Title: *miR-205* targets angiogenesis & EMT concurrently in anaplastic thyroid carcinoma

Running head: *miR-205*, angiogenesis & EMT in thyroid cancer

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Conflict of interest

All the authors of this paper declare that they have no conflict of interest.

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Abstract

Background: The current study aims to evaluate for the first time the inhibitory roles of *miR-205* in the pathogenesis of anaplastic thyroid carcinoma. In addition, we investigated the mechanisms by which miR-205 regulates angiogenesis and epithelial-to-mesenchymal transition (EMT) in cancer.

Methods: Two anaplastic thyroid carcinoma cell lines were transfected with the expression vector pCMV-*MIR-205*. Selected markers of angiogenesis and EMT including vascular endothelial growth factor A (*VEGF-A*) and zinc-finger E-box-binding homeobox 1 (*ZEB1*) were investigated by Western blot. The interaction of *miR-205* expression with EMT and angiogenesis were also investigated by assessment of matrix metalloproteinases 2 and 9 (MMP2 and MMP 9), SNAI1 (Snai1 family zinc finger 1), vimentin, E-cadherin and N-cadherin. The function of *miR-205* was further tested with VEGF enzyme-linked immunosorbent assay (ELISA), wound healing, invasion and tube formation assays. Using an animal model, we studied the association of *miR-205* with angiogenesis, proliferation and invasion.

Results: Permanent overexpression of *miR-205* significantly suppressed angiogenesis and EMT through simultaneously targeting *VEGF-A*, *ZEB1* and downstream products. Ectopic expression of *miR-205* in cancer cells led to decreased migration, invasion and tube formation of endothelial cells. In addition, inhibition of tumour growth, vascularization and invasion were noted in the mouse tumour xenografts.

Conclusion: Our findings provide insights into simultaneous regulatory role of *miR-205* in the pathogenesis of anaplastic thyroid carcinoma by suppressing both angiogenesis and EMT. This may open avenues to exploit *miR-205* as an alternative cancer therapeutic strategy in the future.

Key words: miRNA-205, anaplastic thyroid carcinoma, angiogenesis, EMT, invasion

Introduction

Anaplastic thyroid carcinoma is one of the most aggressive and chemotherapy-resistant types of all thyroid carcinomas. The majority of patients with anaplastic thyroid carcinoma die within a year of diagnosis (Lam, et al. 2000; Lo, et al. 1999). In the current World Health Organization (WHO) classification of endocrine tumours, it is acknowledged that the genetic mechanisms and molecular profiles in the development and progression of anaplastic thyroid carcinoma is complex, which is consistent with the aggressive nature of this lethal malignancy (Lam 2017). Amongst the molecular changes occurring in anaplastic thyroid carcinoma development, angiogenesis and epithelial-to-mesenchymal transition (EMT) are important factors in overall pathogenesis (Braun, et al. 2010; Giatromanolaki, et al. 2010).

Angiogenesis is the process of the development of new blood vessels, which facilitates cancer growth and metastases (Folkman and Shing 1992). In this process, hypoxia plays an important role in activating the proteins and pro-angiogenic factors involved (Figure 1). Vascular endothelial growth factor A (*VEGF-A*), above all other known pro-angiogenic factors, induces sprouting of new blood vessels. In thyroid carcinomas, *VEGF-A* is the principal regulator of angiogenesis (Salajegheh, et al. 2013). In many cancers, the roles of matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) in dissolving extracellular matrix (ECM) and assisting and promoting angiogenesis have been suggested (Araujo, et al. 2015; Hoeben, et al. 2004; Sang 1998). The interactions of these proteins and their potential roles in the invasive behaviour of anaplastic thyroid carcinoma have yet to be addressed properly (Alfano, et al. 2010; Jia, et al. 2013; Wajner, et al. 2014).

EMT as a crucial process for cancer metastasis starts at earliest stages of tumour progression. The process leads to the loss of epithelial-specific characteristics, cell-cell adhesions, gain of stem-like properties of cancer cells and a migratory phenotype (De Craene

and Berx 2013; van Zijl, et al. 2011). Well described EMT inducer factors like *ZEB1* (Zinc-finger E-box-binding homeobox 1), *Snai1*(SNAI1 family zinc finger 1), N-cadherin and vimentin govern EMT through repression of epithelial markers like E-cadherin (Tsai and Yang 2013). Interestingly, there is a link between angiogenesis and EMT-induced cancer cell stemness, which can activate tumorigenicity and malignant transformation mechanisms (Fantozzi, et al. 2014). In addition, *ZEB1* overexpression has been correlated with increased formation of new blood vessels and tumorigeneses through the induction of VEGF-A expression (Liu, et al. 2016). Hypoxia is also one of the main inducers of EMT via up-regulation of *Snai1, ZEB1/2, Twist, vimentin and N-cadherin* (Figure 1) (De Craene and Berx 2013; Lamouille, et al. 2014; Salnikov, et al. 2012).

In thyroid carcinomas, several miRNAs have been used to predict clinical aggressiveness and the prognosis of patients with cancer (Chruscik and Lam 2015). miRNA-205-5p (*miR-205*) is implicated in numerous signalling pathways, in particular angiogenesis and EMT (Qin, et al. 2013; Vosgha, et al. 2014). *miR-205* plays a central role in tumour vascularisation as well as tumour invasion through targeting *VEGF-A* and *ZEB1* in cancers including melanoma, glioblastoma, ovarian carcinoma and breast carcinoma (Figure 1) (Gregory, et al. 2008; Li, et al. 2015a; Xu, et al. 2012; Yue, et al. 2012; Zhang, et al. 2014).

Induction of *miR-205* expression could potentially be used as an anti-angiogenic method to inhibit *VEGF-A* expression and tumour angiogenesis in thyroid carcinoma (Salajegheh, et al. 2015). As there is commonly resistance to current conventional therapies by anaplastic thyroid carcinoma, miRNA targeted therapy may offer hope to develop molecular therapeutic approaches for patients with this cancer. Therefore, the aim of this research was to take advantage of the capability of *miR-205* to impede angiogenic and EMT mechanisms as two powerful events in the pathogenesis of thyroid cancer. In this study, inhibition of pathways of angiogenesis and metastasis via partial targeting the involved genes

of these pathways were examined and demonstrated after permanent re-introduction of *miR-205 in-vitro* and *in-vivo*.

Methods

Cell culture

MB-1 (ACC638, from a human anaplastic thyroid carcinoma growing in the thyroid) and BHT-101 (ACC279, from a metastatic human anaplastic thyroid carcinoma growing in a lymph node) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). STR data for authenticity of the respective cell lines was generated by DNA profiling by the DSMZ company. MB-1 cancer cells were cultured in 80% Roswell Park Memorial Institute 1640 (RPMI 1640), 20% fetal bovine serum (FBS) and 2 mM L-glutamine. BHT-101 cells were maintained in 80% Dulbecco's Modified Eagle Medium (DMEM), 20% FBS and 0.5% human serum. Both media contained 100 U/ml penicillin and 100 µg /ml streptomycin. For cell expansion and transfection manipulation, cells were incubated in a humidified incubator with 5% CO2 and at 37^{o}C. When angiogenic events were studied, cells were incubated in hypoxic conditions (1% O2) at 37°C to create similarity to the microenvironment of malignant lesions. Cancer cells that were sub-cultured more than 5 times were excluded in this study. In addition, an experienced scientist of the team preserved the authenticity of cells by routinely monitoring growth curves, cell morphology and absence of any mycoplasma contamination.

Cell transfection

pCMV-*MIR-205* expression vector (OriGene, Rockville, MD, USA) containing green fluorescent protein (GFP) as a reporter gene and the empty vector pCMV-MIR as a mock control vector were transfected using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 2×10^5 MB1 and BHT-101 cells were seeded in a 12-well plate to reach 70-90% confluency. DNA (1 µg) - Lipofectamine

3000 (3 µl) complex was diluted in Opti-MEM reduced serum medium (Life Technologies). They were gently mixed and incubated for 5 minutes at room temperature. A total of 100µl of vector and lipofectamine was directly added to cells in a final volume of 1 ml per well. The cells were incubated for 24 hours and monitored under a fluorescent microscope. This was performed to check the expression of GFP and transfection efficiency. Stable cell lines were created using 600 µg /ml Geneticin® Selective antibiotic (G418 Sulfate) (Thermofisher Scientific).

RNA extraction and Quantitative Real-Time PCR (RT-PCR)

Total RNA and miRNA from the MB1 and BHT-101 pCMV-MIR-205-transfected cells, pCMV-MIR mock and un-transfected cells were extracted (RNeasy Mini Kit; Qiagen, Hilden, Germany). cDNA was synthesised (Qiagen miScript Reverse Transcription kit). RT-PCR was performed to estimate the expression of miR-205 in selected cancer cells as described previously (Salajegheh et al. 2015; Salajegheh, et al. 2016).

Western blot

Total cell lysate collection and protein extraction was performed and the concentration was measured (Protein quantification assay Macherey-Nagel GmbH & Co. KG, Düren, Germany) and 30 µg of extracted protein from cell lysates were run and separated on a 4% to 15% precast polyacrylamide gel (Mini-PROTEAN TGX Precast Gel; Bio-Rad). The proteins were then transferred onto polyvinylidene difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs; Bio-Rad) using a blotting instrument (Trans-Blot Turbo Transfer Starter System; Bio-Rad). Blocking of the membrane was performed using 5% milk in Tris-buffered saline and Tween 20 (120 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl and 0.05% Tween 20: TBST) for 1 hour at room temperature. The membrane

was incubated with antibodies overnight at 4°C. The antibodies used were VEGF-A (sc-152, a rabbit polyclonal antibody at 1:100 concentration), ZEB1 (sc-81428, a mouse monoclonal antibody at 1:200 concentration), MMP-2 (sc-13594, a mouse monoclonal antibody at 1:200 concentration), MMP-9 (sc-21733, a mouse monoclonal antibody at 1:200 concentration), E-cadherin (sc-7870, a rabbit polyclonal antibody at 1:200 concentration), N-cadherin (sc-393933, a mouse monoclonal antibody at 1:200 concentration), vimentin (sc-7557, a goat polyclonal antibody at 1:200 concentration) and SNAI1 (21-35, a rabbit polyclonal antibody at 1:500 concentration). VEGF-A, ZEB1, MMP-2, MMP-9, E-cadherin, N-cadherin and vimentin were obtained from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA) whereas SNAI1 was obtained from Sigma-Aldrich. Following antibody incubation, the membrane was washed three times with TBST. Then, it was incubated with the secondary antibody conjugated to horseradish peroxidase for 1 hour. Signals were developed using the Western Lighting Plus-ECL substrate (Bio-Rad). ImageStudio (LI-COR, Cambridge, UK) was used for image analyses and densitometry measurement.

Wound healing

To determine cell migration ability, a wound-healing assay was performed. pCMV-MIR-205-transfected cells, pCMV-MIR mock and un-transfected cells were cultured overnight. After making the scratch with a p200 pipette tip, the wound area was measured within three days in the transfected and control groups. The cells were gently washed with 1X phosphate buffer saline (PBS) two times and then incubated with media containing reduced fetal bovine serum (FBS). This was to confirm that the results were based on the migratory ability of the cancer cells, not their proliferation (starvation was optimised at 5%). The images of wound areas were captured after 24, 48 and 72 hours by an inverted microscope at 10X magnification. Three different fields per well were selected randomly and

captured. The mean wound area between one side of scratch to the other side was calculated and analysed using ImageJ software (Version 1.51; Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016).

Cell invasion assay

MB1 and BHT-101 pCMV-MIR-205-transfected cells, pCMV-MIR mock and un-transfected cells were incubated in transwell inserts (Corning, NY, USA) with 8-µm pore size and 6.5-mm polycarbonate membranes coated with 0.3 mg/ml matrigel (ECM gel, Sigma-Aldrich) diluted in 50µl serum-free media. 2×10^5 pCMV-MIR-205-transfected cells, pCMV-MIR mock and un-transfected cells in 100µl serum-free media cells were seeded into the upper compartment of the transwell chambers, and 650µl complete media was added to the lower chambers. After 24 hours, the number of invading cells were calculated and compared in these groups. The images of five random locations from three replicates of each transwell insert were captured using 10X magnification of microscope. The mean number of invasive cells for each condition was then measured and calculated using ImageJ software.

ELISA assay

The amount of VEGF-A protein concentration in the extracellular medium (culture media) was measured by means of Human VEGF-A-165 enzyme-linked immunosorbent assay (ELISA) novex kit (Life Technologies). pCMV-*MIR-205*-transfected cells, pCMV-MIR mock and un-transfected cells were seeded at the density of 5×10^5 cells/ml into the 10 cm^2 tissue culture dishes and incubated in hypoxic conditions (1% O2) at 37°C. Culture media were then collected after 24, 48 and 72 hours, and VEGF absorbance was quantified with a PolarStar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at a wavelength of 450 nm and according to kit instructions.

Tube formation assay

Transwell cell culture chambers (Corning, NY, USA) with polycarbonate filters (4 μm pore size; 0.33 cm2 area) were used for a co-culture assay with human umbilical vein endothelial cells (HUVEC; Lonza, Basel, Switzerland) and cancer cells. Endothelial Growth Medium-2 (EGM-2) media was diluted with growth factor reduced matrigel (ECM gel, from Engelbreth-Holm-Swarm mouse sarcoma, Sigma) without VEGF at a final concentration of 1 mg/ml. 1×10^5/ml cancer cells were seeded into the transwell inserts (upper chambers) and incubated for 24 hours at hypoxic conditions (1% O2). 3×10^4 HUVEC cells were suspended in 500 μl VEGF-free media and added to each well pre-coated with matrigel (lower chambers) in triplicate. Then the transwell inserts were transferred to the top of the HUVEC cells overnight. After incubation, the tube formation ability of endothelial cells was imaged using an inverted microscope 4X magnification. Wimasis WimTube (Wimasis GmbH Munich, Germany) software was used to analyse the number of loops and number of branching points.

Tumour xenografts studies in mice

In order to perform xenograft studies, animal ethical approval was obtained from Griffith University Animal Ethics Committee in 2016 (MED/02/15/AEC). Six to eight-week old female NU/NU nude mice were purchased through the animal facility at Griffith University. They were maintained in a well-ventilated cage in accordance with the Australian code for the care and use of animals for scientific purposes published by the National Health and Medical Research Council. 2×10^6 MB-1 pCMV-*MIR-205*-transfected cells, pCMV-MIR mock cells and un-transfected cells as well as 2×10^6 BHT-101 pCMV-*MIR-205*-transfected cells, pCMV-MIR mock and un-transfected cells were suspended in cell media and matrigel (1:1). According to the Guidelines for the welfare and use of animals in

cancer research and the power calculator suggested in the British Journal of Cancer in 2010 (Workman, et al. 2010), the population size was set to 80% power to produce significant results. Based on possible variations in the formation of new blood vessels within three experimental groups, 7 mice were entered into each group. The cancer cells were injected subcutaneously into the flanks of the nude mice. Tumours' growth in the mice were monitored and measured with a calliper ruler at different time points. The tumour volumes were also calculated using the formula: $(\text{width})^2 \times \text{length} \times 1/2$. After 28 days and when progressive tumour growth was evident, euthanasia was performed. Resected tumour tissues were weighted (grams) and immersed in formaldehyde for paraffin embedding, sectioning and immunohistochemical staining.

Immunohistochemical staining

Resected tumour tissues were fixed in formalin and embedded in paraffin. Sections were cut from the tissues and stained with haematoxylin & eosin staining for histological examination by a pathologist (AKL). A marker of thyroid gland differentiation (thyroid transcription factor 1: TTF-1) was used to assess the differentiation of the thyroid cancer cells. Furthermore, the proliferative index, distribution of endothelial cells and EMT processes were evaluated by Ki-67, CD34, E-cadherin and vimentin staining respectively.

Micro-vessel density quantification (MVD)

Tumour-associated vessel formation was examined in samples stained with CD34 as the marker of endothelial cells. Assessment of micro-vessel density (MVD) in anaplastic thyroid carcinoma was performed by light microscopy. Positive staining of CD34 in endothelial cells was examined and identified by the pathologist of the team (AKL). In addition, the number of endothelial tubes formed in the tumour tissues were calculated and

determined by counting five high-power (×40) fields of the highest vascular density. MVD was expressed as the mean value of the micro-vessels counted in sum of those fields. We also considered any stained endothelial cell or clusters of endothelial cells separated from other micro-vessel structures as a countable micro-vessel.

Data Analysis

All the experiments and assays were repeated in triplicates. The data retrieved from miRNA and protein expressions as well as the values measured by different imaging software and instruments described were entered into SPSS (statistical analysis in social science) software (version 24; IMB Corp. NY, USA) for statistical analysis and Prism 3 GraphPad software (La Jolla, CA, USA) for graphs and charts demonstration. Final normalised data were analysed using paired and independent t-tests and analysis of variance (ANOVA), using Bonferroni and LSD correction. The significance threshold was taken at $p < 0.05$. Finally, experimental results were demonstrated as mean values ± SEM.

Results

Stable expression of *miR-205* precursor in anaplastic thyroid carcinoma cell lines

After successful transfection of pCMV-*MIR-205* expression vector and the empty vector pCMV-MIR, the level of *miR-205* expression was evaluated by performing qRT-PCR. Significant overexpression of *miR-205* was observed in both anaplastic thyroid carcinomas (MB-1 and BHT-101) compared with mock transfected and un-transfected cancer cells ($p < 0.05$) (Figure 2 A, B). This result confirmed the permanent ectopic expression of *miR-205* in the anaplastic carcinoma cells (MB-1 and BHT-101).

miR-205 potentially acts against malignant angiogenesis and invasion in anaplastic thyroid carcinoma cell lines

As shown in figure 1, *VEGF-A* and *ZEB1* have been considered to be targets of *miR-205*. In concordance with previous reports, we similarly observed repression of luciferase activity after co-transfection of *VEGFA 3'-UTR* and *ZEB1 3'-UTR* into the luciferase reporter vector (not published) confirming that these sites have functions in anaplastic thyroid carcinoma cells (Gregory et al. 2008; Li et al. 2015a; Xu et al. 2012; Yue et al. 2012; Zhang et al. 2014).

Following confirmatory qRT-PCR experiments to show the ectopic expression of *miR-205*, Western blot was performed to check the expression of VEGF-A as a major angiogenic growth factor in cancer cells, as well as MMP2 and MMP9 as the proposed downstream products involved in angiogenesis (Figure 2C and 2D). The pCMV-*MIR-205*-transfected MB-1 and BHT-101 thyroid carcinoma cells illustrated a dramatic down-regulation of VEGF-A expression ($p<0.05$) and significant drop in MMP2 and MMP9 ($p<0.05$).

It was also observed that overexpression of *miR-205* led to significant reduction of ZEB1, SNAI1, N-cadherin and vimentin expression in the anaplastic thyroid carcinoma cells (MB-1 and BHT-101) ($p<0.05$) (Figure 2C and 2D). Furthermore, we found that E-cadherin expression had been up-regulated after *miR-205* transfection in both anaplastic thyroid carcinomas when compared to pCMV-MIR mock and un-transfected cancer cells ($p<0.05$). These results indicate that *miR-205* could act as an anti-angiogenic agent to target VEGF-A hence affecting MMP2 and MMP9. As a result of the MMP family's role in the process of EMT, *miR-205* could also regulate invasive and metastatic behaviour of a malignant lesion by significantly manipulating the expression of ZEB1, SNAI1, N-cadherin, vimentin and E-cadherin.

miR-205 profoundly suppresses human VEGF-A secretion and endothelial tube formation ability

Taking advantage of the Human VEGFA-165 ELISA kit, we determined the inhibitory effect of *miR-205* expression on VEGF-A production in anaplastic thyroid carcinoma cells. Following three days of incubation of MB-1 and BHT-101 pCMV-*MIR-205*-transfected cells, pCMV-MIR mock and un-transfected cells in hypoxic conditions, the expression of soluble forms of VEGF-A was gradually reduced by 36% and 30%, in the pCMV-*MIR-205*-transfected cells, respectively ($p <0.05$). This indicates that permanent *miR-205*-transfected cancer cells released significantly lower levels of VEGF-A into culture media when compared to control cell lines (Figure 3A).

Given that our previous data shows *miR-205* can target *VEGF-A* (Salajegheh et al. 2015) and following the significant reduction of VEGF-A secretion in MB-1 and BHT-101 thyroid carcinoma cells, we further determined a potentially anti-angiogenic role of *miR-205* using a co-culture system and HUVEC tube formation assay *in vitro*. As shown in Figure

3B, depletion of VEGF-A expression as a result of stable transfection of the *miR-205* precursor in thyroid carcinoma cell lines significantly repressed HUVEC tube formation ability when compared with mock transfected and un-transfected cancer cell lines ($p<0.05$). A 42% and 49% decrease in total loop numbers were observed in *miR-205* transfected MB-1 and BHT-101 cells, respectively, when compared to mock transfected and un-transfected controls. Similar significant trends were also noticed in the number of branching points. Co-cultured HUVECs with the two anaplastic thyroid carcinoma cell lines (MB-1 and BHT-101) overexpressing *miR-205* showed significantly fewer branching points in comparison with HUVECs co-cultured with mock transfected groups and un-transfected cancer cell lines ($p<0.05$) (Figure 3C, D).

Reduction of migration ability of cancer cells by *miR-205*

The effect of *miR-205* on tumour migration was assessed and quantified using a scratch wound healing assay. Cells transfected with *miR-205* as well as the control cancer cells were seeded on 6-well plates and grown in standard complete growth media to reach 80% confluence. After making a scratch on each confluent cell monolayer and replacing the complete media with 5% FBS-media, the open wound area was monitored for three days. The results demonstrated that *miR-205* could noticeably suppress cancer cell mobility and migration ability in MB-1 and BHT-101 when compared to controls ($p <0.05$) (Figure 4A-4D).

Due to a significant drop of MB-1 and BHT-101 cancer cell proliferation in ideal conditions (20% FBS) in the presence of *miR-205* (Salajegheh et al. 2015), it was considered that the cancer cell migratory behaviour in 5% FBS condition was solely a result of their invasive characteristics and any inevitable proliferation would be minor and could be

ignored. MB-1 and BHT-101 cancer cells could not survive in condition below 5% for this test.

Permanent transfection of *miR-205* inhibits the invasive characteristics of cancer cells

As invasion through the extracellular matrix is a vital stage in tumour metastasis, the invasiveness of cancer cells was evaluated using a transwell chamber invasion assay. For this purpose, we cultured cancer cells on transwell inserts with 8-µm pore size and illustrated the anti-invasive role of *miR-205* in cancer cells. As shown in Figure 4E and 4G, overexpression of *miR-205* noticeably attenuates the number of invading pCMV-*MIR-205*-transfected cells compared with pCMV-MIR mock and un-transfected cells in MB-1 and BHT-101cancer cell lines ($p<0.05$) (Figure 4F, H).

Elevated expression of *miR-205* suppresses angiogenesis, invasion and tumour growth *in vivo*

We investigated whether ectopic expression of *miR-205* in anaplastic thyroid carcinoma cells affects tumour angiogenesis, growth and EMT *in-vivo.* MB-1 and BHT-101 cancer cells stably overexpressing *miR-205* showed slower growth compared with controls. In the four weeks after injection, animals with *miR-205* expressing tumour showed approximately 40% smaller volume of the tumour compared with controls ($p<0.05$) (Figure 5A-E). At the end point of the *in-vivo* experiment, the weight of removed lesions was measured. MB-1 cancer lesions with pCMV-*MIR-205* showed a significant 50% weight reduction and BHT-101 lesions overexpressing *miR-205* presented with a 55% lower weight in comparison to control and mock groups ($p<0.05$) (Figure 5F).

To explore the tumour suppressive role of *miR-205* in angiogenesis, growth and EMT/invasion *in-vivo*, cancer tissues in the mice were examined for morphological changes and immunohistochemical analysis (Figures 6 and 7). The cancers derived from MB-1 and

BHT-101 transfected with *miR-205* were often of epithelioid morphology , and had less mitotic/necrotic features and spindle-shaped appearance when compared with mock transfected and control tissues (Figure 6A). Initiation of a MET event (mesenchymal epithelial transition) occurs when undifferentiated malignant cell transforms towards a differentiated phenotype, obtaining epithelial cell characteristics. This was investigated by examining the expression of a marker of thyroid differentiation (TTF-1). Although no significant difference could be established, a raising trend of focal TTF1 expression was noticed in both MB-1 and BHT-101 lesions transfected with *miR-205*) (Figure 6B). Both MB-1 and BHT-101 lesions transfected with *miR-205* showed an approximately 52% drop in nuclear staining of Ki67 ($p<0.05$) (Figure 6C), which is in concordance with lower tumour weight results presented previously.

Significant down-regulation of CD34 as evidence of reduced vascularization in transfected tumour xenografts confirmed the anti-angiogenic potential of *miR-205* ($p<0.05$) (Figure 7A). This result exhibited a significantly reduced microvascular density (MVD) in *miR-205* transfected lesions compared to controls ($p<0.05$) (Figure 7A graphs). Additionally, a comparison of the E-cadherin and vimentin staining in tumour xenografts showed significantly higher expression of E-cadherin and lower expression of vimentin in those tumours overexpressing *miR-205* overexpression ($p<0.05$) (Figure 7B, 7C).

Discussion

The leading cause of mortality in patients with anaplastic thyroid carcinoma is linked to its profound metastatic and invasive characteristics (Guo, et al. 2014). Therefore, this study investigated the simultaneous suppression of angiogenesis, proliferation and invasion of this aggressive tumour through microRNA expression, which could be utilised for cancer treatment.

Our previous findings indicated that the level of *miR-205* expression is notably down-regulated in papillary thyroid carcinoma and anaplastic thyroid carcinoma when compared to non-neoplastic thyroid tissues. Located in chromosome 1q32.2, microRNA-205 (*miR-205*) is one of the well-studied miRNAs, which can be over-expressed or down regulated in different cancers, based on the cell type and its targets. Its dual function as a tumour suppressor or an oncogene suggests that *miR-205* could have an effective role in the induction or repression of tumorigenesis through regulation of numerous signalling pathways such as angiogenesis, cancer cell apoptosis, proliferation, and EMT (Salajegheh et al. 2015; Vosgha et al. 2014).

Various genes can be targeted by *miR-205* including *VEGFA*, *ZEB1*, *BCL2* (B-cell lymphoma 2), *E2F1* (E2F transcription factor 1) and *HER2* (receptor tyrosine-protein kinase-2) which can modulate many cellular mechanisms (Vosgha et al. 2014). miRNAs only need to be partially complementary to their targets to regulate their activity as a result of imperfect base-pairing. Therefore, our research utilised this property of *miR-205* to halt angiogenesis and EMT of anaplastic thyroid carcinoma through targeting VEGFA and ZEB1.

VEGF-A is an essential inducer of tumour angiogenesis in thyroid carcinoma (Salajegheh et al. 2013; Salajegheh, et al. 2011). Overexpression of VEGF-A and its receptor have been reported in a variety of tumours such as thyroid, skin, breast, lung and oesophageal cancers, etc. (Das and Wakelee 2012; Rajabi, et al. 2012; Salajegheh et al. 2011; Salajegheh et al. 2016; Xu, et al. 2015). In this pathway of tumour angiogenesis and in the downstream

of VEGF, many proteins such as MMP2 and MMP9 have been reported to be involved in enhancement and expansion of neo-angiogenesis process in different malignant lesions (Lin, et al. 2015; Mahecha and Wang 2017; Turner, et al. 2003). Interaction of VEGF and MMPs have been reported in lung cancer, endometrial cancer and breast cancer (Lin et al. 2015; Mahecha and Wang 2017; Radisky and Radisky 2010). Likewise, in this study, we noticed that, following the inhibition of VEGF in anaplastic thyroid carcinoma, expression of MMP2 and MMP9 were significantly reduced.

Although there are many modalities of therapies including surgery, chemotherapy, radiation therapy and combination treatments available in the treatment of anaplastic thyroid carcinoma, the majority of patients with anaplastic thyroid carcinoma survive for only a few months, and their survival rates have not significantly changed over time (Smith and Nucera 2015). Unsurprisingly, anaplastic thyroid carcinomas often show drug resistance to conventional therapies (Harris and Bible 2011). Due to the highly angiogenic and lymphangiogenic characteristic of thyroid carcinoma, there have been several targeted-therapy approaches proposed to hinder angiogenesis and metastasis mechanisms, specifically. Anti-angiogenic drugs, such as Bevacizumab and Sorafenib, are not able to significantly reduce the progression of anaplastic thyroid carcinoma. They also have several side effects including cardiovascular toxicity and dyspnoea, which make their use less desireable (Smith and Nucera 2015).

In this study, we have shown that *miR-205* acts as one of the key regulators of multiple neoplastic mechanisms in anaplastic thyroid carcinoma. This was evidenced when permanent over-expression of *miR-205* resulted in significant reduction in production of VEGF-A, a noticeable drop in vascular formation *in-vitro* and microvascular density *in-vivo*. Similar results showing the role of *miR-205* in targeting VEGF-A and angiogenesis have been reported previously in other cancers (Hu, et al. 2016; Yue et al. 2012). *miR-205* is

potentially able to reduce the speed of proliferation and growth in different thyroid cancer cell lines (Salajegheh et al. 2015). In this study, we further validated and enhanced those results, demonstrating a significant reduction in the size and weight of anaplastic thyroid carcinoma in tumour xenografts derived from *miR-205* expressing stable cell lines. Therefore, *miR-205* induction or introduction could be a promising anti-angiogenic and anti-proliferative strategy in the management of patients with anaplastic thyroid carcinoma.

Angiogenesis is one of the crucial stages of tumour metastasis and correlates with aggressiveness and invasiveness in thyroid carcinoma (Salajegheh et al. 2015; Salajegheh et al. 2016). Tumour metastasis is an angiogenesis-dependent mechanism and any strategy to block angiogenesis could be a potential method to stop cancer progression (Ferrara and Kerbel 2005). In the current study, we linked *miR-205* to the invasive behaviour of anaplastic thyroid carcinoma in two different cell lines. First, we confirmed a significant reduction in the migratory abilities of anaplastic thyroid carcinoma in *miR-205* stable cell lines compared to control cells. This result was further supported by performing invasion assays, indicating a decreased number of invasive cells transferred through transwell membranes due to *miR-205* overexpression. These data are consistent with previous studies showing an anti-invasive and anti-migratory role of *miR-205* in breast and colon cancer (Li, et al. 2015b; Wu, et al. 2009).

Numerous studies suggest that epithelial-to-mesenchymal transition (EMT) is strongly associated with thyroid carcinoma progression and metastasis (Buehler, et al. 2013; Fantozzi et al. 2014; Guo et al. 2014; Hardin, et al. 2013; Montemayor-Garcia, et al. 2013). EMT, as an initiator of cancer metastasis, is closely linked to down-regulation of E-cadherin, which is a key cell-cell adhesion molecule, and the simultaneous upregulation of several transcription factors including ZEB1, ZEB2, SNAI1, Slug, vimentin and several MMPs (De Craene and Berx 2013). It has been reported that anaplastic thyroid carcinoma showed

reduced expression of E-cadherin and a high expression of ZEB1 which is connected to the invasiveness and metastatic properties of anaplastic thyroid carcinoma (Montemayor-Garcia et al. 2013).

To further investigate the inhibitory function of *miR-205* in the migration and invasion of anaplastic thyroid carcinoma, the expression of *ZEB1*(a target of *miR-205*) and consequently its downstream interacting proteins such as E-cadherin, N-cadherin, SNAI1 and vimentin were assessed and compared in *miR-205* transfected and control cell lines. Those transfected anaplastic thyroid carcinoma cells overexpressing *miR-205* showed significant reduction of ZEB1, SNAI1, vimentin and N-cadherin and increased expression of membranous E-cadherin when compared to control anaplastic carcinoma cells. A significant anti-invasion role of *miR-205* in anaplastic thyroid carcinoma was also observed in the migration assay and invasion assay experiments. These observations could be associated with the reduction of EMT markers (ZEB1, SNAIl, vimentin and N-cadherin) in addition to inhibition of MMP2 and MMP9, following the VEGF drop, and the increase of the adhesion molecule, E-cadherin, that we have seen previously. Further evidence was added from *in-vivo* studies, where tumours expressing *miR-205* showed reduced invasive characteristics by presenting with less vascularisation, lower proliferation and more cell-to-cell adhesion due to increased membranous E-cadherin. Finally, those tumours had more epithelioid morphology, less mitotic/necrotic features and less spindle-shaped appearance.

Due to the limitations of the xenograft study model, cancer cells implanted in mice s are not highly metastatic. Thus, injection of cell lines into the blood stream seems to be unrealistic or unrepresentative to the nature and morphological needs of real cancer metastasis (Holliday and Speirs 2011). Likewise, neither MB-1 nor BHT-101 with highly invasive characteristics showed any metastasis to distant sites. This suggests that a shift of transition is taking place, due to an increase of *miR-205* and decrease of ZEB1 and other

EMT markers, where the mesenchymal morphology is changing towards an epithelial pattern (MET). These findings are in concordance with previous studies in breast cancer where down-regulation of *miR-205* was correlated with overexpression of ZEB1/2 and poor survival of the patient (Gregory et al. 2008; Zhang et al. 2014).

In conclusion, our data demonstrated for the first time that elevated *miR-205* regulates two hallmarks of anaplastic thyroid carcinoma, angiogenesis and EMT, through targeting *VEGFA* and *ZEB1 in-vitro* and *in-vivo*. The high level of *miR-205* is associated with less biologically aggressive features of anaplastic thyroid carcinoma, suppressing thyroid cancer cell growth and invasion. Hence, our functional analysis of *miR-205* suggests that targeting multiple pathways via partial matches between *miR-205* and its targets can be employed for the development of a *miR-205*-based therapeutic agent against angiogenesis and EMT in anaplastic thyroid carcinoma.

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Author Contributions

AA, AL conceived the idea of the study; HV, AA designed and ran experiments and wrote the manuscript; HV, AA, RS and AL were involved in the data analysis, interpretation of data and revision of the manuscript.

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Figure Legends:

Figure 1. miR-205 targets *VEGFA, ZEB1* and potentially other cross-talking EMT markers. Schematic diagram of hypoxic-induced angiogenesis and EMT, and potential role of miR-205 in inhibition of vascularization and invasion by targeting *VEGFA* and *ZEB1*. Schematic diagram illustrating predicted target sites of miR-205 within the 3'UTR of *VEGFA* and *ZEB1*.

Figure 2. (A, B) Normalised relative expression of mir-205 by qRT-PCR to confirm over-expression of miR-205 after permanent transfection of anaplastic thyroid carcinoma cells (MB-1 and BHT-101) with pCMV-MIR-205 vector ($p<0.05$). (C, D) Protein levels of VEGFA, ZEB1, MMP2, MMP9, E-cadherin and SNAI1, vimentin and N-cadherin were evaluated by Western blot. There was significant down-regulation of VEGF-A, ZEB1, MMP2, MMP9, SNAI1, vimentin and N-cadherin and elevated expression of E-cadherin because of over-expression of miR-205 in both transfected cancer cells (MB1 and BHT-101) compared to negative controls. ß-actin was used as a housekeeping control ($p<0.05$).

Figure 3. Up-regulation of miR-205 diminishes VEGFA secretion and endothelial tube formation. (A) Ectopic expression of miR-205 significantly reduced secreted levels of VEGFA in miR-205 transfected MB-1 and BHT-101 cancer cells culture media collected after 72-hour incubation in hypoxic condition ($p<0.05$). (B) HUVEC endotube formation decreased in the presence of miR-205 expressing MB-1 and BHT-101 cancer cells compared to untransfected and mock cancer cells. (C, D) Quantitative analysis of the morphological tube formation parameters was evaluated. Total loop numbers and branching points were significantly reduced in HUVECs co-cultured with miR-205 expressing MB-1 and BHT-101 cancer cells compared to untreated cancer cells ($p<0.05$).

Figure 4. Over-expression of miR-205 inhibits invasive characteristics of anaplastic thyroid carcinoma cells. (A-D) Wound healing assay showed that elevated expression of miR-205 significantly hindered wound closure area over three days in MB-1 and BHT-101 ($p<0.05$). (E-H) Invasion assay was utilised to confirm anti-invasive role of miR-205. Ectopic expression of miR-205 in transfected MB-1 and BHT-101 cells significantly inhibited the number of invading cells when compared with control cells ($p<0.05$).

Figure 5. miR-205 impedes the growth of MB-1 and BHT-101 thyroid tumour *in-vivo.* (A, B) Comparison of samples of MB-1and BHT-101 miR-205 transfected, mock and control xenografts are shown. (C) Samples of implanted xenografts after 28 days of proliferation. Differences in lesions' sizes and vascularised appearance were noticeable in miR-205 treated and control groups. (D, E) Lesions' volumes were measured in mm^3 over time (4 weeks). Significant reduction of tumour volume was noted in both miR-205 transfected xenografts. (F) A noticeable drop in average tumour weight of miR-205 expressing thyroid carcinoma cells (MB-1 and BHT-101) was noted in comparison with mock and control cells ($p<0.05$).

Figure 6. Histological appearance of tumour xenograft in different experiments and a study of immunological markers for differentiation and proliferation index. (A) Representative histological appearance of tumour xenograft transfected with miR-205 compared to mock and untransfected control. The cancer lesions from control and mock mice reveal anaplastic carcinoma with more frequent mitotic figures, prominent nuclear pleomorphism and spindle cell morphology whereas transfected tumour xenografts show anaplastic carcinoma with more organoid arrangement of tumour cells with epithelioid morphology. (B) Focal overexpression of thyroid transcription factor 1 (TTF-1) in miR-205 transfected MB-1 and

BHT-101 xenografts reflects the potential role of miR-205 in forcing the undifferentiated cancer cell to shift towards more epithelial appearance and characteristics, leading to reduction of EMT and invasive morphology in anaplastic thyroid carcinoma. (C) Significant reduction of Ki-67 expression, as a proliferation marker in MB-1 and BHT-101 expressing miR-205, showed the tumour suppressive role of miR-205 in thyroid tumour xenografts. Magnification: 20X; NS: Not significant; (*) Significant.

Figure 7. High level of miR-205 is associated with less angiogenic and invasive properties of anaplastic thyroid carcinoma *in-vivo*. (A) Immunostaining of CD34 indicated significant reduction of micro-vessel density (MVD) in miR-205 expressing MB-1and BHT-101 xenografts compared to mock and control xenografts. (B, C) Significant expression changes of E-cadherin and vimentin in miR-205 transfected MB-1and BHT-101 xenografts reflected the role of miR205 in reduction of EMT and invasive morphology in anaplastic thyroid carcinoma. Magnification: 20X; (*) Significant.