Total RNA, including small RNA species, was extracted from fresh frozen tissue using RNEasy Mini Kit (Qiagen), as previously described (Meyer-Rochow *et al.* 2010) and formalin-fixed and paraffin-embedded tissue using the RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies). cDNA was synthesised from 10 ng RNA using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). miR-210 expression was measured in triplicate using quantitative PCR according to the methods supplied by the manufacturer (Applied Biosystems). Expression of miR-210 was defined based on the threshold cycle (*C*t) and the relative expression levels were calculated as $2^{-\Delta\Delta Ct}$, with ΔCt defined as the difference between miR-210 and RNU44 or RNU6B expression (PC/PGL and GIST samples respectively). In PC/PGL, $\Delta\Delta C$t was defined as the difference between ΔCt of the tumour and ΔCt of a representative single normal adrenal tissue, which served as the referent sample for all analyses, including other normal adrenal medulla tissue. Normal interstitial pacemaker cells of Cajal, from which GISTs arise, are difficult to isolate from normal tissue and were not available in this study. Hence, in GISTs, miR-210 levels for the SDH-deficient and SDH-proficient GISTs are expressed as $2^{-\Delta Ct}$.

Generation of SDHB-mutant neurosphere cell lines

Generation of neurosphere cell lines from the olfactory mucosal biopsies in patients with *SDHB* mutations was approved by the Northern Sydney Health Human Research and Ethics Committee and patients provided written informed consent. The biopsies were taken by an Ear Nose and Throat Surgeon (D V) from two patients with *SDHB* mutation. One biopsy was carried out at the time of surgery for spinal metastases from a patient with germline *SDHB* mutation (c.72+1G>T) who originally presented with PC. The other biopsy was carried out under local

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Table 2 Patient and tumour characteristics: gastrointestinal stromal tumours (GISTs)

ID	Age[a]	Gender	Tumour	Tumour status[b]	Germline/somatic mutation
G1	41	F	GIST	SDH-deficient	SDHB[c]
G2	27	F	GIST	SDH-deficient	NC[c]
G3	22	F	GIST	SDH-deficient	NC[c]
G4	46	M	GIST	SDH-deficient	NC[c]
G5	45	F	GIST	SDH-deficient	SDHA[c]
G6	13	F	GIST	SDH-deficient	NC[c]
G7	63	F	GIST	SDH-deficient	NC[c]
G8	42	M	GIST	SDH-deficient	NC[c]
G9	41	M	GIST	SDH-deficient	SDHA[c]
G10	61	M	GIST	SDH-proficient	KIT[d]
G11	53	F	GIST	SDH-proficient	KIT[d]
G12	66	M	GIST	SDH-proficient	KIT[d]
G13	61	M	GIST	SDH-proficient	PDGFRA[d]
G14	77	M	GIST	SDH-proficient	PDGFRA[d]
G15	57	M	GIST	SDH-proficient	KIT[d]
G16	53	M	GIST	SDH-proficient	Neg[d]
G17	75	F	GIST	SDH-proficient	PDGFRA[d]
G18	63	M	GIST	SDH-proficient	KIT[d]

NC, not complete; F, female; M, male; GIST, gastrointestinal stromal tumour; SDHB, succinate dehydrogenase subunit B; Neg, negative.
[a]Age at first presentation of GIST.
[b]Based on SDHB staining by IHC, as follows: SDH-deficient tumours exhibit loss of SDHB expression, while SDH-proficient tumours retain SDHB expression.
[c]Mutation analysis was carried out for SDHA, SDHB, SDHC and SDHD. It should be noted that amplification of some exons in SDHA, SDHB, SDHC and SDHD was not achieved, hence mutation analysis in these samples has been deemed 'not complete' (NC). Specifically, amplification was not achieved for SDHA, as previously described (Dwight et al. 2013); SDHB – G1 (exons 2, 7), G2 (exon 2), G3 (exons 2, 7, 8), G4 (exons 2, 4, 5, 7, 8), G6 (exons 4, 6, 7), G7 (exon 7), G8 (exon 4); SDHC – G1 (exon 5), G2 (exon 2), G3 (exons 4, 5), G4 (exons 1, 2, 4, 5), G6 (exons 3, 5), G7 (exon 2), G8 (exon 2); SDHD – G1 (exon 4), G3 (exons 3, 4), G4 (exons 2, 3, 4), G7 (exon 4). No mutations were identified in any of the exons amplified in G2–4 and G6–8. Inability to amplify these samples was attributed to lower quality DNA obtained from formalin-fixed, paraffin-embedded tissue. Each of the SDH deficient GISTs were SDHB IHC negative and confirmed WT for KIT (exons 9, 11, 13, 17) and PDGFRA (exon 18).
[d]For each of the SDH-proficient GISTs (i.e. SDHB IHC positive), mutation analysis encompassed KIT (exons 9, 11, 13, 17) and PDGFRA (exon 18).

anaesthetic in a patient with germline *SDHB* mutation (c.494_497delAAGG) who had no biochemical or radiological evidence of PC, PGL, GIST or renal cell cancer. The normal controls are age-matched unaffected subjects, unrelated to the two *SDHB*-mutation patients. The basement membrane of the olfactory mucosa contains multipotent stem cells, which were grown as primary cultures following collagenase digest, according to a previously described protocol (Matigian *et al.* 2010). Briefly, primary cultures were stimulated to form neurospheres by culturing in serum-free media supplemented with insulin, transferrin, selenium solution (Gibco), human recombinant epidermal growth factor (Millipore, Billerica, MA, USA) and human recombinant basic fibroblast growth factor (Millipore), and plated on a poly L-lysine-coated surface (Sigma–Aldrich). The neurospheres were then collected and grown in high glucose pyruvate DMEM (Gibco) with 10% fetal bovine serum and are used between passages 4 and 12. To observe the effects of hypoxia on miR-210, neurospheres were grown under normoxic (21% O_2, 5% CO_2 at 37 °C) or hypoxic (1% O_2, 5% CO_2 at 37 °C) conditions for 72 h before RNA extraction using RNEasy Mini Kit (Qiagen), as per manufacturer's instructions.

Confirmation of the phenotype of the neurospheres was made using flow cytometry. Trypsinised and washed cells were suspended in a buffer of 10% bovine calf serum and PBS. The cells were stained with 2 µl of antibodies (CD29 (Biolegend), CD44 (Milteni), CD 73 (Milteni), and CD90 (Milteni) which are markers of neural crest lineage described in previous papers (Matigian *et al.* 2010), and incubated for 10 min. Cell staining was compared with unstained controls.

siRNA of SDHB and RT-qPCR of SDHB and miR-210 expression

Hek293 cells were cultured in DMEM plus 10% fetal bovine serum in an incubator at 37 °C and 5% CO_2 in a 24 well plate at a density of 0.5×10^5 cells/well and allowed to settle overnight. The cells were transfected with siRNA

SDHB (Qiagen) or siRNA Allstar (Qiagen) as a negative control, with a final concentration of 150 nmol/l in a total volume of 600 µl/well, and lipofectamine 2000 (Invitrogen) as the transfection reagent at 1 µl/well. The media was changed at 12 h after transfection, and hypoxic cells were incubated at 1% O_2, 5% CO_2 and 37 °C for a total of 72 h. The cells were washed with PBS and lysed according to the miRNEasy protocol (Qiagen). RNA concentration was quantified using Nanodrop.

To measure the expression of SDHB, cDNA was synthesised using Superscript III First Strand Synthesis (Invitrogen).

To measure miR-210 levels, cDNA was generated using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), using the TaqMan probes for miR-210 (Applied Biosystems, ID no. 00512, Cat no. 4427975) and RNU44 (Applied Biosystems, ID no. 001094, Cat no. 4427975). Quantitative PCR was carried out using TaqMan mastermix, and TaqMan probes for *SDHB*, 18S, miR-210, and RNU-44. The results are expressed as $2^{-\Delta\Delta Ct}$, compared with Allstar control at normoxia. Experiments were carried out in four independent experiments in triplicate.

Statistical analysis

Mann–Whitney *U* tests were used to compare miR-210 expression in i) PC/PGL between different diagnostic subgroups (by genotype or by malignant status); ii) GISTs between SDH-deficient and SDH-proficient subgroups and iii) fold change between transfected and untransfected cells. Unless specified, then median is expressed with interquartile range. Multivariate regression was performed including genotype (*SDHx*/*VHL* mutated vs non-*SDHx*/*VHL*-mutated), pathology (benign, malignant) and tumour type (PC, PGL) with miR210 (elevated or normal) as the dependent variable.

Results

miR-210 is overexpressed in PC/PGL compared with normal adrenal medulla, in *SDHB*- or *VHL*-mutated tumours compared with *RET*, *NF1*, or sporadic tumours, and in malignant tumours compared with benign

Patient and tumour characteristics are shown in Table 1. Of the 39 PC/PGL patients, five had germline mutation in *SDHB*, one in *SDHA*, seven had *VHL* germline mutation and seven were found to have *RET* mutation. Three patients had clinical features of neurofibromatosis and were therefore designated *NF1*-mutated. In 15 patients, no

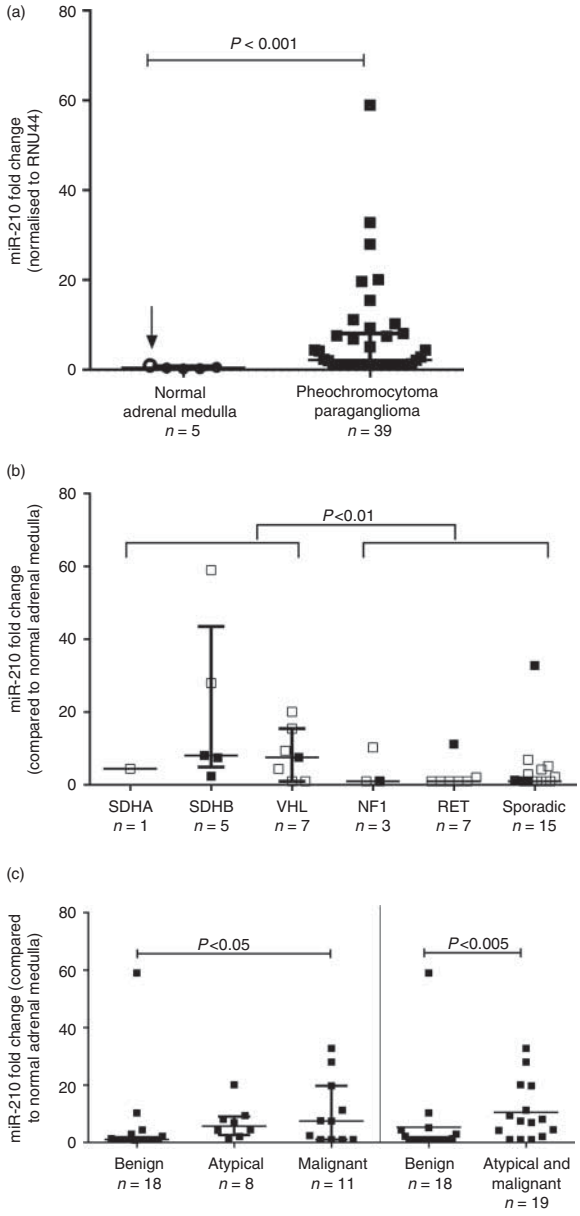

Figure 1
Expression of miR-210 in PC/PGLs according to genotype and pathology. (a) miR-210 expression was significantly higher in PC/PGLs compared with normal adrenal medulla (median 2.1-fold). The control adrenal medulla used throughout all experiments is marked with an open circle and arrow. (b) Tumours that contained germline mutations in either *SDHB* or *VHL* (i.e. exhibited pseudohypoxic gene signature) had significantly higher miR-210 expression (median 7.6-fold) compared with tumours without *SDHB* or *VHL* mutations (median onefold). Closed squares denote malignant tumours, whilst open squares denote benign or atypical tumours. (c) Malignant tumours had higher miR-210 expression (median 7.5-fold) compared with benign tumours (median 1.0-fold); whilst atypical tumours also had higher miR-210 expression (median 5.1-fold) compared with benign tumours ($P<0.005$). miR-210 expression was normalised to RNU44 and expressed as median fold change (±inter quartile range) relative to a referent normal adrenal medulla sample (arrow). PC, pheochromocytoma; PGL, paraganglioma; IQR, interquartile range.

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mutations were detected in *SDHB, SDHD, VHL* or *RET* and were therefore designated as sporadic. Tumour sample was available in 11 of these 15 sporadic samples, and IHC for SDHA and SDHB was positive in all of these samples making the possibility of mutation in any *SDHx* gene unlikely (Gill *et al*. 2010*a*). Somatic *VHL* mutations were not found by direct sequencing in any of these 11 sporadic tumours. The genotype of one patient and pathology of two patients were not available.

Expression of miR-210 in PC/PGL is shown in Fig. 1a. Overall, 23 of 39 PC/PGL samples demonstrated miR-210 overexpression (defined as a miR-210 level at least 1.5-fold above the mean value for normal adrenal samples), with a median 2.2-fold increase (IQR 1.0–7.8) compared with normal adrenal medulla ($P<0.0001$). When tumours were classified according to germline mutation and compared with normal adrenal medulla, *SDHB*-mutated tumours had the highest levels of miR-210 expression (median 8.1-fold, IQR 7.45–28.0), followed by *VHL*-mutated tumours (median 7.6-fold, IQR 2.7–12.4), and sporadic tumours (median 1.0-fold, IQR 1.0–3.56; Fig. 1b). The single *SDHA*-mutated tumour had a 4.4-fold increase compared with normal adrenal medulla. *NF1*-mutated tumours (median onefold, IQR 1.0–5.6) and *RET*-mutated tumours (median onefold, IQR 1.0–1.0) did not have statistically significant elevation of miR-210 expression compared with normal adrenal medulla (Fig. 1b). *SDHB*-mutated PC/PGLs had significantly higher expression of miR-210 compared with *RET*-related tumours or sporadic tumours ($P=0.01$ and $P=0.005$, respectively; Fig. 1b), whereas miR-210 overexpression in *VHL*-mutated PC/PGLs was significant only when compared with *RET*-mutated tumours ($P=0.01$; Fig. 1b). The tumours that contained germline mutations in either *SDHx* or *VHL* had significantly higher miR-210 expression (median 7.6-fold, IQR 4.36–15.45) compared with non *SDHx*/non-*VHL*-mutated tumours (median onefold, IQR 1.0–2.88; $P=0.0016$; Fig. 1b). Moreover, miR-210 was elevated above normal in 11/13 tumours that contained *SDHx* or *VHL* mutations compared with only 9/25 non-*SDHx*/non-*VHL* mutated tumours ($P=0.015$, Fisher exact).

Malignant tumours ($n=11$) had significantly higher miR-210 expression (median 7.5-fold, IQR 1.0–15.5) compared with benign tumours ($n=18$; median 1.0-fold, IQR 1.0–1.1; $P=0.048$; Fig. 1c). Atypical tumours ($n=9$; median 5.1-fold, IQR 4.2–8.1) also had significantly higher expression compared with benign tumours ($P=0.005$; Fig. 1c). There was, however, no difference between malignant tumours only and the combined group of benign and atypical tumours ($P=0.08$). In multivariate analysis, miR-210 was only significantly associated with *SDHx*/*VHL*-mutated genotype ($P=0.02$) but not with malignant pathology or tumour type (PC, PGL).

miR-210 is overexpressed in SDH-deficient GISTs compared with SDH-proficient GISTs

Patient and tumour characteristics are shown in Table 1. Of the 18 GISTs, nine were positive for SDHB by IHC, referred to as SDH-proficient GISTs; while nine showed loss of SDHB staining by IHC, referred to as SDH-deficient GISTs. miR-210 expression was significantly ($P=0.0078$) higher in SDH-deficient GISTs (median 2.58, IQR 1.65–4.15) compared with SDH-proficient GISTs (median 0.60, IQR 0.14–1.44; Fig. 2).

miR-210 is overexpressed in novel cell lines with *SDHB* mutation

miR-210 expression was determined in neurospheres generated from olfactory biopsies in two patients with known *SDHB* germline mutations, compared with age-matched controls without *SDHB* mutations. The first patient was a woman, then 37-year-old, with germline *SDHB* mutation (c.494_497delAAGG) identified on predictive testing, and clinically without evidence of PC/PGL. The second was a man then 49-year-old with germline *SDHB* mutation (c.72+1G>T) diagnosed after discovery of PC, and who had spinal metastases.

The novel cell line was characterised and confirmed as being of neural crest lineage using surface biomarkers for

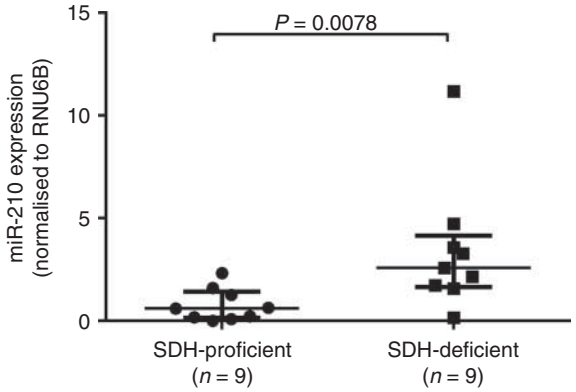

Figure 2
Expression of miR-210 in GISTs. miR-210 expression was significantly higher in SDH-deficient GISTs (median 2.58) compared with SDH-proficient GISTs (median 0.60; $P=0.0078$, Mann–Whitney *U* test). miR-210 expression was normalised to RNU6B and expressed as $2^{-\Delta Ct}$ (median ± IQR). GIST, gastrointestinal stromal tumour; IQR, interquartile range; SDH, succinate dehydrogenase.

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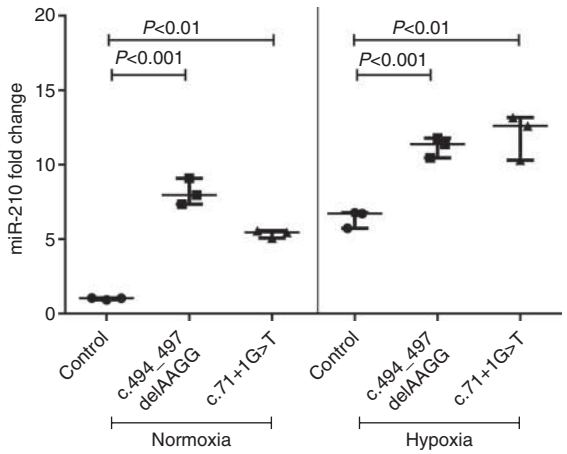

Figure 3
Expression of miR-210 in neurosphere cell lines derived from two patients with germline *SDHB* mutations and age-matched controls without *SDHB* mutations. Under normoxic conditions (21% O_2), miR-210 expression was significantly higher in the mutant *SDHB*-containing neurosphere cell lines (median 7.97-fold in c.494_497delAAGG and median 5.56-fold in c.72+1G>T) compared with the age-matched control neurospheres ($P<0.01$). Under hypoxic conditions (1% O_2), miR-210 was elevated in all neurosphere cell lines when compared with normoxia. However, expression of miR-210 remained significantly higher in the mutant *SDHB*-containing neurosphere cell lines (median 11.37-fold in c.494_497delAAGG and median 12.60-fold in c.72+1G>T) compared with the age-matched control neurospheres ($P<0.01$).

CD29, CD44, CD73 and CD90 which were all positive. *SDHB* sequencing confirmed that the germline mutations were present in these cell lines (data not shown).

miR-210 levels were significantly higher in both mutant *SDHB*-containing neurosphere cell lines compared with normal subjects when cultured under normoxic conditions ($P<0.01$; Fig. 3). In the neurosphere cell line containing c.494_497delAAGG, miR-210 expression was 7.97-fold increased (IQR 7.66–8.52) when compared with the control ($P<0.001$). Further, in the neurosphere cell line containing c.72+1G>T, miR-210 expression was 5.56-fold higher (IQR 5.27–5.51) when compared with the control ($P<0.01$). Following culture under hypoxic conditions (1% O_2 for 72 h), miR-210 expression increased as expected in both neurosphere cell lines (11.37-fold, IQR 11.37–10.91 in c.494_497delAAGG, $P<0.001$; and 12.60-fold increased, IQR 10.3–13.17 in c.72+1G>T, $P<0.01$; Fig. 3).

SDHB knockdown is associated with increased miR-210 levels

To investigate whether SDHB deficiency directly results in increased miR-210, we next used siRNA-mediated knockdown in HEK293 cells. SiRNA against *SDHB* resulted in a knockdown to 63.5% under normoxic conditions ($P=0.0013$), and knockdown to 43.7% in hypoxic conditions ($P=0.0012$) (Fig. 4a). With induction of *SDHB* deficiency in normoxia, miR-210 expression increased by a mean ± S.E.M. of 2.7±0.64-fold above baseline ($P=0.0005$; Fig. 4b). The strong stimulation of miR-210 expression with hypoxia (17.3±2.81-fold) was not further enhanced by *SDHB* knockdown ($P=0.79$; Fig. 4b).

Discussion

miR-210 is considered to be a master regulator of hypoxic responses (Fasanaro *et al.* 2009) and has been shown to be overexpressed in a variety of malignancies, including head and neck squamous cell carcinomas (Gee *et al.* 2010), pancreatic cancer (Greither *et al.* 2010), renal cell cancer

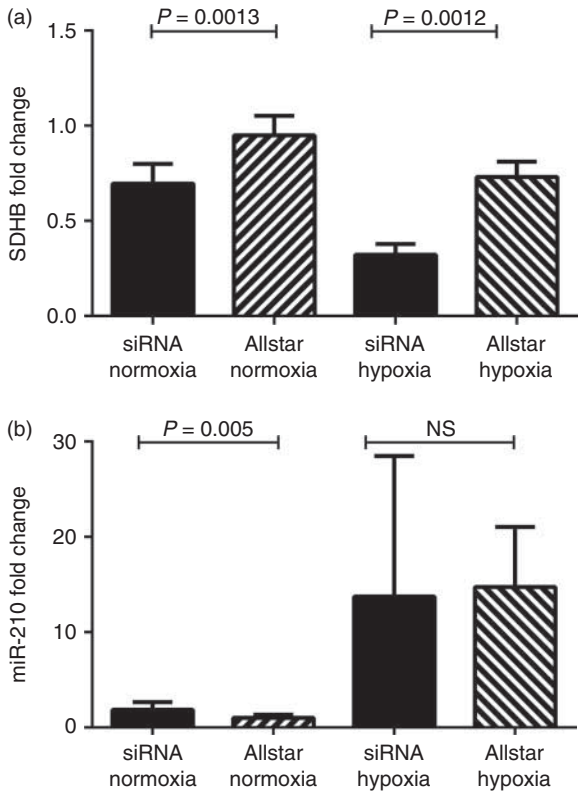

Figure 4
(a) Expression of *SDHB* normalised to 18S was significantly lower in the HEK293 cells transfected with siRNA compared with negative control, in normoxic (reduced to 63.5%, $P=0.0013$) and hypoxic conditions (reduced to 43.7%, $P=0.0012$). (b) Expression of miR-210 normalised to RNU44 was significantly elevated under normoxic conditions (2.7±0.63-fold in knocked down sample compared with negative control) but not under hypoxic conditions as miR-210 expression was already maximally induced (17.29±2.81-fold in knocked down sample and 17.09±8.76-fold in negative control, compared with negative control at normoxia).

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(Juan *et al*. 2010, Neal *et al*. 2010), breast cancer (Foekens *et al*. 2008) and lung cancer (Puissegur *et al*. 2010). Since PCs and PGLs with germline *SDH* subunit or *VHL* mutations exhibit a pseudohypoxic gene expression signature (Dahia & Familial Pheochromocytoma 2006), we hypothesised that *SDH*- and *VHL*-related tumours would have higher miR-210 expression compared with tumours lacking these mutations. In this study, we have shown that miR-210 overexpression is associated with SDH-related PCs, PGLs and SDHB-deficient GISTs. We also found that miR-210 is overexpressed in neurosphere (i.e. non-tumoral) cell lines derived from two patients with germline *SDHB* mutations, suggesting that miR-210 is an early marker for dysregulated hypoxia responses in response to SDH deficiency.

Overall, higher expression of miR-210 was observed in our cohort of PC/PGL when compared with normal adrenal medulla tissue. When grouped according to genotype, miR-210 was most highly expressed in those PC/PGLs associated with germline mutations in either *SDHB* or *VHL*. This is consistent with previous gene array findings in which tumours containing either *SDHB* or *VHL* mutations exhibited a hypoxia-response expression pattern (Dahia & Familial Pheochromocytoma 2006). In contrast, the *NF1* and *RET*-mutated PC/PGLs in this study had miR-210 levels not different from normal adrenal medulla tissue, again consistent with the distinct, non-hypoxic gene expression signature previously described in these tumours (Dahia & Familial Pheochromocytoma 2006). Interestingly, a previous report showed miR-210 overexpression in parasympathetic PGL of the head and neck (HNPGLs) which was independent of germline *SDH* mutations (Merlo *et al*. 2012), a finding which is consistent with more universal association of HNPGLs with hypoxic gene expression regardless of whether *SDH* deficiency is present or not. Our study did not include HNPGLs, and we cannot therefore state whether miR-210 is different in PGL arising from chief cells (HNPGLs) compared with chromaffin cells (sympathetic PGLs/PCs).

When PC/PGLs were grouped according to pathology, miR-210 levels were significantly higher in malignant compared with unequivocally benign tumours, but not to the combined group of benign and atypical tumours. Multivariate analysis suggested that miR-210 associated with genotype (*SDHx* or *VHL* mutated) rather than pathology, and although our numbers are small, this suggests that the borderline association between miR210 and malignant PC/PGL is confounded by genotype and in particular by *SDHB* mutation which is more likely to be associated with malignancy (Amar *et al*. 2007). The prognostic utility of miR-210 has been assessed in a number of tumour types, with elevated miR-210 correlating with poor prognosis in some tissue types and good prognosis in others. Our data support miR-210 as being a marker for hypoxia gene expression rather than malignant potential in PC/PGLs.

miR-210 expression was elevated in 85% of *SDHx*/*VHL*-mutated tumours, but also elevated in 40% of non-*SDHx*/*VHL* mutated tumours (1/3 *NF1*-mutated, 2/7 *RET*-mutated and 7/15 'sporadic' tumours). We carefully examined the sporadic tumours to exclude somatic mutations in *VHL* (by direct sequencing) and *SDHx* (by IHC). Our results are not dissimilar from a recent report in clear cell renal carcinoma, which exhibits markedly elevated miR-210 expression in *VHL*-mutated cancers but also in cancers with WT *VHL* sequence (McCormick *et al*. 2013). The authors of that study speculated that this could occur due to alternative mechanisms of VHL inactivation by methylation or loss of heterozygosity; VHL promoter hypermethylation has been described in renal cell cancers without *VHL* mutation (Herman *et al*. 1994). Whether such epigenetic silencing of *VHL* (or of the *SDHx* subunit genes) occurs in PC/PGLs will require further study.

In a recent paper (de Cubas *et al*. 2013) looking at the miR profiles of PCs and paraganglioms by genotype, it has been found that miR-210 was exclusively elevated in *SDHB*- and *VHL*-mutated tumours. However in that series, the number of malignant tumours was small, and no individual miRNA distinguished between benign and malignant tumours. In our cohort, miR-210 was elevated significantly in two malignant tumours that were not associated with *VHL* or *SDHx* mutation – one associated with *RET* mutation and another apparently sporadic. Larges studies will be required to determine whether elevated miR210 may be associated with aggressive behaviour.

SDH-deficient GISTs, being the component tumours of PC/PGL syndromes and associated with mitochondrial complex II deficiency, were also assessed for miR-210 expression in this study. Similar findings to those observed in the PC/PGLs were observed in our cohort of GISTs, with significantly higher miR-210 expression detected in SDH-deficient GISTs (i.e. tumours exhibiting loss of SDHB by IHC) when compared with SDH-proficient GISTs (i.e. tumours retaining SDHB by IHC). This pattern of overexpression of miR-210 in SDH-deficient GISTs is consistent with SDH-deficiency leading to hypoxia gene expression regardless of tumour tissue-of-origin. To our knowledge, this is the first report of miR-210 expression analysis in GISTs. However, HIF1α, a hypoxia inducible factor known to target

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and induce miR-210 expression, has previously been studied in GISTs (Takahashi *et al.* 2003, Chen *et al.* 2005). In those studies, overexpression of HIF1α correlated with significantly poorer prognosis (Takahashi *et al.* 2003) and tumour recurrence or distant metastasis (Chen *et al.* 2005).

We confirmed that miR-210 was overexpressed in two neurosphere cell lines containing endogenous *SDHB* mutations. As far as we are aware, these neurosphere cell lines represent the first published human cell line with a *SDHB* mutation, and the first use of the neurosphere technique in the study of cancer. Our findings suggest that the association between *SDHB* mutations and pseudohypoxia (i.e. hypoxia gene expression despite normoxic conditions) occurs even before tumour development occurs.

Finally, we directly assessed whether SDH deficiency induced miR-210 expression using siRNA knockdown in HEK293 cells. Our results showed that silencing of *SDHB* modestly increased miR-210 levels. Overall, our results support a conclusion that SDH deficiency is directly associated with elevated miR-210 expression although the mechanism by which this occurs requires further study. One theory proposes that accumulation of metabolic intermediates (especially succinate) leads to stabilisation of HIF-1 via succinate-inhibition of prolyl hydroxylation (Gimenez-Roqueplo *et al.* 2001, Selak *et al.* 2005, Hobert *et al.* 2012).

Interestingly, miR-210 has a number of validated targets that are already associated with PC/PGL including SDHD (Puissegur *et al.* 2010) and the iron sulphur cluster unit (ISCU) (Chan *et al.* 2009), and another putative target in inhibin β B (INHBB) (Puissegur *et al.* 2010) (predicted by Microtop table). miR-210 was recently shown to directly target and downregulate SDHD mRNA in a lung adenocarcinoma cell line (Puissegur *et al.* 2010). Similarly, miR-210 was shown to target ISCUs in human pulmonary artery endothelial cells, potentially interfering with the activity of SDHB that contains three iron sulphur clusters (Chan *et al.* 2009). Most intriguingly, a miR-210 target sequence is present within the 3′UTR of INHBB, a glycoprotein of the growth factor β superfamily that was reported to be downregulated in malignant compared with benign PC/PGLs (Salmenkivi *et al.* 2001). Whether INHBB acts as a tumour suppressor in PC/PGL itself, or is merely an indirect biomarker for miR-210 expression, requires further study.

Taken together, our data suggest that miR-210 overexpression occurs as a direct consequence of mutations in *SDH* subunits or *VHL*, and is (at least for *SDH*) independent of the tissue in which these tumours develop. Further, our data confirms that miR-210 is a robust marker for pseudohypoxia in *SDHB*- and *VHL*-mutated PC/PGLs. Whether miR-210-mediated gene dysregulation plays a direct role in pathogenesis of these tumours requires further study. The mechanism by which miR-210 is upregulated in SDH-related tumours also requires further study, although it would be plausible to implicate HIF1α which is a known positive regulator of miR-210 (Chan *et al.* 2012).

Conclusion

Overexpression of miR-210 in *SDH*-associated PC, PGL and GISTs further substantiates the role of aberrant hypoxic cellular responses in the development of these tumours.

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 project was supported by the Royal North Shore Hospital Staff Specialist Trust Fund, Hillcrest Foundation (administered by Perpetual Trustees), and Pheo-Para Alliance.

Acknowledgements

The authors acknowledge the work of Dr Juan Carlos Cassano and Mr Lyndsay Peters in the preparation of the neurospheres and assistance with flow cytometry.

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Received in final form 11 February 2014
Accepted 24 February 2014
Made available online as an Accepted Preprint
28 February 2014

Published by Bioscientifica Ltd.