MicroRNA profiling of benign and malignant pheochromocytomas identifies novel diagnostic and therapeutic targets

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Abstract

MicroRNAs (miRNAs) are small RNAs (∼22 bp) that post-transcriptionally regulate protein expression and are found to be differentially expressed in a number of human cancers. There is increasing evidence to suggest that miRNAs could be useful in cancer diagnosis, prognosis, and therapy. We performed miRNA microarray expression profiling on a cohort of 12 benign and 12 malignant pheochromocytomas and identified a number of differentially expressed miRNAs. These results were validated in a separate cohort of ten benign and ten malignant samples using real-time quantitative RT-PCR; benign samples had a minimum follow-up of at least 2 years. It was found that IGF2 as well as its intronic miR-483-5p was over-expressed, while miR-15a and miR-16 were under-expressed in malignant tumours compared with benign tumours. These miRNAs were found to be diagnostic and prognostic markers for malignant pheochromocytoma. The functional role of miR-15a and miR-16 was investigated *in vitro* in the rat PC12 pheochromocytoma cell line, and these miRNAs were found to regulate cell proliferation via their effect on cyclin D1 and apoptosis. These data indicate that miRNAs play a pivotal role in the biology of malignant pheochromocytoma, and represent an important class of diagnostic and prognostic biomarkers and therapeutic targets warranting further investigation.

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Introduction

Pheochromocytomas are catecholamine-producing neuroendocrine tumours of chromaffin cell origin arising from the adrenal medulla and less commonly from extra-adrenal sympathetic paraganglia. Approximately 24% of pheochromocytoma cases are caused

by germ line mutations in genes associated with multiple endocrine neoplasia type 2, von Hippel–Lindau disease, and the pheochromocytoma/paraganglioma familial syndromes, but the remaining 76% are sporadic and the pathogenesis remains unknown (Neumann *et al*. 2002, Adler *et al*. 2008). Approximately 10% of pheochromocytoma cases are malignant (DeLellis *et al*. 2004); however, this malignant potential cannot be determined histologically, and there is currently no diagnostic test that can predict whether a tumour is benign or malignant. A definitive diagnosis of malignancy can therefore only be made after metastasis has occurred (DeLellis *et al*. 2004). Previously investigated cancer-associated markers such as p53 and Ki-67 and catecholamine-related markers such as chromogranin A are not reliable diagnostic markers of malignant pheochromocytoma (Brouwers *et al*. 2005, Strong *et al*. 2008). In addition, patients with malignant pheochromocytoma respond poorly to chemotherapy or radiotherapy with a poor overall survival of 50% at 5 years (Adler *et al*. 2008). The identification of reliable diagnostic and prognostic markers and the development of effective molecular therapeutic targets for the treatment of malignant disease have the potential to significantly improve the outcome for patients with malignant pheochromocytoma.

MicroRNAs (miRNAs) are a class of small non-protein-coding RNAs ($\sim$ 22 bp) that negatively regulate protein expression. These single stranded RNA fragments have hundreds of predicted targets and act by binding to the $3'$ untranslated region (UTR) of the mRNA, leading to either mRNA cleavage or translational repression (Engels & Hutvagner 2006). The first study implicating miRNAs in the development of cancer came from the observation that the most common deletions in human chronic lymphocytic leukaemia (CLL) occur in the 13q14 chromosomal region (Calin *et al*. 2002). Two miRNAs (miR-15 and miR-16) are located in this region and have been found to be deleted or down-regulated in $\sim$ 68% of all cases of CLL (Calin *et al*. 2002). Down-regulation of miR-15a and miR-16 has also been reported in pituitary adenomas, prostate cancer, and non-small cell lung cancer (Bottoni *et al*. 2005, Cimmino *et al*. 2005, Bonci *et al*. 2008, Bandi *et al*. 2009).

MiRNA expression is tissue specific and has been demonstrated to be altered in a number of human cancers including colon (Bandres *et al*. 2006), prostate (Porkka *et al*. 2007, Bonci *et al*. 2008), lung (Takamizawa *et al*. 2004, Yanaihara *et al*. 2006), thyroid (He *et al*. 2005, Pallante *et al*. 2006, Weber *et al*. 2006), hepatocellular (Murakami *et al*. 2006,

Huang *et al*. 2008, Li *et al*. 2008, Wang *et al*. 2008, Wong *et al*. 2008), pancreas (Roldo *et al*. 2006, Bloomston *et al*. 2007, Lee *et al*. 2007, Szafranska *et al*. 2007), brain (Bottoni *et al*. 2005, Ciafre *et al*. 2005), ovarian (Iorio *et al*. 2007, Dahiya *et al*. 2008, Nam *et al*. 2008, Yang *et al*. 2008), and cervical (Lui *et al*. 2007, Lee *et al*. 2008) cancers. However, miRNA expression in pheochromocytoma has not been reported.

Here, we report the results of miRNA microarray expression profiling of a large cohort of benign and malignant pheochromocytoma tumours where we identified a number of differentially expressed miRNAs. These differences in expression were validated in a separate cohort of benign and malignant pheochromocytoma samples using real-time quantitative RT-PCR (qPCR). The functional role of miR-15a and miR-16 was also investigated using the rat PC12 malignant pheochromocytoma cell line, in which these miRNAs were shown to regulate proliferation and apoptosis.

Materials and methods

Definitions

Pheochromocytoma (adrenal or extra-adrenal) – catecholamine-producing tumours of chromaffin cell origin; benign pheochromocytoma – solitary chromaffin tumour with no evidence of metastasis with at least 2 years of follow-up after resection; malignant pheochromocytoma – chromaffin tumour with metastasis to a region where chromaffin tissue would not be expected either at initial presentation or during follow-up (DeLellis *et al*. 2004); sporadic pheochromocytoma – tumour where no familial history of pheochromocytoma or syndrome-related tumours were present, and/or no germ line mutations were identified. Locally invasive or recurrent tumours were not considered malignant and were excluded from the benign sample cohort in this study in order to avoid ambiguity.

Pheochromocytoma and normal control tissue samples

Ethical approval for the collection of tissue from patients and clinical data were obtained from the Northern Sydney Area Health Ethics Committee and from participating Institutional Human Research Ethics Committees. Tissue samples were obtained at the time of surgery, snap frozen in liquid nitrogen and stored at $-80\,^\circ$C. MiRNA microarray expression profiling was carried out using a primary cohort of 12 benign and 12 malignant tumours, and five normal

adrenal medulla tissue samples were used as a normal reference (internal cohort). Of the malignant tumours in the internal cohort, ten were primary tumours and two were metastases from a primary tumour in that cohort. An independent cohort of ten benign and ten primary malignant tumours (external cohort) was used to validate the miRNA microarray results using qPCR. Clinical details of patients in the internal cohort and external cohort are given in Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article. Normal adrenal medulla was sourced from uninvolved adrenal gland adjacent to a small (<4 cm) non-functioning adrenocortical incidentaloma or small aldosterone-producing adenoma (Conn's syndrome). Tumour specimens and normal medulla were examined histologically and confirmed to contain at least 80% tumour cells or adrenal medulla before being used in the study. Germ line mutations were analysed using dHPLC as described previously (Meyer-Rochow *et al*. 2009), and any variants were sequenced.

RNA extraction

Total RNA was extracted from fresh-frozen tissue (50 mg) of normal adrenal medulla, pheochromocytoma, or the rat PC12 cell line (passage 17) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA was further purified by precipitation with 7.5 M lithium chloride (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol and stored at $-80\ ^{\circ}$C. RNA concentration and purity were measured by u.v. absorbance at 260/280 nm (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE, USA), and quality was assessed using the Agilent Bioanalyser 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA).

MiRNA microarray profiling

MiRNA microarray profiling was performed by Exiqon Services (Vedbaek, Denmark) using miRCURY LNA Arrays. All the RNA tumour samples met the minimum quality criteria for miRNA microarray analysis requested by Exiqon (260/280 nm ratios >1.7, 260/230 nm ratios >1.4 and a RNA integrity number >7.0). One hundred nanograms of total RNA from each sample and reference (pooled RNA of all samples) were labelled with Hy3 and Hy5 fluorescent labels respectively using the miRCURY LNA Array power labelling kit (Exiqon) following the procedure described by the manufacturer. The Hy3-labelled samples and a Hy5-labelled reference RNA sample

were mixed pairwise and hybridised to the miRCURY LNA array version 11.0 (Exiqon), which contains capture probes targeting all miRNAs for human, mouse or rat registered in the miRBase version 13.0 at the Sanger Institute. The hybridisation was performed according to the miRCURY LNA array manual using a Tecan HS4800 hybridisation station (Tecan, Grodig, Austria). After hybridisation, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.), and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery, Inc., El Segundo, CA, USA). The quantified signals were background corrected (Normexp with offset value 10 (Ritchie *et al*. 2007)) and normalised using the global LOcally WEighted Scatterplot Smoothing (Lowess) regression algorithm.

MiRNA qPCR analysis

Differential expression was confirmed in selected miRNAs (miR-15a, miR-16, and miR-483-5p) from total RNA by qPCR using TaqMan miRNA assay probes (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. qPCR was performed on a Corbett Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using gene-specific TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems). RNU48 was used as the internal control for normalisation (Applied Biosystems). All the samples were run in triplicate and repeated a minimum of two times. qPCR results were analysed using the relative expression software tool (REST, 2005, Corbett Research, Sydney, NSW, Australia).

Co-expression of insulin-like growth factor 2 with miR-483-5p

Relative expression of insulin-like growth factor 2 (*IGF2*) was evaluated by qPCR and immunohistochemistry. Total RNA from each tumour and normal tissue sample was reverse transcribed into cDNA using random hexamers and the Superscript III first-strand synthesis system (Invitrogen). Each PCR was performed as a duplex reaction with the gene-specific FAM-TAMRA-labelled TaqMan probe and a ribosomal 18S RNA VIC-labelled TaqMan probe as the internal control for normalisation (Applied Biosystems).

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sectioned at 4 µm using a mouse monoclonal anti-*IGF2* primary antibody (Clone S1F2; Upstate Cell Signalling Solutions, Lake Placid, NY, USA) at a dilution of 1 in 400. All slides were processed with the Vision Biosystems Bondma X autostainer (Vision Biosystems, Mount Waverley, VIC, Australia) according to the manufacturer's protocol and with the manufacturer's retrieval solutions. Scoring of *IGF2* was performed by histological examination and graded semi-quantitatively by a blinded observer from 0 to 4+.

Survival and receiver operating characteristic analysis in malignant pheochromocytoma

The ability of *IGF2*, miR-483-5p, miR-15a, and miR-16 to provide diagnostic and prognostic information for patients with malignant disease was evaluated using follow-up data available from 17 of the 22 patients with malignant disease. Kaplan–Meier survival curves, receiver operating characteristic (ROC) curves, and statistical analysis were performed using SPSS (SPSS v16.0, Chicago, IL, USA). $P < 0.05$ was considered significant.

Cell culture

Rat pheochromocytoma cells (PC12) were obtained from American Tissue Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 10% v/v horse serum (HS) and 5% v/v fetal bovine serum (FBS) and maintained in a humidified chamber (5% CO_2 and 37 °C). Cells were confirmed mycoplasma free.

MiRNA transfection

Fifty microlitres of pre-miR hsa-miR-15a, pre-miR hsa-miR-16, or pre-miR-negative control #1 (pre-miR-NC1; Ambion) in OptiMEM I (Invitrogen), final concentration of 100 nM, were mixed with 50 µl of Lipofectamine 2000 (Invitrogen) ($25 \times$ dilution in OptiMEM I) per well, incubated at room temperature for 20 min, then added to each well of a 24-well plate (100 µl). PC12 cells (400 µl of 6.25×10^5 cells/ml) were subsequently added to each well. The transfection mixture was incubated (5% CO_2 and 37 °C) for 24 h, then either the cells were used immediately in assays or the media was replaced (500 µl DMEM/10% v/v HS/5% v/v FBS) and incubated further. To determine the transfection efficiency, Cy3-labelled pre-miR-NC1 was transfected into PC12 cells using Lipofectamine as described above, and

the cells were analysed using BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The homology between rat and human mature miR-15a or miR-16 is 100%.

Cell cycle and cell death analysis

Changes in cell cycle and apoptosis in response to pre-miR treatment were analysed by flow cytometry using propidium iodide (PI) or Annexin V respectively. For cell cycle analysis, cells treated with pre-miR-NC1, pre-miR-15a, or pre-miR-16 were washed with PBS, resuspended in 5% v/v Triton X-100 (75 µl) and RNase was added (10 mg/ml, 25 µl), followed by the addition of PI (0.5 mg/ml, 25 µl; Sigma–Aldrich). PBS was then added to a final volume of 250 µl. Induction of apoptosis was measured using the PE Annexin V apoptosis detection kit I (BD Biosciences) as per the manufacturer's instructions. Cell counts and viability were assessed using trypan blue exclusion.

Western blot analysis

The effect of pre-miR transfection on protein expression was analysed by western blot. Twenty micrograms of total protein from triplicate samples of pre-miR-NC1-, pre-miR-15a-, or pre-miR-16-treated PC12 cells were separated on a 12% v/v SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking overnight at 4 °C with 5% w/v skim milk powder/0.1% v/v Tween 20/Tris-buffered saline (TBS), membranes were incubated with either 1 µg/ml CCND1 (clone DCS-6, BD Biosciences) or 1 µg/ml BCL2 (clone 7/BCL-2, BD Biosciences) (5% w/v skim milk powder/0.1% v/v Tween 20/TBS, 1 h, RT). Bound primary antibody was detected using a goat anti-mouse HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) diluted $1000 \times$ (30 min, RT), followed by exposure to a HRP chemiluminescence substrate for 30 s (ECL Plus Western Blotting Detection System, GE Healthcare, Chalfont St Giles, UK). The resulting bands were visualised using the LAS 4000 system (FujiFilm, Tokyo, Japan).

Statistical analyses of functional assays

In cell viability, cell cycle analysis and western blotting assays, statistical significance was determined using an one-way ANOVA, with Tukey's test or Bonferroni's as the post-test; $P < 0.05$ was considered significant. Data were collected from three independent experiments performed in triplicate and expressed as the s.e.m. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Table 1 Significantly differentially expressed miRNAs between 12 benign and 12 malignant human pheochromocytoma tumours

Up-regulated in malignant compared with benign pheochromocytoma			Down-regulated in malignant compared with benign pheochromocytoma		
miRNA	*P*	Fold change	miRNA	*P*	Fold change
hsa-miR-603	8.69×10^{-4}	+2.3	hsa-miR-451	1.34×10^{-3}	−9.0
hsa-miR-483-5p	9.57×10^{-3}	+2.1	hsa-miR-144	1.36×10^{-4}	−6.5
hsa-miR-431*	5.12×10^{-3}	+2.0	hsa-miR-16	9.92×10^{-4}	−3.4
hsa-miR-138-1*	1.94×10^{-3}	+1.7	hsa-miR-19b	9.45×10^{-3}	−3.1
hsa-miR-574-3p	4.07×10^{-3}	+1.7	hsa-miR-15a	2.89×10^{-4}	−3.0
hsa-miR-483-3p	5.87×10^{-4}	+1.6	hsa-miR-557	2.59×10^{-4}	−2.6
hsa-miR-877	7.61×10^{-3}	+1.6	hsa-miR-33a	7.85×10^{-3}	−2.4
hsa-miR-766	7.46×10^{-3}	+1.4	hsa-miR-887	5.25×10^{-3}	−2.1
miRPlus_42530[a]	8.62×10^{-4}	+1.3	miRPlus_28302[a]	8.11×10^{-3}	−1.3

[a]miRPlus_42530 and miRPlus_28302 annotations represent putative miRNA-licensed human sequences not yet annotated in miRBase.

Results

Comparison of miRNA expression in benign and malignant pheochromocytomas

MiRNA expression was compared between benign and malignant pheochromocytoma tumours, and a total of 18 miRNAs were identified as being significantly differentially expressed with $P < 0.001$ after correction for multiple comparisons (Table 1). The heat map shows a distinct difference between benign and malignant samples which also clustered into two groups (unsupervised hierarchical clustering; Fig. 1). There was good separation between the benign and malignant tumour groups with three exceptions: patient sample 12 which is a benign tumour that clustered with the malignant tumours, and the malignant patient samples 22 and 23, both of which clustered with the benign samples. Unsupervised hierarchical clustering of miRNA expression demonstrated tight clustering of

miRNAs derived from the same stem-loop sequence (miR-483-5p/miR-483-3p) or in close genomic proximity (miR-15a/miR-16 and miR-144/miR-451). These miRNA pairs also showed similar patterns of expression. In a separate analysis, the rat PC12 malignant pheochromocytoma cell line clustered with the group of malignant tumour samples. The normal samples clustered separately from both the benign and malignant samples, with the exception of one benign tumour, patient sample 7, which clustered with the malignant tumours.

Validation of differentially expressed miRNAs in benign and malignant pheochromocytomas

Three miRNAs (miR-483-5p, miR-15a, and miR-16) were selected for qPCR validation using the original miRNA microarray cohort (internal) or the external cohort of ten benign and ten malignant tumours.
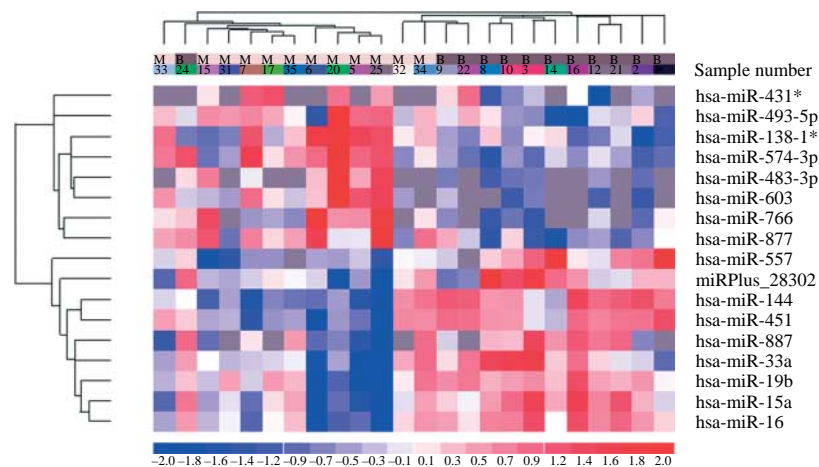
Figure 1 Heat map of differentially expressed miRNAs between 12 benign (B) and 12 malignant (M) pheochromocytoma tissues with unsupervised hierarchical clustering by miRNA and tumour type; red indicates increased expression, and blue indicates decreased expression relative to pooled reference RNA; grey indicates inadequate signal for assessment.
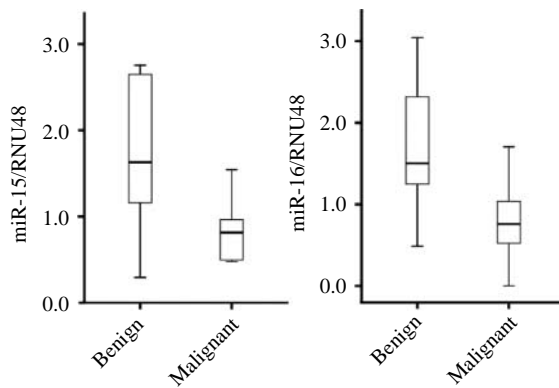
Figure 2 Decreased expression of miR-15a (A) and miR-16 (B) in malignant ($n=10$) tumours compared with benign ($n=10$) pheochromocytoma tumours ($P=0.007$ and $P=0.029$ respectively, Mann–Whitney U test).

These miRs were selected on the basis that they have been reported to be involved in the pathogenesis of other cancers, specifically miR-15a and miR-16 have been found to be under-expressed in prostate, lung, and ovarian cancer, and miR-483-5p has been over-expressed in adrenocortical cancer (Bonci *et al*. 2008, Bandi *et al*. 2009, Bhattacharya *et al*. 2009, Soon *et al*. 2009*a*). Analysing the external cohort alone, there was a significant reduction in miR-15a and miR-16 expression in malignant tumours compared with benign tumours ($P=0.007$ and $P=0.029$ respectively; Fig. 2). There was also a trend towards increased miR-483-5p expression in the malignant tumours in the external cohort alone (data not shown), but this was not statistically significant ($P=0.199$). However, when quantification of miR-483-5p relative expression was extended to include both internal and external cohorts of benign and malignant tumours, as well as of normal medulla, there was a statistically significant increase in expression in malignant tumours compared with normal medulla ($P<0.01$) and in malignant tumours compared with benign tumours ($P<0.01$; Fig. 3A).

Co-expression of IGF2 mRNA and miR-483-5p

The genomic location of miR-483-5p which is within the second intron of *IGF2* at 11p15.5 suggests a high likelihood of co-expression, which has not previously been reported; however, we have previously observed this in adrenocortical carcinoma (Soon *et al*. 2009*b*). To test this hypothesis, we measured *IGF2* mRNA expression by qPCR. There was a significant increase in expression of *IGF2* in malignant tumours compared with benign tumours ($P<0.01$) and in malignant tumours compared with normal medulla ($P<0.05$;

Fig. 3B). The relative expression of *IGF2* and miR-483-5p were positively correlated (Spearman correlation coefficient of 0.72 ($P<0.001$); Fig. 3C). To determine if IGF2 protein expression could be used as a predictor of malignant disease, 15 benign and 10 malignant tissue samples were stained for *IGF2* (where tissue was available). There was a statistically significant increase in *IGF2* expression in malignant tumours compared with benign tumours ($P=0.001$). Strong ($3+$ or $4+$) *IGF2* immunohistochemical
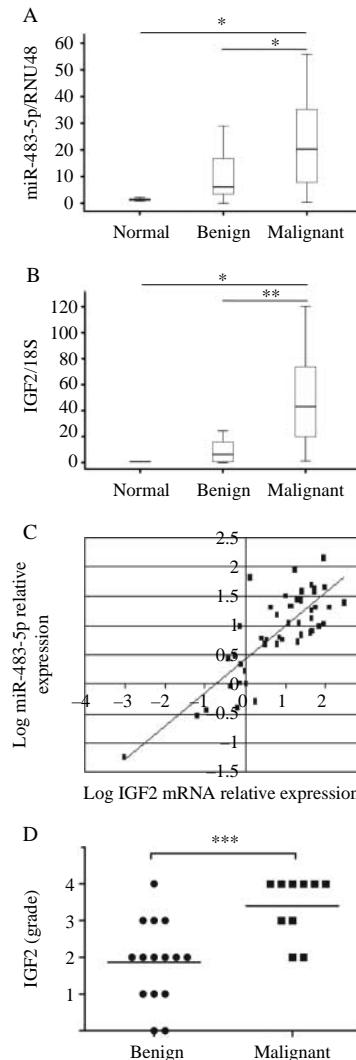
Figure 3 MiR-483-5p and *IGF2* mRNA are increased in malignant pheochromocytoma. Relative expression of (A) miR-483-5p and (B) *IGF2* mRNA in normal adrenal medulla ($n=5$) and benign ($n=22$) and malignant ($n=21$) pheochromocytomas by qPCR (*$P<0.05$ and **$P<0.01$, ANOVA & Dunn's). (C) Positive correlation between miR-483-5p and *IGF2* mRNA (Spearman Correlation, $P<0.001$). (D) IGF2 protein is increased in malignant pheochromocytoma compared with benign pheochromocytoma; immunohistochemical grading was performed (Grades 0–4, ***$P=0.001$, Spearman correlation).
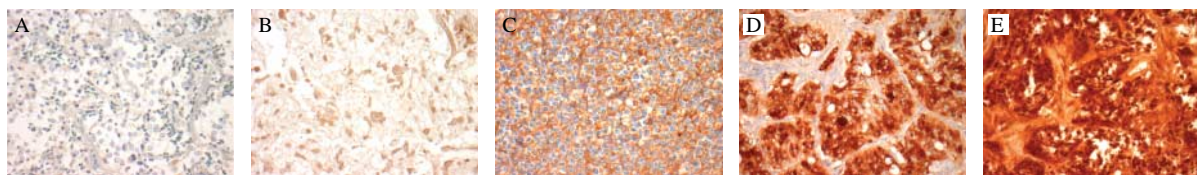
Figure 4 Immunohistochemical staining (IHC) for *IGF2*. IHC score: 0 – no staining (A), 1 – intra-cytoplasmic specks only (B), 2 – focal peri-nuclear dot-like pattern of staining (C), 3 – peri-nuclear dot-like and homogeneous cytoplasmic staining in some cells only (D), 4 – peri-nuclear dot-like and homogeneous cytoplasmic staining in all or nearly all tumour cells (E).

staining was present in 80% of the malignant tumours (Fig. 3D) compared with 27% of the benign tumours. Examples of each category of immunohistochemical grading are shown in Fig. 4.

Clinical utility of miR-483-5p, IGF2, miR-15a, and miR-16 expression in the diagnosis and prognosis of malignant pheochromocytoma

Disease-free survival and patient survival were compared using Kaplan–Meier analysis according to high or low expression of *IGF2*, miR-483-5p, miR-15a, or miR-16 in 17/20 patients where outcome data were available using qPCR data. Of these, miR-483-5p was significant for predicting patients who were more likely to have metastatic disease at the time of initial presentation and have worse disease-free survival ($P = 0.05$) but not overall survival (Fig. 5).

To determine if *IGF2*, miR-483-5p, miR-15a, or miR-16 could be used to differentiate benign from malignant tumours at initial surgical resection, ROC analysis was performed on qPCR data. Both high *IGF2* and low miR-15a had an area under the curve value > 0.85 indicating high diagnostic accuracy
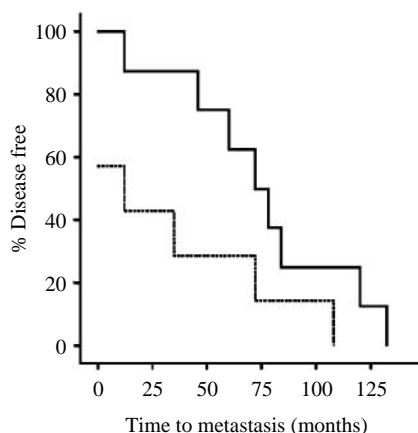

Figure 5 Disease-free survival of patients with malignant pheochromocytoma relative to miR-483-5p expression dichotomised to the median value of the primary malignant samples (log-rank 0.05); solid line indicates high expression, and dotted line indicates low expression.

($IGF2$, $P < 0.001$ and miR-15a, $P = 0.008$ respectively; Supplementary Figure 1, see section on supplementary data given at the end of this article). *IGF2* had a sensitivity of 100% and specificity of 70%, while miR-15a had a sensitivity of 90% and specificity of 80%. Based on ROC analysis, a cut-off for each was chosen, and the ability of a high *IGF2* level and low miR-15a level to distinguish malignant from benign tumours was investigated. The area under the ROC curve for both in combination was 0.9 ($P = 0.002$, 80% sensitivity and 100% specificity), meaning that all of benign tumours were called correctly using this method, and that 20% of the malignant tumours were identified as benign. In addition, we found that for immunohistochemical staining of *IGF2* protein in benign and malignant tumours the area under the curve in ROC analysis was 0.85, $P = 0.003$ (80% sensitive and 73% specific).

Pre-miR-15a and pre-miR-16 inhibit proliferation and induce cell death in the rat PC12 pheochromocytoma cell line

To determine the functional effect and thus their potential use in therapy, miR-15a and miR-16 were reintroduced as either pre-miR-15a or pre-miR-16 transfection into the rat PC12 malignant cell line. The homology between rat and human mature miR-15a or miR-16 is 100%. Reintroduction of pre-miR-15a or pre-miR-16, compared with the scrambled control pre-miR-NC1, both led to a decrease in cell viability (Fig. 6A) and cell number (Fig. 6B) as determined by trypan blue exclusion. A significant decrease in viability was observed at 48 h (pre-miR-15a and pre-miR-16, $P < 0.01$) and 72 h (pre-miR-15a, $P < 0.001$ and pre-miR-16, $P < 0.01$), but not at 24 h ($P > 0.05$) compared with the scrambled control, pre-miR-NC1. Similarly, a significant decrease in cell number was observed at 48 h (pre-miR-15a and pre-miR-16, $P < 0.05$) and 72 h (pre-miR-15a and pre-miR-16, $P < 0.01$), but not at 24 h ($P > 0.05$) compared with the scrambled control, pre-miR-NC1. The transfection efficiency was determined as 91% using Cy3-labelled
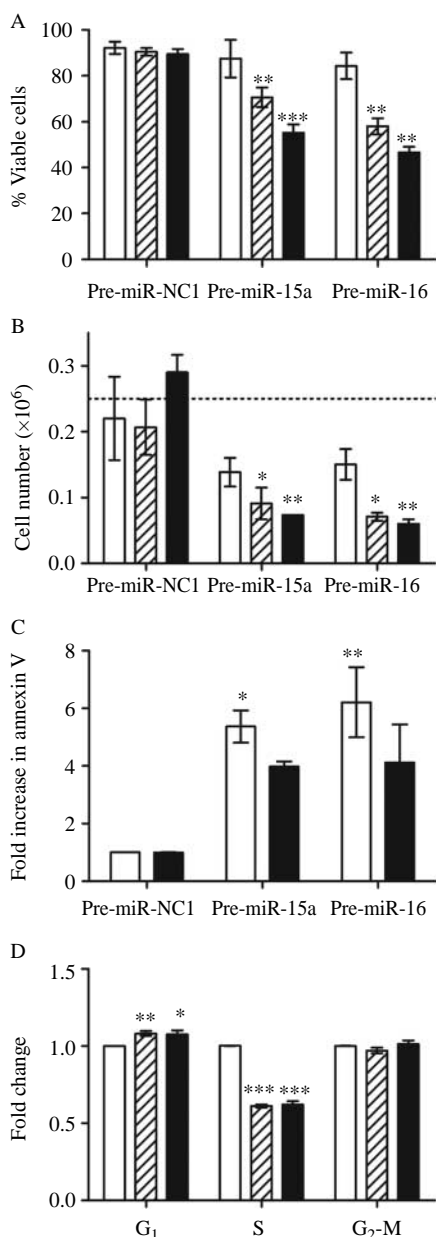
Figure 6 Pre-miR-15a and pre-miR-16 induce cell death and inhibit proliferation in rat PC12 cells. Decrease in cell viability (A), cell number (B), increase in apoptosis by Annexin V staining (C); white bar – 24, hatched bar – 48 or black bar – 72 h post transfection with pre-miR-15a, pre-miR-16, or pre-miR-NC1 scrambled control. (D) Cell cycle analysis by propidium iodide staining; white bar – pre-miR-NC1, hatched bar – pre-miR-15a, and black bar – pre-miR-16. Data are expressed as the mean and s.e.m. of three independent experiments performed in triplicate. Significant decrease in viability is indicated (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, ANOVA & Tukey's test).

pre-miR-NC1 (Supplementary Figure 2, see section on supplementary data given at the end of this article).

To determine if cell death was due to the induction of apoptosis, Annexin V cell surface expression was investigated. A significant increase in Annexin V cell surface expression was observed at 48 h ($P < 0.05$) after transfection with pre-miR-15a or pre-miR-16 compared with the scrambled control, pre-miR-NC1 (Fig. 6C).

To determine if pre-miR-15a or pre-miR-16 transfection of the rat PC12 cell line would affect their proliferation, cell cycle analysis was performed. DNA synthesis was significantly reduced in pre-miR-15a- and pre-miR-16-transfected cells compared with scrambled control, pre-miR-NC1 (Fig. 6D and Supplementary Figure 3, see section on supplementary data given at the end of this article). In addition, there was a significant increase in the number of cells in the G_1 phase of cell cycle, indicating that there is inhibition of the G_1 to S phase transition.

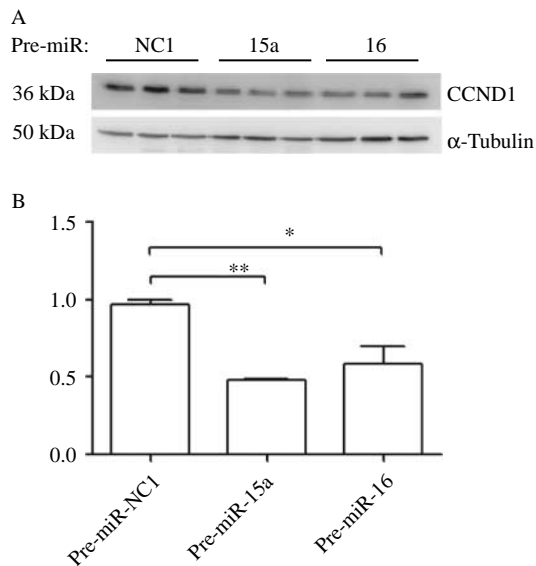## Pre-miR-15a and pre-miR-16 decrease CCND1 protein expression

The change in the level of expression of CCND1 (a cell cycle regulatory protein), a known target of miR-15a and miR-16, was analysed by western blotting and densitometry. There was a significant decrease in CCND1 expression in PC12 cells after transfection with pre-miR-15a ($P < 0.01$) or pre-miR-16 ($P < 0.05$; Fig. 7A and B). BCL2 was found not to be expressed in PC12 cells by western blotting.

Discussion

Currently, it is not possible to predict malignancy in patients with pheochromocytoma, and many investigated cancer-related genes have not proven to be reliable diagnostic or prognostic markers (Brouwers *et al*. 2005, Strong *et al*. 2008). MiRNAs have been shown to be differentially expressed in tumours compared with normal tissues (Lu *et al*. 2005), as well as in benign tumours compared with malignant tumours (Schultz *et al*. 2008, Soon *et al*. 2009a), and therefore have potential as novel diagnostic and prognostic markers. MiRNAs have also been implicated in tumourigenesis and therefore represent potential therapeutic targets. This is the first report of miRNA expression profiling of a large cohort of benign and malignant pheochromocytomas with the identification of diagnostic and prognostic biomarkers and potential therapeutic targets.

Here we report that 18 miRNAs were found to be differentially expressed between benign and malignant tumours by microarray analysis (Table 1). Of these, miR-15a and miR-16 were confirmed to have reduced expression in malignant tumours compared with benign tumours, and miR-483-5p was

A

Pre-miR: NC1   15a   16

36 kDa —————————————————— CCND1

50 kDa —————————————————— α-Tubulin

B

Figure 7 Pre-miR-15a and pre-miR-6 decrease CCND1 protein expression in rat PC12 cells. Western blot analysis of CCND1 (A) protein in PC12 cells 24 h after transfection with pre-miR-15a or pre-miR-16, relative to pre-miR-NC1scrambled control. Analysis by densitometry (B) showed that this decrease was significant; each lane was normalised using α-tubulin and is expressed relative to the scrambled control. Data are expressed as the mean and S.E.M. of three independent experiments performed in triplicate. Significant inhibition is indicated (*$P < 0.05$ and **$P < 0.01$, ANOVA & Tukey's test).

confirmed to have increased expression by qPCR (Figs 2 and 3). Of the three validated miRNAs, miR483-5p appeared to be predictive of metastasis at initial surgery and worse disease-free survival (Fig. 5) but not overall survival.

An increased level of miR-483-5p in malignant tumours was correlated with an increase in *IGF2* mRNA (Fig. 3). MiR-483-5p is located at 11p15.5 within the second intron of *IGF2*, and here we report its co-expression with *IGF2*. Therefore, we investigated whether *IGF2* could also be used to differentiate benign from malignant tumours. There was a statistically significant increase in both mRNA and protein expression of *IGF2* in malignant tumours compared with benign tumours which has not been previously reported in pheochromocytoma. IGF2 protein is known to be increased in a number of tumours including Wilms' tumour (Ravenel *et al.* 2001) and adrenocortical cancers (Schmitt *et al.* 2006), and we have previously shown that *IGF2* immunohistochemistry can be used to differentiate adrenal cortical adenomas from carcinomas in combination with Ki-67 (Soon *et al.* 2009*a*). Moreover, we have also observed that miR-483-5p is up-regulated in adrenocortical

carcinoma, which is consistent with our current finding of *IGF2* and miR-483-5p co-expression.

We further analysed by ROC analysis whether these three validated differentially expressed miRNAs with the addition of *IGF2* mRNA and protein levels could distinguish malignant from benign pheochromocytoma. *IGF2* and miR-15a produced an area under the curve value of 0.86 identifying them as having high diagnostic accuracy, i.e. a high level of *IGF2* and a low level of miR-15a would be predictive of a malignant tumour. A cut-off for each was then defined based on ROC analysis, and it was found that a high *IGF2* level and a low miR-15a level were able to differentiate benign from malignant tumours (80% sensitive and 100% specific). This was confirmed for IGF2 protein by immunohistochemistry, where the area under the curve in ROC analysis was 0.85 (80% sensitive and 73% specific). This identifies the combination of *IGF2* mRNA/protein and miR-15a expression levels as a potential diagnostic test to differentiate benign from malignant tumours, and would require further validation in a larger cohort.

Reintroduction of miR-15a and miR-16 in the malignant rat PC12 pheochromocytoma cell line was found to induce cell death and inhibit proliferation (Fig. 6) – to date there is no human malignant pheochromocytoma cell line available. This is the first time that this has been reported, and given the high homology between rat and human miRNAs, this suggests that miR-15a and miR-16 may be novel therapeutic targets for treatment of patients with metastatic pheochromocytoma. Previous studies have shown a decreased expression of miR-15a and miR-16 in prostate (Bonci *et al.* 2008), pituitary (Bottoni *et al.* 2005), lung (Bandi *et al.* 2009), and ovarian cancers (Bhattacharya *et al.* 2009). In addition, reintroduction of miR-15a and miR-16 has been reported to reduce tumour size in a mouse model of prostate cancer (Bonci *et al.* 2008, Takeshita *et al.* 2010), providing proof-of-principle for their use as a therapeutic target.

Previous studies have demonstrated that miR-15a and miR-16 regulate CCND1 and BCL2 expression in other cancers (Bonci *et al.* 2008, Roccaro *et al.* 2009). We have shown that in a rat malignant pheochromocytoma cell line, restoration of miR-15a and miR-16 results in a decrease in CCND1 (a cell cycle regulator) expression and leads to inhibition of proliferation (Fig. 7). BCL2 was found not to be expressed in the rat PC12 cell line, as has been shown in the literature (Díaz-Prieto *et al.* 2008), and thus the mechanism of cell death after miR-15a/miR-16 reintroduction warrants further investigation.

The inability to differentiate benign from malignant pheochromocytoma, as well as the paucity of effective treatments for metastatic disease, highlights the urgent need to identify biomarkers of malignancy and novel therapeutic targets. We report for the first time that several miRNAs are differentially expressed in malignant tumours compared with benign pheochromocytoma tumours. *IGF2* and miR-15a have the potential to be utilised as diagnostic markers of malignancy following surgical resection of the primary tumour. MiR-483-5p is a marker of worse disease-free survival in malignant pheochromocytoma. Restoration of miR-15a and miR-16 in a rat cell line of malignant pheochromocytoma has an effect of inducing apoptosis and inhibiting cell proliferation, which can be partially explained via their effect on the known target CCND1. Further work is required to expand the role for these miRNAs as a potential therapeutic intervention.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0142.

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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