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

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. 2010a). 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 miRNAs. miR-210 has become known as a key regulator of hypoxia, acting on a number of targets including, E2F3, NPTX1, RAD52, ACVR1B, MNT, CASP8AP2, FGFR1 and HOXA1 and -9 (Fasano 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 (Fasano 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.
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

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<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>Tumour</th>
<th>Pathology</th>
<th>Germline mutation</th>
<th>Follow Up (years)</th>
<th>Size (mm)</th>
<th>SDHA IHC</th>
<th>SDHB IHC</th>
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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.

*Age at time of surgery.

*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.

*Germline tests performed included SDHB (all exons), SDHD (all exons), RET (exon 11 only), and VHL (all exons).
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. 2010a); 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°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. 2010b, 2011a,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, Aguia 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 (Ct) and the relative expression levels were calculated as $2^{-\Delta\Delta Ct}$, with $\Delta Ct$ defined as the difference between miR-210 and RNU44 or RNU6B expression (PC/PGL and GIST samples respectively). In PC/PGL, $\Delta\Delta Ct$ was defined as the difference between $\Delta Ct$ of the tumour and $\Delta Ct$ 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...
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% O₂, 5% CO₂ at 37 °C) or hypoxic (1% O₂, 5% CO₂ at 37 °C) conditions for 72 h before RNA extraction using RNEasy Mini Kit (Qiagen), as per manufacturer’s instructions.

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

<table>
<thead>
<tr>
<th>ID</th>
<th>Age*</th>
<th>Gender</th>
<th>Tumour</th>
<th>Tumour status</th>
<th>Germline/somatic mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>41</td>
<td>F</td>
<td>GIST</td>
<td>SDH-deficient</td>
<td>SDHB&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G2</td>
<td>27</td>
<td>F</td>
<td>GIST</td>
<td>SDH-deficient</td>
<td>NC&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G3</td>
<td>22</td>
<td>F</td>
<td>GIST</td>
<td>SDH-deficient</td>
<td>NC&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G4</td>
<td>46</td>
<td>M</td>
<td>GIST</td>
<td>SDH-deficient</td>
<td>NC&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G5</td>
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<td>SDHA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
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<td>GIST</td>
<td>SDH-deficient</td>
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</tr>
<tr>
<td>G7</td>
<td>63</td>
<td>F</td>
<td>GIST</td>
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<td>NC&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G8</td>
<td>42</td>
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<td>SDH-deficient</td>
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<tr>
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<td>KIT&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>F</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>KIT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>G12</td>
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<td>KIT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>G13</td>
<td>61</td>
<td>M</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>PDGFRA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
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<td>M</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>PDGFRA&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>G15</td>
<td>57</td>
<td>M</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>KIT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>G16</td>
<td>53</td>
<td>M</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>Neg&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
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<td>F</td>
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<td>SDH-proficient</td>
<td>PDGFRA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>G18</td>
<td>63</td>
<td>M</td>
<td>GIST</td>
<td>SDH-proficient</td>
<td>KIT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NC, not complete; F, female; M, male; GIST, gastrointestinal stromal tumour; SDHB, succinate dehydrogenase subunit B; Neg, negative.

*Age at first presentation of GIST.

<sup>b</sup>Based on SDHB staining by IHC, as follows: SDH-deficient tumours exhibit loss of SDHB expression, while SDH-proficient tumours retain SDHB expression.

<sup>c</sup>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, 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).

<sup>d</sup>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).

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% CO₂ in a 24 well plate at a density of 0.5 × 10⁵ 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% O2, 5% CO2 and 37 °C for a total of 72 h. The cells were washed with PBS and lysed according to the miRNENasy 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^{-ΔΔCt}, 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 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.
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. 2010a). 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
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 $\pm$ S.E.M. of 2.7 $\pm$ 0.64-fold above baseline ($P=0.0005$; Fig. 4b). The strong stimulation of miR-210 expression with hypoxia (17.3 $\pm$ 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

\[
\begin{align*}
\text{SDHB knockdown is associated with increased miR-210 levels} \\
\end{align*}
\]

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).
miR-210 overexpression in SDH-related tumours

V H M Tsang, R J Clifton-Bligh, N R Parker et al.

miR-210 overexpression in GISTs

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miR-210 overexpression in GISTs
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 SDHB mRNA in a lung adenocarcinoma cell line (Puissegur et al. 2010). Similarly, miR-210 was shown to target ISCU5 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.

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References


Benn DE, Croxson MS, Tucker K, Bambach CP, Richardson AL, Delbridge L, Pullan PT, Hammond J, Marsh DJ & Robinson BG 2003 Novel succinate dehydrogenase subunit B (SDHB) mutations in familial...
phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas. Oncogene 22 1358–1364. (doi:10.1038/sj.onc.1206300)


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