RESEARCH ARTICLE

Interactions of Vascular Endothelial Growth Factor and p53 with miR-195 in Thyroid Carcinoma: Possible Therapeutic Targets in Aggressive Thyroid Cancers

Hamidrea Maroof\textsuperscript{a}, Soussan Irani\textsuperscript{a,b}, Armin Arianna\textsuperscript{a}, Jelena Vider\textsuperscript{c}, Vinod Gopalan\textsuperscript{a,c} and Alfred King-yin Lam\textsuperscript{a,*}

\textsuperscript{a}Cancer Molecular Pathology, School of Medicine, Griffith University, Gold Coast, Queensland, Australia; \textsuperscript{b}Associate Professor, Dental Research Centre, Research Centre for Molecular Medicine, Oral Pathology Department, Dental Faculty, Hamadan University of Medical Sciences, Hamadan, Iran; \textsuperscript{c}School of Medical Science, Griffith University, Gold Coast, Queensland, Australia

Abstract: Background: The clinical pathological features, as well as the cellular mechanisms of miR-195, have not been investigated in thyroid carcinoma.

Objective: The aim of this study is to identify the interactions of vascular endothelial growth factor (VEGF), p53 and miR-195 in thyroid carcinoma. The clinical and pathological features of miR-195 were also investigated.

Method: The expression levels of miR-195 were identified in 123 primary thyroid carcinomas, 40 lymph nodes with metastatic thyroid carcinomas and seven non-neoplastic thyroid tissues (controls) as well as two thyroid carcinoma cell lines, B-CPAP (from metastasizing human papillary thyroid carcinoma) and MB-1 (from anaplastic thyroid carcinoma), by the real-time polymerase chain reaction. Using Western blot and immunofluorescence, the effects of exogenous miR-195 on VEGF-A and p53 protein expression levels were examined. Then, cell cycle and apoptosis assays were performed to evaluate the roles of miR-195 in cell cycle progression and apoptosis.

Results: The expression of miR-195 was downregulated in majority of the papillary thyroid carcinoma tissue as well as in cells. Introduction of exogenous miR-195 resulted in downregulation of VEGF-A and upregulation of p53 protein expressions. Upregulation of miR-195 in thyroid carcinoma cells resulted in cell cycle arrest. Moreover, we demonstrated that miR-195 inhibits cell cycle progression by induction of apoptosis in the thyroid carcinoma cells.

Conclusion: Our findings showed for the first time that miR-195 acts as a tumour suppressor and regulates cell cycle progression and apoptosis by targeting VEGF-A and p53 in thyroid carcinoma. The current study exhibited that miR-195 might represent a potential therapeutic target for patients with thyroid carcinomas having aggressive clinical behaviour.

Keywords: VEGF-A, p53, microRNA, miR-195, angiogenesis, thyroid carcinoma.

1. INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most commonly diagnosed thyroid cancer accounting for approximately 80% of all thyroid tumours [1]. Previous studies have shown that vascular endothelial growth factor (VEGF) exerts a pivotal role in the control of angiogenesis and biological aggressiveness in thyroid carcinomas [2-6]. It is worth stating that, in sustained hypoxic conditions \textit{in vitro/in vivo}, p53 downregulates VEGF expression through the retinoblastoma (Rb) pathway in a p21-dependent manner [7, 8].

In thyroid and other carcinomas, miRNAs regulate cell growth, differentiation, proliferation, cell metabolism and cancer metastasis by directly or by regulating its downstream target proteins [9-19]. miR-195, a member of the miR-15/16 family, has reported to play a pivotal role as a tumour suppressor in many human cancers. It could promote apoptosis, mainly through targeting p53 expression [20, 21]. miR-195 is located at chromosome 17p13.1, the same genomic locus as p53. P53 is the most frequently mutated gene in cancers and its mutated form is involved in some thyroid carcinomas [22-26]. Recently, the regulatory role of miR-195 in angiogenesis and specifically its direct effect on VEGF-A has been described in hepatocellular carcinomas [27, 28]. Taken together, it can be hypothesised that miR-195 acts as a key regulator for the VEGF-A/p53 crosstalk in thyroid carcinoma cells.

The underlying cellular mechanisms responsible for the increased expression of p53 following miR-195 activation in thyroid cancers are still unknown. In addition, the clinical and pathological implications of miR-195 have not been in-
vestigated in patients with thyroid carcinomas. In this study, we aimed to identify the cellular implications of miR-195 as well as the regulatory effects of miR-195 on p53, VEGF-A and clinicopathological correlations in thyroid carcinomas.

2. MATERIALS AND METHODS

2.1. Patients and Tissue Samples

Patients with papillary thyroid carcinomas were recruited from different collaborating hospitals in Australia. A pathologist (AKL) reviewed the histological sections from these carcinomas to confirm the diagnosis and classify the carcinoma. The presence of co-existing lymphocytic thyroiditis, psammoma bodies, calcification or ossification in the tumour stroma was also noted. The clinical and pathological features were recorded in database. The thyroid carcinomas were classified concerning the criteria defined by the World Health Organization classification of endocrine tumours [29, 30]. Only conventional and follicular variant of papillary thyroid carcinoma are included in this study [31-34]. The eighth edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system was used to stage the thyroid tumours [35].

In total, 123 primary thyroid papillary carcinomas (including 79 conventional papillary thyroid carcinomas and 44 follicular variants of papillary thyroid carcinoma), 40 lymph nodes with metastatic papillary thyroid carcinomas and seven non-neoplastic thyroid tissues were selected from archival formalin-fixed and paraffin embedded tissue. For the use of tissue samples in this study, ethical approval was obtained from Griffith University (MED/19/08/HREC). Histologic sections (4 µm thick) were cut from the selected tissue blocks. The sections were stained with haematoxylin & eosin. The author (AKL), who is a pathologist, reviewed the histology of the thyroid carcinomas. All blocks containing over 90% of cancer (with < 10% stromal tissue contamination) were selected.

2.2. Cell Culture

The thyroid cancer cell lines used in this study, B-CPAP (from metastasizing human papillary thyroid carcinoma) and MB-1 (from anaplastic thyroid carcinoma) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH-German Collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). Anaplastic thyroid carcinoma is the most clinical and biological aggressive form of thyroid carcinoma. Many of the anaplastic thyroid carcinomas had co-existing papillary thyroid carcinoma implying that the anaplastic carcinoma differentiated from papillary thyroid carcinoma [36]. A non-neoplastic thyroid follicular cell line (Nthy-ori 3-1) was obtained from the European Collection of Cell Cultures (ECACC) as a control. B-CPAP and Nthy-ori3-1 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Invitrogen Carlsbad, CA, USA), 2 mM l-glutamine (Invitrogen) supplemented with 10% heat & inactivated fetal bovine serum (Invitrogen). MB-1 cells were cultured in 80% RPMI 1640 (Invitrogen), 10% heat & inactivated fetal bovine serum (Invitrogen) and 2 mM l-glutamine (Invitrogen). The cell lines were authenticated in the standard protocol (using multiplex polymerase chain reaction of mini-satellite markers for DNA fingerprinting and identification of short tandem repeats of cell lines and cytogenetics). The passage numbers of these cell lines were less than eight.

2.3. Isolation of miRNA

Total miRNA was extracted from formalin fixed paraffin embedded tissue samples using miRNeasy extraction kits (Qiagen Pty. Ltd., Hilden, NRW, Germany). In brief, the sections were deparaffinised by suspending in 1 ml xylene and then centrifuged. After adding one ml of ethanol (96-100%), 240µl proteinase K digestion buffer was added to the sections. Then, 10µl proteinase K was added. After incubation at 56°C for 15 minutes and then at 80°C for 15 minutes, 25µl DNase booster buffer and 10 µl DNaseI were added. In the next step, 1200 µl ethanol (100%) was applied to the sample. Then, 500µl buffer RPE (Qiagen) was added. Finally, 30µl RNase-free water was added and centrifuged to elute the RNA. Extraction of total miRNA from the cells was performed using NucleoSpin® miRNA Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). cDNA synthesis was done using the miScript reverse transcription kit (Qiagen) according to the manufacturer’s protocol.

2.4. Quantification of miR-195 Expression

The miR-195 expression level was quantified by real-time quantitative PCR (qRT-PCR) using Hs_miR-195 miScript Primer Assay (Qiagen) following the suggested protocol. A total volume of 20µl reaction mixture containing 10 µl QuantiTect SYBR Green, 2 µl of each primer (miScript Universal Primer (Qiagen) and miR-195-5p 5’ UACGAC- CACAGAAAUAUUGGC 3’), 2 µl of RNase-free water and 4 µl of template cDNA at 1.5 ng/µl were obtained. Samples were normalised using the housekeeping gene RNU6B (Hs_ RNU6B_2 miScript Primer Assay, Qiagen). All qRT-PCR reactions were carried out in triplicates with non-template controls as previously published protocol [6]. The ΔCt method was used to calculate miRNA expression levels. The relative expression level of miR-195 was calculated and quantified with the 2^-ΔΔCt method after normalisation with reference to expression of RNU6B. Normalised final data was analysed using one-way (ANOVA) to determine if there were significant differences in miRNA-195 expression between thyroid tissue in primary and metastatic sites. Additional comparisons were to determine whether there were significant differences in other thyroid cancer subgroups, staging, patients’ gender and other clinicopathological characteristics in the expression of miRNA-195.

2.5. Transient Transfection with miR-195 mimic

The miR-195 mimic sequence (guide strand) 5’-UAGCAGCAGAAAUAUUGGC-3’ and HiPerFect transfection reagent were purchased from Qiagen. The cell lines were transiently transfected with miR-195 mimics (+ miR-195), a non-targeting control (positive control) (+ miR-1) and AllStars negative control siRNA (scramble control) (Qiagen) immediately after being seeded at a density of 20×10⁴ cells / well in 6 well plate, using the HiPerFect transfection reagent (Qiagen). Based on the Qiagen’s protocol and time-course experiment, a final concentration of 5nM
and a 48-hours transfection time was chosen for all transfections. miR-195 mimic was added in 200 µl of serum-free, antibiotic-free medium, supplemented with 5 µl of HiperFect. The mixture was allowed to stand for 15 minutes at room temperature. The resulting 200 µl of transfection reagent was added dropwise to each well containing 2 ml of medium. Cells were maintained at 37 °C and 5% CO₂ and monitored for 48 hours afterwards. The same protocol was performed for miR-1 and scramble transfections.

2.6. Immunofluorescence

In the immunofluorescence analysis, cells were cultured on a glass culture slide and transiently transfected for 48 hours. Then, cells were fixed in 4% cold paraformaldehyde/phosphate buffered saline (PBS) for 30 minutes. After permeabilization with 0.4% Triton X-100 for 10 minutes, cells were blocked with 5% normal goat serum/PBS (Sigma–Aldrich St. Louis, MO, USA) for 45 minutes. Then, they were incubated with antibodies against p53 (Pab 1801, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), VEGF-A (A-20, 1:200 dilution; Santa Cruz Biotechnology) overnight at 4 ºC. Thereafter, the cells were incubated with Texas Red-labelled secondary antibody (1:3000 dilution; Life technologies, St. Louis, MO, USA) for 2 hours at room temperature. As negative control for each stain, the staining was performed without the primary antibody. After being counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich), confocal laser scanning microscopy images were performed with an Eclipse Ti-E microscope (Nikon Pty. Ltd., Melville, NY, USA) using a plan apochromat 60×/1.40 objective and NIS-Elements imaging software platform (Nikon) with the following setting: image Size 2,048 x 2,048 and 16 bit; Pixel/dwell of 25.2 µs; Pixel Size 0.31 µm; laser power 10%; Master gain 600–1,000. After the images were captured, the original image files were converted into tagged image file format (TIFF) files.

2.7. Western Blot Analysis

After 48 hours of transient transfection, the cells were lysed in Cell Lysis Buffer NP40 (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40, 0.02% NaN3) (Invitrogen) supplemented with protease inhibitor cocktail (Sigma–Aldrich), phenylmethanesulfonyl fluoride solution (PMSF) (Sigma–Aldrich) and phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA, USA). Then, whole protein lysates were quantified using the Macherey-Nagel protein assay kit (MACHEREY-NAGEL). Equal quantities of 30 µg protein samples were run on a 4–15% precast polyacrylamide gel (Mini-PROTEAN® TGX™ Precast Gel, BIO-RAD, Hercules, CA, USA). Blocking was performed with 5% non-fat milk in TBST (Tris-buffered saline-Tween 20: 120 mmol/l Tris–HCl, pH 7.4, 150 mmol/l sodium chloride, and 0.05% Tween 20) for 2 hours at room temperature. The membrane was incubated with anti-p53, 1:100 dilution; anti-VEGF-A, 1:300 dilution and anti-β-actin, 1:5000 dilution; Santa Cruz Biotechnology) overnight at 4 ºC. According to the manufacturer’s protocol, blots were washed three times with TBST. Then, the blots were incubated for 2 hours with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution; Santa Cruz Biotechnology) for 1.5 hours at room temperature. Bolts were developed using Clarity™ Western ECL Blotting Substrate kit (BIO-RAD). They were then visualised by using VersaDoc-MP Imaging System (BIO-RAD) and analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Cell Cycle Analysis

For cell cycle analysis by flow cytometry, cells were cultured in 6-well plate. After 48 hours of transient transfection, the cancer cells were trypsinized and washed with ice-cold PBS. They were then fixed in 70% ice-cold ethanol at -20ºC for one hour. After centrifugation, the cells were washed twice with PBS, stained with propidium iodide (PI) (50 mg/ml in PBS), RNase (50 mg/ml) and Triton X-100 (0.1%). They were incubated for 40 minutes at 37 ºC and analysed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Each histogram was constructed with the data from at least 10,000 events in triplicate. Data were analysed to calculate the percentage of the cell population in each phase using the FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

2.9. Apoptosis Assay

Apoptosis assay was performed to measure the percentage of apoptotic cells using a Membrane Permeability/Dead Cell Apoptosis Kit (Invitrogen). After 48 hours of transient transfection, cells were harvested and washed twice with ice-cold PBS. They were resuspended at the 25×10⁴ cells/ml in PBS. For staining, 1 µl of YO-PRO®-1 and 1 µl of PI were added and kept in the dark for 20 minutes at room temperature. Cells were analysed for the number of apoptotic cells using a FACS Calibur flow cytometer (BD Biosciences) and calculated with FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

2.10. Statistical Analysis

All experiments were performed at least three times. All the clinical information, pathological data and miRNA expression changes were computerised. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 25.0; IBM SPSS Inc., Armonk, 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 chi-square or likelihood ratio for categorical variables. Experimental results were expressed as means ± SD (standard deviation). In addition, Pearson correlation (2-tailed) test used for correlation analysis. A p value of < 0.05 was considered statistically significant and individual p-value was shown in the figures. GraphPad Prism (Prism 7.0; Graph Pad Software, San Diego, California, USA) was used to show the charts and graph.

3. RESULTS

3.1. Expression Profiles of miR-195 in Primary Thyroid Carcinomas and Clinicopathological Parameters

Downregulation of miR-195 expression was noted to be predominant in papillary thyroid carcinoma as ~ 70% (n=86)
Table 1. The relationship of miR-195 expression levels and clinicopathological characteristics of 123 papillary thyroid carcinomas.

<table>
<thead>
<tr>
<th>Clinical &amp; Pathological Data</th>
<th>Total Number</th>
<th>miR-195 Expression</th>
<th></th>
<th></th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41</td>
<td>7 (17.1%)</td>
<td>32 (78%)</td>
<td>2 (4.9%)</td>
<td></td>
<td>0.002 *</td>
</tr>
<tr>
<td>Female</td>
<td>82</td>
<td>16 (19.5%)</td>
<td>54 (65.9%)</td>
<td>12 (14.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 45</td>
<td>67</td>
<td>11 (16.4%)</td>
<td>49 (73.1%)</td>
<td>7 (10.4%)</td>
<td></td>
<td>0.691</td>
</tr>
<tr>
<td>≥ 45</td>
<td>56</td>
<td>12 (21.4%)</td>
<td>37 (66.1%)</td>
<td>7 (12.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour size (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40 mm</td>
<td>110</td>
<td>13 (13%)</td>
<td>73 (73%)</td>
<td>14 (14%)</td>
<td></td>
<td>0.001*</td>
</tr>
<tr>
<td>&gt; 40 mm</td>
<td>13</td>
<td>10 (43.5%)</td>
<td>13 (56.5%)</td>
<td>0 (0.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 or T2</td>
<td>78</td>
<td>9 (11.5%)</td>
<td>58 (74.4%)</td>
<td>11 (14.1%)</td>
<td></td>
<td>0.020*</td>
</tr>
<tr>
<td>T3</td>
<td>45</td>
<td>14 (31.1%)</td>
<td>28 (62.2%)</td>
<td>3 (6.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>40</td>
<td>7 (17.5%)</td>
<td>33 (82.5%)</td>
<td>0 (0.0%)</td>
<td></td>
<td>0.017*</td>
</tr>
<tr>
<td>Negative</td>
<td>83</td>
<td>16 (19.3%)</td>
<td>53 (63.9%)</td>
<td>14 (16.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM staging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages I or II</td>
<td>87</td>
<td>13 (14.9%)</td>
<td>62 (71.3%)</td>
<td>12 (13.8%)</td>
<td></td>
<td>0.147</td>
</tr>
<tr>
<td>Stage III</td>
<td>36</td>
<td>10 (27.8%)</td>
<td>24 (66.7%)</td>
<td>2 (5.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological variant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional</td>
<td>79</td>
<td>16 (20.3%)</td>
<td>52 (65.8%)</td>
<td>11 (13.9%)</td>
<td></td>
<td>0.355</td>
</tr>
<tr>
<td>Follicular</td>
<td>44</td>
<td>7 (15.9%)</td>
<td>34 (77.3%)</td>
<td>3 (6.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psammoma body</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>52</td>
<td>9 (17.3%)</td>
<td>38 (73.1%)</td>
<td>5 (9.6%)</td>
<td></td>
<td>0.791</td>
</tr>
<tr>
<td>Absent</td>
<td>71</td>
<td>14 (19.7%)</td>
<td>48 (67.6%)</td>
<td>9 (12.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcification in stroma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>62</td>
<td>14 (22.6%)</td>
<td>42 (67.7%)</td>
<td>6 (9.7%)</td>
<td></td>
<td>0.494</td>
</tr>
<tr>
<td>Absent</td>
<td>61</td>
<td>9 (14.8%)</td>
<td>44 (72.1%)</td>
<td>8 (13.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osseous metaplasia in stroma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>6</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
<td>0 (0%)</td>
<td></td>
<td>0.639</td>
</tr>
<tr>
<td>Absent</td>
<td>117</td>
<td>22 (18.8%)</td>
<td>81 (69.2%)</td>
<td>14 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytic thyroiditis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>40</td>
<td>9 (22.5%)</td>
<td>27 (67.5%)</td>
<td>4 (10%)</td>
<td></td>
<td>0.738</td>
</tr>
<tr>
<td>Absent</td>
<td>83</td>
<td>14 (16.9%)</td>
<td>59 (71.1%)</td>
<td>10 (12%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant; High expression: fold change greater than 2; Low expression: fold change = less than 0.5; Normal expression = fold changes between 0.5 and 2

of the thyroid carcinomas exhibited low expression of miR-195. Only 19% (n=23) of the papillary thyroid carcinoma had miR-195 restoration. The other 11% (n=14) had a similar expression as non-neoplastic thyroid tissue.

Table 1 showed the correlation between miR-195 expressions and various clinicopathological features of papillary thyroid carcinomas. A significant difference between miR-195 expression level and gender of the patient was detected (p=0.002). High prevalence of miR-195 downregulation was noted in male patients with papillary thyroid carcinomas (78% versus 65.9%). Additionally, the miR-195 downregulation was highly prevalent in patients with small thyroid carcinomas (maximum dimension ≤40mm) when compared to those of other larger carcinomas (73% versus 56.5%, p=0.001). Similarly, patients with early T stages (T1 or T2) cancers showed a slight high prevalence of high expression of miR-195 high expression when compared to patients with advanced T stage (T3) cancers (74.4% versus 62.2%, p=0.020).
Of the 123 patients, 33% (n=40) had lymph node metastases. miR-195 expression was often down-regulated in papillary thyroid carcinomas with lymph node metastases when compared to those without lymph node metastases (82.5% versus 63.9%, p=0.017). In these 40 patients with lymph node metastases, we compared the expression of miR-195 in primary cancer and metastatic cancer in the lymph node. There was also a significant difference in miR-195 expression between them. In primary carcinoma, 82.5% (33 of 40) showed low expression of miR-195 whereas, in metastatic carcinoma in lymph node, 45% (18 of 40) revealed low expression of miR-195 (p=0.0001). Thus, lower expression levels were more commonly detected in primary cancers when compared with metastatic cancers.

miR-195 expression level was down-regulated in thyroid carcinoma cells (B-CPAP and MB-1). These findings are compatible with miR-195 expression levels in tissues examined, as miR-195 was more often down-regulated in those thyroid carcinomas with lymph node metastases when compared to thyroid carcinomas without lymph node.

3.2. miR-195 Expression and its Target Effects on VEGF-A and p53 Proteins

miR-195 was significantly downregulated in B-CPAP (Metastasizing human papillary thyroid carcinoma) and MB-1 (human anaplastic thyroid carcinoma), when compared to the non-cancer, immortalised thyroid cell line (Nthy-ori3-1 cell line). The relative expression (ratio of expression) of miR-195 in Nthy-ori3-1 cells was equal to “1” whereas the ratios of expression were 0.44 ± 0.05, 0.19 ± 0.01, in B-CPAP and MB-1 cell lines respectively (Fig. 1) (p< 0.05). To investigate the biological effects of miR-195 restoration on thyroid cancer cells, B-CPAP and MB-1 cell lines were transfected with an exogenous miR-195 (mimics). Cells treated with the miR-195 showed significant restoration of miR-195 levels (shown as a ratio of expression; 2.79 ± 0.01, 1.72 ± 0.33) in B-CPAP and MB-1 cells respectively when compared to the Nthy-ori3-1 cells (Fig. 1) (P<0.05). miR-195 was significantly overexpressed to investigate its specific regulatory function on VEGF-A and p53 expression levels.

Western blot (Fig. 2) and immunofluorescence analysis (Fig. 3) showed that miR-195 over-expression results in the downregulation of VEGF-A protein expression. In addition, thyroid cancer cells with high miR-195 lead to upregulation of p53 protein expression. Control cell groups did not show any significant changes in VEGF and p53 protein expressions after miR-195 transfection.

4. DISCUSSION

In the present study, we assessed the miR-195 expression in a large cohort of patients with papillary thyroid carcinoma and correlated its expression with various clinicopathological features. The miR-195 was downregulated in ~70% of cancer tissues indicating its tumour suppressor role in the pathogenesis of thyroid carcinomas. In thyroid cancers, the size of the tumour and extent of local invasion of cancer cells (T stage) plays a key role in predicting the biological aggressive of cancer [36]. We believe that our results showed for the first time that miR-195 is overexpressed in 19% of papillary thyroid carcinomas. This means that miR-195 expression pathway is complex. For instance, a study conducted by Wang et al., displayed that miR-195 applies its function through modulation of Raf1 gene, which is upregulated in papillary thyroid carcinoma, and dysfunction of this gene results in upregulation of miR-195 in papillary thyroid carcinoma [37].

In this study, low expression of miR-195 expression was predominantly noted in thyroid carcinomas of smaller size and lower T stages (Table 1). Therefore, miR-195 downregulation enhances cancer progression or establishment of the early growth of tumour masses. These clinical correlations of miR-195 were not previously reported in thyroid carcinomas. However, the association of miR-195 downregulation with increased cancer progression is demonstrated in several cancers [38, 39].

In some other cancers, similar findings were often noted in the study of miR-195. High circulating levels of miR-195 correlated with early pathological stages (T1 and T2) in breast cancer [40]. Downregulation of miR-195 was also reported in colorectal cancer [41]. However, in a study on tongue squamous cell carcinoma tissues, downregulation of miR-195 was significantly associated with larger and pathologically advanced tumours (T3, T4) [42]. Thus, the expression level of miR-195 in cancer could be cancerspecific. Further studies matching the expression profiling between carcinoma tissue and blood samples could be done to confirm these findings in thyroid carcinomas.

Lymph node metastasis is an important predictor of survival of patients with cancer. It is present in the advanced stages of patients with cancer. In the current study, a significant sub-population of metastatic thyroid cancers showed decreased miR-195 expression level. Thus, miR-195 could be a potential diagnostic and prognostic marker for thyroid
Fig. (2). Restoration of miR-195 regulates VEGF-A and p53 expressions in carcinoma cells. miR-195 restoration down-regulates target protein VEGF-A and p53 in B-CPAP (metastasizing human papillary thyroid carcinoma) and in MB-1 (human anaplastic thyroid carcinoma) cells when compared to miR-1 transfected and scramble group. B-CPAP and MB-1 were transfected for 48 hours. The effect of this restoration was examined in thyroid cancer cells using Western blotting. In both carcinomas, expression of VEGF-A was decreased in miR-195 mimic transfected group when compared to miR-1 transfected and scramble group. Expression of p53 was increased in miR-195 mimic transfected group when compared to miR-1 transfected and scramble group. Sample loading control was β-actin; y-axis on Western blot comparison diagrams show VEGF-A and p53 protein expressions based on signal absorption. Results were representative of three independent experiments. They were shown as mean ± SD; and (*) implies as probability value p < 0.05, when compared to controls.

Fig. (3). Confirmation of miR-195 mediated alteration of targets proteins in B-CPAP and MB-1 cells via immunofluorescence microscopy. VEGF-A and p53 protein expressions in B-CPAP and MB-1 cells at 48 hours after the transfection by immunofluorescence. Similar to Western blot analysis, miR-195 restoration significantly reduced the expression level of VEGF-A protein in B-CPAP and MB-1 cells when miR-195 mimic transfected group, miR-1 transfected (positive control) and scramble group (negative group) were compared. The expression level of p53 was increased in transfected cells when compared to miR-1 transfected and scramble group. Immunofluorescence images were captured by a Nikon A1R+ confocal microscope using 60× objective with immersion oil. VEGF-A and p53 are stained red, and nuclei are blue; Scale in the immunofluorescence images shows 5 and 10 µm. B-CPAP (metastasizing human papillary thyroid carcinoma) and in MB-1(human anaplastic thyroid carcinoma).

cancer. In addition, a study on oesophageal squamous cell carcinoma using microarray reported that down-regulated miRNAs including miR-195 were significantly associated with cancer cell invasion and lymph node metastasis [43]. These results further confirm the tumour suppressor role for miR-195 in the progression of papillary thyroid carcinoma, which is consistent with previous findings of miR-195 in different cancers [44-48].

To investigate the interactive factors of miR-195 in metastatic thyroid cancer carcinoma and tumorigenesis, we have analysed its modulatory effects on VEGF-A and p53 protein expressions in thyroid carcinoma cells. VEGF-A is a key proangiogenic factor secreted by carcinoma cells while p53 act as a potent apoptotic and tumour suppressor factor in human cancer [28, 49]. This study noted a significant down-regulation of VEGF-A protein and upregulation of p53.
Fig. (4). Effect of miR-195 on cell cycle distribution of thyroid carcinoma. Metastasizing human papillary thyroid carcinoma cells (B-CPAP) (A) and human anaplastic thyroid carcinoma cells (MB-1) (B) were transfected for 48 hours. Propidium iodide (PI) solution was used to stain nuclei so that they can be analysed for DNA content by flow cytometry. Results showed that the cell number increased in G0-G1 phase and decreased in S and G2-M phases in the miR-195 transfected group when compared to miR-1 and scramble group. An asterisk (*) indicate statistically significant differences (p < 0.05, Student’s t-test) when compared to control cells. Data shown as mean ± SD of three independent experiments. They represent percentage cells in different phases of the cell cycle with miR-195 related to controls.

Fig. (5). Induction of apoptotic by re-expression of miR-195 in thyroid carcinoma. Following transfection of metastasizing human papillary thyroid carcinoma cells (B-CPAP) (A) and human anaplastic thyroid carcinoma cells (MB-1) (B) for 48 hours, cells were subjected to Annexin V/propidium iodide (PI) staining and flow cytometry analysis. Data shown as mean ± SD of three independent experiments. They represent percentage of Annexin V-positive cells with miR-195 related to controls. The percentage of dead cells (Q1; upper left quadrant), late apoptosis cells (Q2; PI+/Annexin V+; upper right quadrant), early apoptosis cells (Q3; PI-/Annexin V+; lower right quadrant) and live cells (Q4; lower left quadrant), are indicated. Asterisks (*) indicate statistically significant differences (p < 0.05, Student’s t-test) when compared to control cells.
protein following miR-195 restoration. This finding was in agreement with previous data indicated that suppression of VEGF-A and restoration of p53 following application of miR-195 in hepatocellular carcinoma and cervical carcinoma, respectively [28, 41, 50]. It has also been demonstrated that p53 could identify the miR-195 promoter region and overexpressed miR-195 [20], resulting in enhancement of the miR-195 effects [48]. Therefore, miR-195 could act as a potential candidate in controlling the cell proliferation and play a vital role in tumour development by regulating p53 and VEGF protein expressions [51]. Downregulation of miR-195 might contribute to the development of papillary thyroid carcinomas by activating angiogenesis and by evading apoptosis [52].

In glioblastoma (brain tumour), miR-195 suppresses cell proliferation by induction of cell cycle arrest at G0-G1 phase [53]. This study has reported for the first time that the miR-195 induced the cell cycle and apoptotic changes in thyroid carcinoma cells. This effect on cell cycle is similar to results for restorations of p53 functions reported in some cancers [54-56]. Blocking of the thyroid carcinoma cell’s entry into the ‘S’ transitional phase following miR-195 restoration could be attributed to upregulation of p53 gene, and to downregulation of VEGF-A by miR-195 restoration.

In this study, we noted that miR-195 has dual functions in thyroid cancer cells, operating as a tumour suppressor by directly inhibiting both cell proliferation and invasion, and as an angiogenic inhibitor by targeting VEGF-A and p53 [53]. Restoration of miR-195 could rebuild the suppressor function of p53 tumour suppressor function and modulates VEGF-A expression. In tumour microenvironment, the lack of oxygen concentration (hypoxia) because of tumour growth is demonstrated to regulate VEGF-A expression. This hypoxic condition also stimulates the binding of hypoxia-inducible factor (HIF) to the VEGF promoter, promoting VEGF gene transcription [57]. Therefore, it is reasonable to assume that miR-195 also, directly and indirectly, regulates genes, which are involved in tumour microenvironment, invasion and metastasis.

In summary, our results demonstrated for the first time that miR-195 plays a key role in thyroid carcinogenesis by exhibiting its tumour suppressor properties in vitro. Multiple novel clinicopathological associations noted in this study suggests the potential role of miR-195 as a marker in predicting thyroid cancer aggressiveness. Furthermore, modulatory effects of miR-195 on VEGF-A and p53 proteins and its subsequent effects on cell cycle events and apoptosis confirm its significance in targeting molecular pathogenesis in thyroid carcinomas. In addition, we have recently reported that miR-34b-5p and miR-205 suppressed cancer growth in thyroid cancer by suppressing angiogenesis and apoptosis process [12, 58]. Thus, suppression of multiple miRNAs may have synergetic roles that could have the potential for targeting therapy in thyroid carcinoma with aggressive clinical behaviours.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank the funding support of student scholarships from Griffith University and the project grants of the Menzies Health Institute of Queensland from Griffith University. In addition, we would like to thank the staff of Menzies Health Institute of Queensland and Pathology Queensland for their help in the laboratory work.

FUNDING

The authors would like to thank the funding support of student scholarships from Griffith University, grant from Queensland Cancer Council and the project grants of the Menzies Health Institute of Queensland, Griffith University.

AUTHOR CONTRIBUTIONS

Hamidreza Maroof: cellular experimental works and manuscript writing
Soussan Irani: works on the histology experiment
Armin Ariana: supervise histology experiment
Jelena Vider: help with flow cytometry
Vinod Gопalan supervise the cellular experiment and edit of manuscript
Alfred King-yin Lam: supervise the works and manuscript editing

REFERENCES


