Modulatory role of *miR-205* in angiogenesis and progression of thyroid cancer

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Abstract

miR-205 plays a crucial role in angiogenesis and has been found in association with several types of cancers. The aims of this study were to investigate the clinical and functional roles of *miR-205* on as the major initiator and modulator of angiogenesis in thyroid cancer. 101 thyroid carcinomas, including 51 conventional and 37 follicular variants of papillary thyroid carcinomas, and 13 undifferentiated thyroid carcinomas in addition to 13 lymph nodes with metastatic thyroid carcinoma were recruited to be compared with 14 nodular goitre and seven normal thyroid tissues. Five thyroid carcinoma cell lines, of papillary and undifferentiated origin with and without history of metastasis, were also used. Expression of vascular endothelial growth factor A (VEGFA) and *miR-205* were measured and exogenous *miR-205* were transfected to observe the changes of VEGFA (by immunofluorescence and western blot techniques). Proliferation assay, cell cycle analysis and apoptosis assays were also used to evaluate the role of *miR-205* in these events. Significant under-expression of *miR-205* and over-expression of *VEGFA* mRNA and protein were noticed in thyroid cancer tissues and cell lines compared to normal thyroid control. Transfection of *miR-205* into the cancer cell lines caused significant reduction of VEGFA protein and significant inhibition in cell proliferation, arrest in G0-G1 of the cell cycle and promotion of total apoptosis ($P < 0.05$). The angiogenic and tumour-suppressive roles of *miRNA-205* were demonstrated for the first time in thyroid cancer. The current experiments provided specific information on the functional consequences of VEGF manipulation via miRNA on cancer.

Key Words

- VEGF
- miR-205
- microRNA
- Papillary thyroid carcinoma
- Undifferentiated thyroid carcinoma

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Introduction

MicroRNAs (miRNAs) are one of the most crucial classes of angiogenesis regulators (Suarez & Sessa 2009). They represent a family of short, single-stranded, non-coding RNA molecules consisting of only 20–25 nucleotides and participate in various regulatory events by acting upon the genes involved in the control of these processes (Calin &

Croce 2006). miRNAs govern gene expression at the posttranscriptional level by degradation or translational repression of a target mRNA (Gu & Kay 2010). MicroRNA 205 (*miR-205*) is one of the widely reported miRNAs that regulate different cellular pathways such as cell survival, apoptosis, angiogenesis and metastasis (Vosgha *et al.* 2014).

Previous studies suggested that additional *miR-205* can repress cancer proliferation, clonogenic survival, growth and aggressiveness by direct targeting of related genes such as *E2F1* and *BCI2* (Vosgha *et al.* 2014). Nonetheless, the role of this miRNA has not been investigated in the pathophysiology of thyroid carcinoma.

Cancer can be invasive and spread through the process of angiogenesis (Nguyen *et al.* 2009). As a promoter of angiogenesis, vascular endothelial growth factor (VEGF) is a key mediator of tumour-associated angiogenesis and progression in different cancers (Salajegheh *et al.* 2011, 2013, Xu *et al.* 2015). In our previous studies, we have shown that VEGF expression is upregulated and correlated with clinicopathological features in human thyroid carcinoma (Yu *et al.* 2005, 2008a,b, Salajegheh *et al.* 2011, 2014, Xu *et al.* 2015). Lymphangiogenesis and angiogenesis share a number of basal mechanisms, including cross talk between the different VEGFs and their receptors (Salajegheh *et al.* 2013).

The overexpression of VEGFA in our previous research revealed a correlation with the pathological parameters and metastatic status of thyroid carcinomas (Salajegheh *et al.* 2011). These significant correlations add weight to hypotheses concerning the role of VEGFA in cancer progression (Salajegheh *et al.* 2013). The functional role of miRNAs and VEGF in the context of cancer has not yet been extensively explored. Previous studies in breast and lung cancer and melanoma revealed that VEGFA as a potent target for *miR-205* can be suppressed due to their affinity to bind (Fig. 1). Therefore, we herein used a variety of thyroid carcinoma tissues and thyroid cancer cell lines to investigate the role of *miR-205* in dysregulation of VEGFA expression and its potential in regulation of tumour growth and clonogenic survival. This research will build on our previous results to more closely examine the VEGF gene and its regulatory microRNA (*miR-205*) in thyroid cancer angiogenesis and the progression of the disease.

Has-miR-205/VEGF-A alignment:

Figure 1

Alignment of VEGF-A gene with the *miR-205* suggest a potential match. The transcript is positioned and matched with *miR-205* between 138 and 156 base pairs of VEGF-A gene. Diana tool predicted miTG score 0.752 with the threshold set to 0.7; <http://diana.imis.athena-innovation.gr/DianaTools/> and microRNA.org gave the miSVR score of -0.9049 for this match (The smaller the miSVR score gets, the stronger the match will be; <http://www.microrna.org>).

Materials and methods

Tissue samples

One hundred and one thyroid carcinomas consisting of 51 conventional papillary thyroid carcinomas (CPTCs), including 25 metastatic CPTC (CPTC LN+) and 26 non-metastatic (CPTC LN-), 37 follicular variant of papillary thyroid carcinoma (FVPTC) and 13 undifferentiated thyroid carcinomas (UC) along with 13 matched lymph nodes with metastatic thyroid carcinoma (LN) were selected for the study, with full ethical approval (MSC/04/05/HREC). In addition, 21 non-cancer thyroid tissues (14 nodular goitre and seven normal thyroid tissues obtained from non-cancer areas adjacent to benign lesions) were recruited as controls. Diagnoses were made and histological subtype classifications were confirmed by a pathologist (AKYL) with reference to the World Health Organization (WHO) criteria for classification of endocrine tumours (DeLellis *et al.* 2004). For staging of the thyroid carcinomas, the American Joint Committee on Cancer /International Union against Cancer's tumour-node-metastasis (TNM) staging system for cancer was used (Sobin & Wittekind 2009). In addition, five thyroid cancer cell lines and one normal thyroid cell line were recruited to investigate the functional effect of nominated miRNAs and the expression of VEGFA and cancer progression.

Cell lines and culture

Human papillary thyroid carcinoma (K1) and human normal thyroid follicular cell line (Nthy-ori 3-1) were obtained from Sigma-Aldrich. Metastasizing human papillary thyroid carcinoma (B-CPAP), human UC from a lymph node with primary papillary thyroid carcinoma (8505C), human UC (MB-1) and human UC metastases in lymph node (BHT-101) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All cell lines were maintained in 5% CO₂/95% air at 37 °C.

K1 cells were maintained in DMEM, F-12 nutrient medium (Ham's F12), molecular cell developmental biology 105 (MCDB 105) (2:1:1), 2 mM glutamine and 10% foetal bovine serum. Nthy-ori 3-1 cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640), 2mM glutamine and 10% foetal bovine serum. B-CPAP and 8505C cells were cultured in 90% RPMI 1640 and 10% foetal bovine serum. The media for BHT-101 cells were 80% DMEM and 20% foetal bovine serum. The MB-1

cell line was maintained in 80% RPMI 1640, 20% foetal bovine serum and 2mM glutamine. These cells were passaged within 3–5 days. They were routinely tested to confirm the absence of mycoplasma. All transfection experiments were conducted with 60–80% confluent cultures.

Isolation of mRNA and miRNA

RNA and miRNA were extracted from paraffin blocks using FFPE QIAGEN miRNeasy extraction kits (Qiagen Pty. Ltd). Extraction of total RNA, including miRNA from the cells, was performed using MACHEREY-NAGEL NucleoSpin RNA kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany).

Total RNA, including miRNA, underwent cDNA synthesis using miScript Reverse Transcription kit (Qiagen). Preparation and evaluation of these samples has been described previously (Salajegheh *et al.* 2011).

miRNA microarray

A miRNA microarray experiment was performed to identify the miRNAs that interact with VEGF. Extracted miRNAs from selected samples were loaded on a miRNA microarray chip developed by Agilent Technologies, Inc., Santa Clara, CA, USA according to manufacturer's instructions, using a pooling method. Samples selected for this part of the study were chosen from groups of different types of thyroid cancer as well as different histological variants of papillary thyroid carcinomas. These included tissue from ten patients with CPTC having lymph node metastasis (CPTC LN+), ten patients with CPTC without lymph node metastasis (CPTC LN–), ten patients with follicular variant papillary thyroid carcinoma (FVPTC) and ten patients with UC. The tissues in each group had similar morphology and clinicopathological features. This ensured that variations due to phenotypes within the pools was limited and maximised the detection of effect-specific miRNAs. miRNAs from non-cancer thyroid tissues were also used (Control) to normalize the results and identify non-cancer tissue behaviour within this study (Fig. 2).

Real-time PCR of miR-205 and VEGFA

miR-205 was selected for further study based on the result of the miRNA array. Sets of primers for detection of expression of miR-205 (Hs_miR-205_1 miScript Primer Assay) in the whole population of the study were purchased from Qiagen (Qiagen). RNU6B RNA

Figure 2

(A) Whole-genome miRNA expression in different types of thyroid tissues (carcinoma and non-cancerous morphological normal control). The finding reveals that expression of miR-205 is downregulated in all pooled cancer tissues (samples 2–5). Sample 1-Control, morphological normal thyroid tissue; Sample 2- FVPTC, follicular variant of papillary thyroid carcinoma; Sample 3- CPTC LN–, CPTC with no lymph node metastasis; Sample 4- CPTC LN+, CPTC with lymph node metastases; Sample 5- UC, UC, y axis: normalized intensity values; arrow: expression of miR-205 in different population of the study; expression of all detectable miRNAs were measured against the control group and the spectrum shows their differences based on their expression. (B) Hierarchical clustering algorithm of the whole genome miRNA results with attention to miR-205. A heat map comparing the average fold changes in miRNAs showing their expression in different thyroid carcinomas compared to morphological normal thyroid tissues as determined by microarray analysis. The spectrum shows the expression differences and the intensity of each of the microRNAs. Tendency towards the right side of the spectrum (red) show overexpressing miRNAs compared with control. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0182>.

(Hs_RNU6B_2 miScript Primer Assay, Qiagen) was chosen to be used as a ubiquitous control gene. Real-time quantitative PCR (RT-PCR) was performed using IQ5 Multicolour Real-Time PCR detection system (Bio-Rad). All the samples (unknown and standards) were run in triplicate and accompanied by a non-template control. PCR was performed in a total volume of 20 µl reaction mixture containing 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 µl of miScript Universal Primer (Qiagen), 1 µl miScript Primer Assay (Qiagen), and 4 µl of cDNA template at 1.5 ng/µl concentration and 4 µl of RNase-free water. Thermal cycling conditions included initial denaturation in one cycle of 15 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C. Melting curve analysis was also performed using 81 cycles of 30 s increasing from 55 °C. The melting curves of all final real-time PCR products were analysed for determination of genuine products and contamination by non-specific products and primer dimers. The fold changes in the miRNA were calculated for each sample group using the $2^{-\Delta\Delta C_t}$ method. Fold changes <0.5 were considered as low expression, those between 0.5 and 2 were considered as normal expression and those >2 were considered as high expression.

The expression finding of *VEGFA* in the exact same population that has been reported in our previous works (Salajegheh *et al.* 2013) were used in order to compare against the expression outcome of *miR-205*. The PCR primers for *VEGFA* and *GAPDH* were reported previously (Salajegheh *et al.* 2013). PCR was performed using cDNA iQ SYBR green supermix (Bio-Rad). Thermal cycling conditions included initial denaturation in one cycle of 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Melt curve analysis was also performed using eighty-one cycles of 30 s increasing from 55 °C. To ensure that the correct product was amplified in the reaction, all samples were also separated by 2% agarose gel electrophoresis (Salajegheh *et al.* 2011, 2013).

Transfection

The mimic of *miR-205*, a non-targeting control, positive control (miR-1) and AllStars negative control siRNA were obtained from Qiagen. Transfection was optimized and cells were transfected by *miR-205* mimic at the concentration of 5 µM using HiPerFect reagent (Qiagen) at 5 µl/ml concentration and prepared according to the manufacturer's instructions. Transfection medium was replaced 24 h after the transfection and cells were monitored and observed for 48–72 h afterward.

Immunofluorescence staining

Cells were plated, transfected and treated on glass cover-slips. After 72 h, the treated cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Blocking with 1% BSA/PBS buffer for 30 min was followed by a triplicate wash with PBS. Then, cells were incubated for 60 min in 1:100 dilutions of anti-*VEGFA* (VEGF mouse monoclonal antibody (clone VG1), Life Technologies). Cells were washed three times with PBS afterward and incubated with 1:1000 secondary antibodies Alexa Flour 488 goat anti-mouse (Abcam, Cambridge, UK), for green colour. Then cells were stained and mounted with 4',6-diamidino-2-phenylindole (DAPI) with mounting media. The mounted cells were viewed with a two-colour fluorescent Nikon A1R+ confocal laser microscope (Nikon Instruments, Inc., Melville, NY, USA).

Western blot analysis

Whole-cell lysates were collected using Cell Lysis Buffer NP40 (Sigma-Aldrich) supplemented with fresh phenylmethylsulfonyl fluoride at 1 mmol/l. Protein samples (20–30 µg) were run on a 4–15% precast polyacrylamide gel (Mini-PROTEAN TGX Precast Gel, Bio-Rad) and transferred onto polyvinylidene-difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad) using the blotting instrument (Trans-Blot Turbo Transfer Starter System, Bio-Rad). Membranes were blocked with 5% skim milk powder in TBST (Tris buffered saline Tween 20: 120 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, and 0.05% Tween 20) for 1 h at room temperature. Proteins were detected by incubation with *VEGFA* rabbit polyclonal antibody (sc-152) by Santa Cruz (1:100, Santa Cruz biotechnology) overnight at 4 °C. Membranes were then washed three times with TBST and incubated for 1 h with the secondary antibody conjugated to HRP. Blots were developed using the Western Lightning Plus-ECL substrate (Bio-Rad). *GAPDH* served as a loading control.

MTS proliferation assay

The capacity for cellular proliferation was measured with a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS) assay (Promega BioSciences LLC). Thyroid cancer cell lines K1, B-CPAP, BHT-101, 8505C and MB-1 cells were plated at 2000 cells per well in 96-well plates with four replicates wells for each condition, transfected with *miR-205* mimic and non-targeting control, and assayed 24, 48 and 72 h

post-transfection. The absorbance was measured with a PolarStar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at a wavelength of 490 nm. Cell growth inhibition rates were calculated with this method: inhibition of proliferation (%) = (absorbance (untreated) – absorbance (treated))/absorbance (untreated).

Cell cycle analysis

After 48 h of transfection, cells were collected by trypsinization and centrifugation at 300 *g* for 5 min. The supernatant media was discarded. Then, one ml of cold 70% ethanol was added and incubated at –20 °C for 1 h. Cells were centrifuged again at 300 *g* for 5 min and were washed twice with PBS. Cells were then subjected to propidium iodide (50 µg/ml in PBS), RNase (50 µg/ml) and Triton X-100 (0.1%) and incubated for 40 min at 37 °C. A total of 10 000 cells were examined by flow cytometry, with a MUSE cell analyser instrument (Merck Millipore) in triplicates. The results were analysed by FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA). Experiments including transfected cells, mock control and non-targeting control were performed in triplicate.

Cell apoptosis assay

Measurements of viable, apoptotic and necrotic cells before and after transient transfection were performed after 48 h of experiments in studied and control groups. After their detachment and double wash with PBS, the cells were stained with 75 µl of the Muse Annexin V & Dead Cell Assay purchased from Merck Millipore. While viable cells remained unstained in this experiment, Annexin V would identify early and late apoptotic cells and Nexin 7-AAD (7-amino-actinomycin) would detect necrotic cells and nuclear debris. Experiments including transfected cells, mock control and non-targeting control were performed using the flow cytometry, MUSE cell analyser instrument counting a total of 10 000 cells in triplicate.

Data analysis

RNA and protein expression data was entered into the statistical analysis software, SPSS version 22.0 (IBM, New York, NY, USA). Final normalised data were analysed as comparisons of group means using Student's *t*-test and ANOVA (using Bonferroni and LSD correction) for continuous variables and χ^2 or Fisher exact tests for categorical variables. In addition, Pearson correlation

(two-tailed) test was used for correlation analysis. Significance threshold was taken at $P \leq 0.05$. Prism 6 (GraphPad Software version 6.02, La Jolla, CA, USA) was used to show the graph and charts.

Results

Expression of miR-205 is significantly downregulated in thyroid carcinoma tissues

The miRNA microarray (Agilent) produced a variety of differences in over 700 human miRNAs. For the purpose of this research and as a preliminary and confirmatory tool to identify the variety of expression of miR-205 in different thyroid carcinoma, data from this microarray related to miR-205 were extracted only in this report.

A lower expression of miR-205 in different thyroid cancer tissues compared to morphologically normal thyroid tissue was noticed (Fig. 2A). The findings from hierarchical clustering algorithm suggested that miRNA profiles (including miR-205) in FVPTC are closer to non-cancer thyroid tissues. On the other hand, the miRNA profiles of CPTC with or without lymph node metastases are clustered in another group. In addition, miRNA profiles in undifferentiated carcinoma were clustered in a different group from the other carcinomas (Fig. 2B).

The expression of miR-205 was then measured individually by performing qRT-PCR. The normalised data for miR-205 was calculated and the expression of miR-205 was determined in the variants of papillary thyroid carcinoma, the metastatic papillary thyroid carcinoma in lymph nodes, UC and the control samples (nodular hyperplasia and non-cancerous adjacent) (Fig. 3).

Overall, miR-205 expression was noted to be different in various type of thyroid carcinomas compared with the control group ($P < 0.05$). Significant under-expression of miR-205 was noticed in papillary thyroid carcinomas, metastatic papillary thyroid carcinoma in lymph nodes and UC when compared to the non-cancerous controls ($P < 0.05$). There was no significant difference in the expression of miR-205 in nodular hyperplasia and the morphologically less hyperplastic thyroid tissues. Furthermore, the levels of expression of miR-205 in CPTCs with metastasis were significantly lower than those without metastasis ($P < 0.05$). Also, the level of expression of miR-205 in CPTCs with metastasis was significantly lower than the matched metastatic papillary thyroid carcinoma in lymph nodes ($P < 0.05$). In another comparison, there was no significant difference in the expression of miR-205 in CPTCs without metastasis and the FVPTCs.

Figure 3

Expression of VEGF-A (A) and miR-205 (B) in the same population of thyroid cancer tissues and control with RT-PCR. Significant overexpression of VEGF-A ($P < 0.05$) and significant under-expression of miR-205 ($P < 0.05$) were noted in all thyroid cancers. *, significant differences. Bivariate significant correlation between overexpression of VEGF-A and under-expression of miR-205 ($P < 0.05$) in conventional papillary thyroid carcinoma with lymph node metastases (C), conventional papillary thyroid carcinoma with no lymph node metastasis (D), follicular variant of papillary thyroid carcinoma (E) and undifferentiated thyroid carcinoma (F).

Note: For the purpose of better demonstration, expression level of miR-205 has been shown in logarithmic manner (log 10). CPTCLN+, conventional papillary thyroid carcinoma with lymph node metastases; LN Met, lymph node with metastatic papillary thyroid carcinoma; CPTCLN-, conventional papillary thyroid carcinoma with no lymph node metastasis; FVPTC, follicular variant of papillary thyroid carcinoma; UC, undifferentiated thyroid carcinoma; NH, nodular hyperplasia of thyroid; control, morphologically normal thyroid tissue.

Papillary thyroid carcinomas were then divided based on TNM categories and their clinicopathological features into groups as previously described (Salajegheh *et al.* 2011, 2013), and the fold changes of *miR-205* were examined in those. A significant under-expression of *miR-205* was noticed in T3 and T4 carcinomas ($P=0.038$) and those in stage 3 and stage 4 categories ($P=0.043$). Other clinicopathological features such as age, gender of the patient, size of the tumour, presence of calcification, ossification and psammoma bodies in stroma did not show any significant linkage to the expression of *miR-205* ($P>0.05$).

Under-expression of *miR-205* is significantly correlated with overexpression of VEGF in thyroid carcinoma tissues

Expression of *VEGFA* in the same population of this study was measured and described previously (Fig. 3A) (Salajegheh *et al.* 2011). Briefly, it was noted that the levels of expression of *VEGFA* mRNA and protein were high in thyroid carcinomas and in particular in those carcinomas with metastatic nature ($P<0.05$). When the expression levels of *VEGFA* were measured against the expression of *miR-205*, it was established through a bivariate correlation that changes in overexpression of *VEGFA* in thyroid carcinomas significantly co-vary with the under-expression of *miR-205* ($P<0.05$) (Fig. 3C, D, E and F).

Expression of *miR-205* and VEGF in thyroid carcinoma cell lines

The levels of expression of *VEGFA* mRNA were measured by performing qRT-PCR and the expression of VEGF proteins were determined by immunofluorescence staining and confirmed by western blot in different thyroid carcinoma cell lines. The expression of *miR-205* was measured by performing qRT-PCR. The normalised data for *miR-205* and *VEGFA* were calculated and the expressions were determined in different thyroid carcinoma cell lines (Fig. 4).

Overall, *miR-205* was expressed significantly differently in various types of thyroid carcinoma cells compared with a normal immortalised thyroid cell line (N-Thy-ori 3-1) ($P<0.05$) (Fig. 4- Right). Significant under-expression of *miR-205* was noticed in papillary thyroid carcinoma cells (K1 and B-CPAP), metastatic papillary thyroid carcinoma cells (8505C) and UC lines (BHT-101 and MB-1) when compared to the non-cancer control (N-Thy-ori 3-1) ($P<0.05$).

The expression of *VEGFA* was also significantly different in various types of thyroid carcinoma cells

compared with non-cancer control cells (N-Thy-ori 3-1) ($P<0.05$). Significant overexpression of *VEGFA* was noticed in papillary thyroid carcinoma cells (K1 and B-CPAP), metastatic papillary thyroid carcinoma cells (8505C) and UC cells (BHT-101 and MB-1) when

Figure 4

Expression of *VEGF-A* (A) and *miR-205* (B) in thyroid cancer cell lines and control. There is significant overexpression of *VEGF-A* in all thyroid cancer cell lines ($P<0.05$) and simultaneously significant underexpression of *miR-205* ($P<0.05$) in all thyroid cancer cell lines (K1: human papillary thyroid carcinoma; B-CPAP: metastasizing human papillary thyroid carcinoma; 8505C: human undifferentiated thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: human undifferentiated thyroid carcinoma; BHT-101: human undifferentiated thyroid carcinoma metastases in lymph node) compared with the control (Nthy-ori 3-1: human normal thyroid follicular cell line). *, significant differences. Note: Expression levels were based on relative ratio of gene of interest and ubiquitous control gene.

compared to the immortalized non-cancer thyroid cells (N-Thy-ori 3-1) ($P < 0.05$) (Fig. 4- Left).

Immunofluorescence staining and western blotting analysis were carried to check the expression of VEGFA protein. Overall, VEGFA protein was also expressed differently in various types of thyroid carcinoma cells when compared to non-cancer thyroid cells (N-Thy-ori 3-1) (Fig. 5IA and IIA). Overexpression of VEGFA protein was noticed in papillary thyroid carcinoma cells (K1 and B-CPAP), metastatic papillary thyroid carcinoma cell (8505C) and UC (BHT-101 and MB-1) when compared to the immortalized non-cancer control (N-Thy-ori 3-1).

Modulatory role of miR-205 on the expression of VEGFA in thyroid cancer cell lines

After confirmation of the presence of VEGFA protein in various types of thyroid cancer cell lines by immunofluorescence staining confirmed by western blotting (Fig. 5IA and IIA), using confocal laser microscope and western blotting image analysis, the role of exogenous miR-205 on expression level of VEGFA and consequently modulation of angiogenesis were investigated. Transfection with 5 μ M of miR-205 mimic could significantly suppress expression levels of VEGFA (Fig. 5IB, IIC and IIIC) relative to control or scrambled treated cells (Fig. 5IIB and IIIB). Transfected cell lines (K1, B-CPAP, 8505C, MB-1 and BHT-101) exhibited a significant downregulation of VEGFA protein expression when incubated with the anti-VEGFA antibodies, with less fluorescence distributed in the cytoplasm in immunofluorescence images and a decrease of VEGFA signal in thyroid cancer cell lines by western blotting after transfection ($P < 0.05$) (Fig. 5IB, IIC and IIIC).

Suppressive role of miR-205 in tumourigenicity in vitro

To investigate the effect of exogenous miR-205 on proliferation of different thyroid cancer cells, after transfection with miR-205 mimic, cell proliferation was measured using MTS assay (Promega) (Fig. 6A). A significant drop in cell proliferation of cancer cell lines after introduction of miR-205 mimic was noticed mainly in day 1 and day 2 after transfection. The proliferation of K1 (papillary thyroid carcinoma cell) was reduced significantly in day 3 to $15.7\% \pm 2.90$ (Fig. 6AI) whereas proliferation of B-CPAP (papillary thyroid carcinoma cell with metastatic nature) showed the most significant drop after 2 days to $11.73\% \pm 2.69$ (Fig. 6AII). Cell proliferation in 8505C (metastatic papillary thyroid carcinoma cell with undifferentiated appearance) was significantly decreased

by day 3 to $23.51\% \pm 2.15$ of control (Fig. 6AIII). The significant role of miR-205 in the inhibition of proliferation was also noticed in the UC lines of the study (MB1 and BHT-101). It was observed that by day 3, MB-1 proliferation had dropped to $26.75\% \pm 0.74$ and BHT-101 showed a proliferation of $14.75\% \pm 1.44$ by day 3 (Fig. 6AIV and V) ($P < 0.05$).

Role of miR-205 in cell cycle arrest and induction of apoptosis

Following a noticeable drop in proliferation of all thyroid cancer cell lines, in particular within 2 days of the transfection, cell cycle analysis and apoptosis assay by flow cytometry were performed on day 2 of transfection to further investigate the suppressive role of miR-205 in the proliferation of thyroid cancer cells in this study (Fig. 6B and C).

Introduction of miR-205 mimic into papillary thyroid carcinoma (K1) showed a significant arrest and accumulation of cancer cells in the G0-G1 phase ($3.68\% \pm 0.17$) and a significant drop in S phase and G2-M phase after 48 h ($P < 0.05$) (Fig. 6BI). At the same time, the percentage of early and late apoptosis events were significantly increased in the K1 cell line after 2 days of transfection with miR-205 mimic, compared with mock transfected and un-transfected controls ($35.63\% \pm 1.55$) (Fig. 6CI).

Mimic of miR-205 in metastatic papillary thyroid carcinoma cells (B-CPAP) demonstrated its anti-growth abilities with a significant arrest of cancer cells in G0-G1 phase ($6.4\% \pm 0.64$) and a significant drop in G2-M phase after 48 h ($P < 0.05$) (Fig. 6BII). Increased early and late apoptosis events in B-CPAP with miR-205 mimic were also noticed after 2 days of transfection, compared with mock transfected and un-transfected controls ($21.28\% \pm 0.68$) (Fig. 6BIII).

The number of cells in the G0-G1 phase in 8505C (metastatic papillary thyroid carcinoma cell with undifferentiated appearance) transfected with miR-205 mimic was significantly increased by day 2 to $9.7\% \pm 0.23$ with a significant drop in S phase and G2-M phase while the early and late apoptotic features were also noticed to be increased significantly after 2 days of transfection compared to the mock transfected and un-transfected controls ($33.98\% \pm 0.40$) ($P < 0.05$) (Fig. 6BIII and CIII). Similar trends were detected in the undifferentiated carcinoma cell lines (MB-1 and BHT-101) with significant arrest in G0-G1 of $8.03\% \pm 0.15$ and $4.9\% \pm 0.02$ respectively, while cells in the S phase and G2-M phase were also noticeably reduced ($P < 0.05$) (Fig. 6BIV and BV).

Figure 5

Expression of VEGF-A protein in thyroid cancer cell lines by immunofluorescence staining and western blotting. The cancer cells before manipulation (Control: IA, IIA, IIIA), after transfection by mock miRNA (IIB, IIIB), transfection by miR-205 mimic (IB, IIC, IIIC) were compared. Significant under-expression of VEGF-A protein was noticed when exogenous mimic miR-205 was transfected to all cancer cell lines of this study ($P < 0.05$). **, significant differences. (K1: human papillary thyroid carcinoma; B-CPAP: metastasizing

human papillary thyroid carcinoma; 8505C: human undifferentiated thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: human undifferentiated thyroid carcinoma; BHT-101: human undifferentiated thyroid carcinoma metastases in lymph node; Scale in the immunofluorescent images shows 10 µm; y axis on Western Blot comparison diagrams shows VEGF-A protein expression based on signal absorption). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0182>.

Total apoptotic features of these two UC lines (MB-1 and BHT-101) were also noticed increasing significantly up to $14.13\% \pm 0.11$ and $17.53\% \pm 0.36$ respectively (Fig. 6CIV and CV). The schematic sample results of cell cycle analysis and apoptosis assay derived from the MUSE cell analyser instrument are presented in Fig. 7.

Discussion

As the most common endocrine malignancy, thyroid cancer is characterized by the hallmark of a highly

angiogenic and lymphangiogenic type of cancer (Lam *et al.* 2000, 2005, Lam & Lo 2006, Lang *et al.* 2006, Lo *et al.* 2006, Salajegheh *et al.* 2008, Sobin & Wittekind 2009, Pillai *et al.* 2015). These morphological characteristics make it a useful model to obtain in-depth understanding of molecular mechanisms involved in thyroid cancer angiogenesis and to develop the therapeutic paradigms of this disease. In addition, thyroid cancer has different morphological types and variants of diverse biological behaviour (Lam *et al.* 2000, 2005, Lam & Lo 2006, Lang *et al.* 2006, Lo *et al.* 2006, Salajegheh *et al.* 2008, Sobin &

Wittekind 2009, Pillai *et al.* 2015). These different phenotypic types could be used to observe the effect of experimental manipulations on the different stages of thyroid cancers. For instance, papillary thyroid carcinoma

and in particular follicular variant have a relative indolent biological aggressiveness (Lam *et al.* 2006, Lang *et al.* 2006, Lo *et al.* 2006, Salajegheh *et al.* 2008). On the other hand, UC is one of the most biological aggressive cancers and

patients with the cancer practically have no long-term survival (Lam *et al.* 2000).

Angiogenesis, as an essential role-player in tumour survival and activation of quiescent endothelial cells of tumours, is the formation of new blood vessels from pre-existing capillaries (Ramsden 2000, Nikiforova *et al.* 2009). Vascular endothelial growth factor A (VEGFA) has been shown as the most pivotal mediator of tumour angiogenesis in cancer, in particular thyroid cancer (Salajegheh *et al.* 2013). It was also reported that there is a significant correlation between the expression of VEGFA protein and cancer development/progression (Salajegheh *et al.* 2013). In the present study, we found a significant upregulation of VEGFA expression both in thyroid cancer specimens and in thyroid cancer cell lines when compared to normal samples. These findings implied that an elevated expression of VEGFA is necessary for thyroid cancer progression and invasion (Salajegheh *et al.* 2011, 2013). Hence, anti-angiogenic agents to target VEGFA could be useful for treating thyroid cancers with aggressive biological behaviour.

microRNAs are endogenous single-stranded, non-coding RNAs. They are gene regulators in multiple biological and metabolic processes and might be used as therapeutic agents for cancer (Ebrahimi *et al.* 2014, Gopalan *et al.* 2014, Maroof *et al.* 2014a,b, Amin & Lam 2015, Gopalan *et al.* 2015). *miR-205* is a regulatory miRNA whose deregulation may influence initiation of cancer, progression, epithelial mesenchymal transformation (EMT) and metastasis (Vosgha *et al.* 2014). Also, *miR-205* has dual functions. It can play a role as an oncogene in malignancies such as lung cancer, bladder cancer, cervical cancer and head/neck carcinoma (Vosgha *et al.* 2014). On the other hand, it has been also shown to be downregulated in breast cancer, prostatic cancer and

glioma (Vosgha *et al.* 2014). In the present study, we demonstrated for the first time the tumour suppressive role of *miR-205* in thyroid cancer. Expression of *miR-205* was detected to be decreased in different types of thyroid cancer samples and cell lines.

Our further investigation revealed that induced expression of *miR-205* in thyroid cancer can significantly suppress VEGFA expression at the protein level. This finding was in concurrence with the finding in glioblastoma cell lines for a direct interaction between *miR-205* and VEGFA (Yue *et al.* 2012). In the current study, we showed that transfection of a *miR-205* mimic could significantly suppress the expression level of VEGFA relative to control or scrambled treated cells. This result for the first time in thyroid cancer indicates that VEGFA is a direct target of *miR-205* that could be inhibited through *miR-205* binding to 3'-UTR of VEGFA protein in thyroid cancer cell lines.

The significant under-expression of *miR-205* in papillary thyroid carcinoma tissues of advanced T stages and pathological stages as well as the noticeable drop of *miR-205* in the thyroid cancer cell lines of this study also suggests the potential tumour suppressive role of *miR-205* in thyroid cancer. In addition, results from cell proliferation assays, cell cycle analysis and apoptosis assays revealed that the introduction of additional *miR-205* to thyroid cancer cells can inhibit the growth of these cancer cells from 12% up to nearly 27% within the first few days. It is also determined that through *miR-205* induction to thyroid cancer cell lines, more cells will be significantly arrested at the G0-G1 phase, and the cell cycle and growth process will also be slowed down through a reduction in S phase and G2-M phase cell populations. These findings in thyroid cancer were in concurrence with functional studies in other cancers demonstrating tumour

Figure 6

Suppressive role of *miR-205* in thyroid cancer cell lines tumourigenicity. MTS assay of K1 cell line (papillary thyroid carcinoma without metastasis) showed $15.7\% \pm 2.90$ drop in proliferation by day 3 of the transient induction of exogenous *miR-205* treatment (AI). Cell cycle arrest in G0-G1 phase ($3.68\% \pm 0.17$) ($P < 0.05$) (BI) and induction of total apoptosis ($35.63\% \pm 1.55$) ($P < 0.05$) after transfection by exogenous *miR-205* (C1) were noted. MTS assay of B-CPAP cell lines (papillary thyroid carcinoma with metastases) showed $11.73\% \pm 2.69$ drop in proliferation by day 2 of the transient induction of exogenous *miR-205* treatment (AII). Cell cycle arrest in G0-G1 phase ($6.4\% \pm 0.64$) ($P < 0.05$) (BII) and induction of total apoptosis ($21.28\% \pm 0.68$) ($P < 0.05$) after transfection by exogenous *miR-205* (CII) were noted. MTS assay of 8505C cell line (metastatic papillary thyroid carcinoma) showed $23.51\% \pm 2.15$ drop in proliferation by day 3 of the transient induction of exogenous *miR-205* treatment (AIII). Cell cycle

arrest in G0-G1 phase ($9.7\% \pm 0.23$) ($P < 0.05$) (BIII) and induction of total apoptosis ($33.98\% \pm 0.40$) ($P < 0.05$) after transfection by exogenous *miR-205* (CIII) were noted. MTS assay of MB1 (undifferentiated thyroid carcinoma) showed $26.75\% \pm 0.74$ drop in proliferation by day 3 of the transient induction of exogenous *miR-205* treatment (AIV). Cell cycle arrest in G0-G1 phase ($8.03\% \pm 0.15$) ($P < 0.05$) (BIV) and induction of total apoptosis ($14.13\% \pm 0.11$) ($P < 0.05$) after transfection by exogenous *miR-205* (CIV) were noted. MTS assay of BHT-101 (metastatic undifferentiated thyroid carcinoma) showed $14.75\% \pm 1.44$ drop in proliferation by day 3 of the transient induction of exogenous *miR-205* treatment (AV). Cell cycle arrest in G0-G1 phase ($4.9\% \pm 0.02$) ($P < 0.05$) (BV) and induction of total apoptosis ($17.53\% \pm 0.36$) ($P < 0.05$) after transfection by exogenous *miR-205* (CV) were noted. *, significant differences.

A

Cell cycle analysis

B

Apoptosis assay

Figure 7

The schematic sample results of cell cycle analysis and apoptosis assay derived from the MUSE cell analyser instrument shows cell cycle arrest in G0-G1 phase in all the cancer cell lines of this study in addition to induction of apoptosis. (K1: human papillary thyroid carcinoma; B-CPAP:

metastasizing human papillary thyroid carcinoma; 8505C: human undifferentiated thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: human undifferentiated thyroid carcinoma; BHT-101: human undifferentiated thyroid carcinoma metastases in lymph node).

suppressive role of *miR-205* in certain cancer subtypes (Vosgha *et al.* 2014).

The role of *miR-205* in the process of apoptosis moderation was also demonstrated in this research. A significant increase in the percentage of early and late apoptosis in all cancer cell lines of this study has been noticed after the addition of the *miR-205* mimic. The finding of a potential regulatory role of *miR-205* in apoptosis in our thyroid cancer cell lines are in parallel to the previously described role of this miRNA and its interaction with *Bcl2* in prostatic cancer (Verdoodt *et al.* 2013).

Thyroid cancer has different morphological subgroups. In the current study, we noted that *miR-205* had different expression levels in thyroid cancers of different types or variants. From the tissue and cell line analysis, primary thyroid cancer appeared to have more suppression of *miR-205* expression when compared to metastatic thyroid cancer. This difference in expression was noted in both papillary thyroid carcinoma and UC. Due to the known potential role of *miR-205* in moderation of EMT, our finding of a noticeable under-expression of *miR-205* in primary cancer compared to metastatic cancer was in concurrence with this role in thyroid carcinoma, indicating a shift in these mechanisms following metastasis.

Conclusion

In conclusion, we detected for the first time the role of *miR-205* in different thyroid carcinomas and its possible correlation and modulatory effect on the angiogenesis process in thyroid cancer. We also demonstrated the tumour suppressive role of *miR-205* in different thyroid carcinoma cell lines. Introduction of exogenous *miR-205* (mimic) to thyroid carcinoma cell lines revealed inhibition of cancer cell growth and promotion of apoptosis.

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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