The Endothelin Axis and Angiogenesis in 
Papillary Thyroid Carcinoma

Soussan Irani

D.D.S., OMFP

School of Medicine

Griffith Health

Griffith University

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

February 2014
Abstract

Papillary Thyroid Cancer (PTC) is the most common thyroid cancer accounting for 80% of all cases. The prognosis is good, with 5-year survival rates of 95%, but in some cases the tumour behaves in an aggressive manner characterized by local recurrence and/or metastasis, processes contributed to by angiogenesis.

Angiogenesis is an essential physiologic activity involved in normal tissue biology and several pathologic conditions such as cardiac failure, and cancer. During carcinogenesis, tumour cells secrete pro-angiogenic factors to initiate angiogenesis. Angiogenesis also causes the migration of endothelial cells from pre-existing vessels to improve nutrient and oxygen delivery to tumours, angiogenesis has a key role in tumour growth and metastasis.

Vascular endothelial growth factor (VEGF) has a pivotal role in the control of angiogenesis, aggressiveness in thyroid cancers. The endothelins (ETs) are a family of genes inducing DNA synthesis and cellular growth in different cells, affecting vascular tone and angiogenesis. ET-1 has a direct effect on neoplastic cells by inducing cellular proliferation, migration as well as invasion and inhibition of apoptosis. ET-1 induces VEGF expression by increasing hypoxia-inducible factor-1α (HIF-1α) stimulation.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs. miRNAs are involved in cell proliferation, development, differentiation and metabolism. Up-regulation of oncogenically associated miRNAs decreases tumour suppressor gene expression and similarly, down-regulation of other miRNAs allows increased oncogene translation. In addition, miRNAs have a critical role in the development of metastases in a variety of cancers.
In order to examine the ways in which this angiogenic pathway alters in thyroid cancer development, the role of ET-1 axis and miRNA-195 were studied. One hundred and twenty three patients with papillary thyroid cancer were recruited for this study. Forty lymph nodes with confirmed metastases and their matched primary thyroid cancers were also selected to study the variations affecting the cancer behaviour. Seven normal thyroid tissues were also selected as a control group for this study.

*ET*-1 mRNA expression level was elevated in 27% of the primary carcinomas (n=33) compared to the control tissues. *ET_AR* mRNA expression was increased in 21% (n=26) of the primary papillary thyroid carcinomas.

A significant difference between *ET*-1 mRNA expression level and gender was detected (p=0.040), with higher expression levels mostly found in males (37% vs. 22%). *ET*-1 mRNA was more often overexpressed in those thyroid carcinomas with lymph node metastases compared to thyroid carcinomas without lymph node metastases (34% vs. 20%, p=0.0001). Also, stromal calcification was often noted in papillary thyroid carcinoma with higher *ET*-1 mRNA expression (70% vs. 43%, p=0.018). Other than these, *ET*-1 mRNA expression did not correlate with the size, histological subtype, presence of psammoma bodies, osseous metaplasia, pathological stage of thyroid cancer as well as the presence of co-existing lymphocytic thyroiditis (p> 0.05).

Papillary thyroid carcinoma of larger size (diameter > 40mm) had significantly higher levels of *ET_AR* mRNA expression than those with smaller size (46% vs. 18%, p = 0.003). Other than this, the *ET_AR* mRNA expression did not correlate with gender of the patients, histological
subtype, presence of psammoma bodies, calcification, osseous metaplasia, pathological stage of thyroid cancer as well as the presence of co-existing lymphocytic thyroiditis (p > 0.05).

Pearson correlation testing showed a strong and significant positive correlation between ET-1 and ET_{AR} mRNA expression in primary thyroid carcinomas (Pearson’s r=0.522, p<0.001).

For ET-1, high mRNA expression was detected in 30% (12/40) of the metastatic thyroid cancer in lymph nodes. For ET_{AR}, high mRNA expression was detected in 25% (10/40) of the metastatic thyroid cancers in lymph nodes.

Furthermore, elevated ET-1/ ET_{AR} expression levels seen in our research were produced by primary thyroid cancers or metastases compared to primary cancers, and may also be produced by endothelial cells within highly vascularized primary tumours and metastases. These results suggest that ET axis expression could be used as a possible indicator to predict the aggression levels of papillary thyroid carcinomas, identifying which carcinomas have metastasized as well as helping to locate the presence of new and developing metastases in lymph nodes and other locations, due to the role of the axis in establishing tumour vasculature. Although we have identified a link between ET axis expression and cancer aggression, more research is needed to determine whether ET axis levels in thyroid cancer tissues could serve as prognostic markers, or an indicator of response to therapy.

miR-195 expression level was elevated in 18.7% of the primary carcinomas compared to the control tissues. The expression was low in 69.9% and normal in 11.4% of the primary carcinomas. Metastatic thyroid cancers in lymph node mostly showed low expression in 45%.
There was a significant relationship between miR-195 expression level and tumour size (p=0.002), as low expression levels were mostly detected in small tumours (72.7% vs 46.2%). There was also a significant relationship between miR-195 expression level and T staging (p=0.020). Low expression levels were mostly noted in the T1 and T2 category compared to T3 (74.4% vs 62.2%). A significant difference was detected between miR-195 expression in thyroid cancers with lymph node metastasis and thyroid cancers without metastasis, as high expression levels were mostly detected in thyroid cancers without metastasis in 19.3% (n=16) (p=0.017). The other characteristics did not correlate with the age, histological subtype, and presence of psammoma bodies, calcification, osseous metaplasia, and pathological stage of thyroid cancer or the presence of co-existing lymphocytic thyroiditis (p > 0.05).

There was a significant difference between primary cancers and metastatic thyroid cancers in the lymph node (p=0.001). Low expression was noted as being more common in primary thyroid cancers compared to lymph nodes (69.9% vs 45%). miR-195 was more often down-regulated in those thyroid carcinomas with lymph node metastases compared to thyroid carcinomas without lymph node metastases (82.5% vs. 63.9%, p=0.017).

There was also a significant difference between miR-195 expression level in primary cancers with metastasis and metastatic tumours in lymph nodes (p<0.001). Lower expression levels were mostly detected in primary cancers with metastasis (82.5% vs 45%).

Similarly, a significant difference was detected between miR-195 expression level in thyroid carcinomas without lymph node metastases compared to metastatic thyroid cancer in lymph node (p=0.037). miR-195 was mostly down-regulated in thyroid carcinomas without lymph node metastases as compared to the lymph nodes themselves (63.9% vs 45%).
Primary thyroid cancers showed the low expression level of both miR-195 and ET-1 mRNA in 83.1% (49/123) of cases.

Pearson correlation testing showed a significant positive correlation between miR-195 and ET-1 expression in primary thyroid cancers (Pearson r =0.254, p<0.005).

We also examined the expression level of miR-195 in K1 cell line (derived from a papillary thyroid carcinoma). There was no significant difference between miR-195 expression level in normal thyroid cell line (Nthy-ori3-1, derived from human thyroid follicular epithelium) and the K1 cell line. Then, miR-195 expression level was examined in the B-CPAP cell line (derived from a metastasizing papillary thyroid carcinoma). miR-195 expression level was down-regulated in B-CPAP cell line with a fold change of 43. 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 compared to thyroid carcinomas without lymph node metastases.

The results have revealed that the levels of miR-195 are much lower in PTC cell lines compared to normal thyroid cells. In addition, our finding suggest that miR-195 in PTC functions as an anti-angiogenesis and tumour suppressing factor in cancer through knockdown of VEGF and Bcl2, but does not exert effects via HIF-1 α or p53. miR-195 also appears to play a role in invasion and metastasis. Additionally, it could be said that miR-195 as a tumour suppressor gene is not related to manipulation of the ET-1 axis directly in cancer cells, though it may in cells that produce endogenous ET-1 by affecting pathways that ET-1 also affects.
miR-195 was down-regulated in even well differentiated human thyroid cell lines; miR-195 level may thus be a useful diagnostic tool in thyroid neoplasms. The identification of miRNAs as anti-angiogenetic or tumour suppressor factors might help in finding new diagnostic markers for human cancers and possibly for anticancer therapy.
Statement of Originality

The material present in this thesis has not previously been submitted for a degree or diploma in any other university. To the best of my knowledge the presented material has not been published or written by another person.

Soussan Irani
Acknowledgements

This study was performed at the Griffith Health Institute (GHI) Laboratory and the pathology Department, School of Medicine, Griffith University.

My sincere thanks to go to my supervisors Professor Alfred lam, Dr. Robert Smith, and Dr. Ali Salajegheh for their guidance to finish my PhD. I would also like to acknowledge them for reviewing this thesis and for their great constructive criticisms.

I would like to thank all members of Pathology team and my dear colleagues for their scientific collaborations.

Finally, I would like to thank my family to support me emotionally, and special thanks to Farshd Kafi for his great support. Farshad, I really appreciate you for all days and years with your support and kind words.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>II</td>
</tr>
<tr>
<td>Statement of Originality</td>
<td>VIII</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>IX</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>X</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>XVI</td>
</tr>
<tr>
<td>List of Figures</td>
<td>XIX</td>
</tr>
<tr>
<td>List of Tables</td>
<td>XXIV</td>
</tr>
<tr>
<td>Publications Arising From This Thesis</td>
<td>XXV</td>
</tr>
<tr>
<td>Publications Tangential to This Thesis</td>
<td>XXV</td>
</tr>
<tr>
<td>Conference Presentations</td>
<td>XXV</td>
</tr>
<tr>
<td>Chapter 1: Principles: Aims</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Aims</td>
<td>2</td>
</tr>
<tr>
<td>1.2. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Chapter 2: Literature Review</td>
<td>9</td>
</tr>
<tr>
<td>2.1: Thyroid gland</td>
<td>10</td>
</tr>
<tr>
<td>2.1.1: Thyroid Gland Anatomy and Biology</td>
<td>10</td>
</tr>
<tr>
<td>2.1.2: Thyroid Cancer</td>
<td>10</td>
</tr>
<tr>
<td>2.1.3: Clinical Features of Papillary Thyroid Cancer</td>
<td>11</td>
</tr>
<tr>
<td>2.1.4: Pathological Features of Papillary Thyroid Cancer</td>
<td>12</td>
</tr>
<tr>
<td>2.2: Endothelin and its Role in Cancer</td>
<td>14</td>
</tr>
<tr>
<td>2.2.1. Endothelin Types</td>
<td>14</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.2.2. Endothelin Receptors</td>
<td>15</td>
</tr>
<tr>
<td>2.2.3. Molecular Biology Related to ET-1</td>
<td>17</td>
</tr>
<tr>
<td>2.2.4. ET-1 Functions</td>
<td>18</td>
</tr>
<tr>
<td>2.2.4.1. Endothelin’s Role in Carcinogenesis</td>
<td>18</td>
</tr>
<tr>
<td>2.2.4.2. Endothelin’s Role in Angiogenesis in Cancer</td>
<td>19</td>
</tr>
<tr>
<td>2.2.4.3. Endothelin’s Role in Lymph vessels</td>
<td>20</td>
</tr>
<tr>
<td>2.2.4.4. Endothelin’s Role in Development</td>
<td>21</td>
</tr>
<tr>
<td>2.2.4.5. Endothelin’s Role in Apoptosis</td>
<td>22</td>
</tr>
<tr>
<td>2.2.4.6. Endothelin, EMT, Invasion and Metastasis</td>
<td>22</td>
</tr>
<tr>
<td>2.2.4.7. Endothelin’s Role in Inflammation</td>
<td>24</td>
</tr>
<tr>
<td>2.2.4.8. Endothelin’s Role in Wound Healing</td>
<td>25</td>
</tr>
<tr>
<td>2.2.4.9. Endothelin Role in Nociceptive Response and Pain</td>
<td>25</td>
</tr>
<tr>
<td>2.2.4.10. The Role of Stroma in Carcinogenesis Related to Endothelin</td>
<td>26</td>
</tr>
<tr>
<td>2.2.4.11. Endothelins and Bone Formation</td>
<td>28</td>
</tr>
<tr>
<td>2.2.4.12. The ET-1 Axis in Chemo-resistance</td>
<td>29</td>
</tr>
<tr>
<td>2.2.4.13. Endothelin and Cancer Therapeutics</td>
<td>29</td>
</tr>
<tr>
<td>2.2.5. Endothelin Presence in Different Tissues and Diseases</td>
<td>35</td>
</tr>
<tr>
<td>2.2.5.1. Salivary Glands</td>
<td>35</td>
</tr>
<tr>
<td>2.2.5.2. Tooth</td>
<td>36</td>
</tr>
<tr>
<td>2.2.5.3. Brain</td>
<td>36</td>
</tr>
<tr>
<td>2.2.5.4. Lung</td>
<td>37</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>2.2.5.5. Vascular System and Heart</td>
<td>37</td>
</tr>
<tr>
<td>2.2.5.6. Adrenal Glands</td>
<td>38</td>
</tr>
<tr>
<td>2.2.5.7. Myometrium</td>
<td>38</td>
</tr>
<tr>
<td>2.2.5.8. Autoimmune Diseases</td>
<td>38</td>
</tr>
<tr>
<td>2.2.5.9. Atherosclerosis</td>
<td>39</td>
</tr>
<tr>
<td>2.2.5.10. Pancreatic Disease</td>
<td>40</td>
</tr>
<tr>
<td>2.2.5.11. Diabetes</td>
<td>40</td>
</tr>
<tr>
<td>2.2.5.12. Renal Disease</td>
<td>40</td>
</tr>
<tr>
<td>2.2.6. Endothelin Conclusions</td>
<td>40</td>
</tr>
<tr>
<td>2.3. miRNA</td>
<td>42</td>
</tr>
<tr>
<td>Chapter 3: ET Axis Study</td>
<td>46</td>
</tr>
<tr>
<td>3.1. Methodology</td>
<td>47</td>
</tr>
<tr>
<td>3.1.1: Patients and Tissue Samples</td>
<td>47</td>
</tr>
<tr>
<td>3.1.2: Haematoxylin and Eosin (H&amp;E) Staining Protocol</td>
<td>48</td>
</tr>
<tr>
<td>3.1.3: RNA Extraction</td>
<td>49</td>
</tr>
<tr>
<td>3.1.4: cDNA Preparation</td>
<td>53</td>
</tr>
<tr>
<td>3.1.5: PCR Primer Design</td>
<td>55</td>
</tr>
<tr>
<td>3.1.6: Real-time Polymerase Chain Reaction</td>
<td>57</td>
</tr>
<tr>
<td>3.1.7: Generation of a Standard Curve to Assess the Quality of the RT-PCR by Using Universal Human Reference RNA (UHRR)</td>
<td>61</td>
</tr>
<tr>
<td>3.1.8: Agarose Electrophoresis</td>
<td>64</td>
</tr>
<tr>
<td>3.1.9: Tissue Microarray</td>
<td>67</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.1.10: Immunohistochemistry Staining for ET-1 and ET&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>69</td>
</tr>
<tr>
<td>3.1.11: Data Analysis</td>
<td>86</td>
</tr>
<tr>
<td>3.2: Results</td>
<td>87</td>
</tr>
<tr>
<td>3.2.1: Detection of ET Axis mRNAs by RT-PCR in Primary Thyroid Carcinomas</td>
<td>88</td>
</tr>
<tr>
<td>3.2.2: Comparison Between the Expression in Primary Cancer and the Matched Lymph Node with Metastatic Cancer</td>
<td>91</td>
</tr>
<tr>
<td>3.2.3: Immunohistochemical Analysis of ET-1, and ET&lt;sub&gt;A&lt;/sub&gt;R Protein Expression</td>
<td>120</td>
</tr>
<tr>
<td>3.3: Discussion</td>
<td>121</td>
</tr>
<tr>
<td>3.4. Conclusions for ET-1 Study</td>
<td>125</td>
</tr>
<tr>
<td><strong>Chapter 4: miRNA-195 study</strong></td>
<td>126</td>
</tr>
<tr>
<td>4.1: Methodology</td>
<td>127</td>
</tr>
<tr>
<td>4.1.1: miR-195 Primer Design</td>
<td>127</td>
</tr>
<tr>
<td>4.1.2: RT-PCR</td>
<td>128</td>
</tr>
<tr>
<td>4.1.3: Data Analysis</td>
<td>129</td>
</tr>
<tr>
<td>4.1.4: Non-PCR Based Methods</td>
<td>130</td>
</tr>
<tr>
<td>4.1.4.1: Cell Culture</td>
<td>130</td>
</tr>
<tr>
<td>4.1.4.2: Cell Reviving Protocol</td>
<td>131</td>
</tr>
<tr>
<td>4.1.4.3: Cell Splitting Protocol</td>
<td>133</td>
</tr>
<tr>
<td>4.1.4.4: Protocol for Freezing the Cells</td>
<td>133</td>
</tr>
<tr>
<td>4.1.5: mRNA and miRNA Extraction From Cell Lines</td>
<td>134</td>
</tr>
<tr>
<td>4.1.6: Micro RNA Transfection</td>
<td>137</td>
</tr>
</tbody>
</table>
4.1.6.1: Cell Transfection Protocol 137
4.1.6.2: Protein Extraction 138
4.1.6.3: Protein Assay 139
4.1.6.4: Western Blot Analysis 141
  4.1.6.4.1: Protocol for Western Blot 143
  4.1.6.4.2: Protocol for Preparing Coomassie Staining 147
  4.1.6.4.3: Protocol for Destaining of the Gel (Coomassie staining) 148
  4.1.6.5: Immunofluorescence Staining 148

4.2: Results 150
  4.2.1: Detection of miR-195 by RT-PCR in Primary Thyroid Carcinomas 150
  4.2.2: Comparison Between the Expression in Primary Cancer and the Matched Lymph Node with Metastatic Cancer 165
  4.2.3: Correlation Between miR-195 and ET-1 Axis Expression Levels in PTC 166
  4.2.4: Comparison Between the Expression in K1 and B-CPAP Cell Lines 166
  4.2.5: VEGF-A Immunofluorescent Staining Results 167
  4.2.6: Western Blot Results 168
    4.2.6.1: The Decreased Protein Levels 168
    4.2.6.2: The Unchanged Protein Levels 168
    4.2.6.3: Undetected Protein Levels 169
    4.2.6.4: VEGF-A and Bcl2 are Direct Targets of miR-195 169

4.3: Discussion 170
## Chapter 5: Conclusions and Further Studies

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1. Conclusion</td>
<td>178</td>
</tr>
<tr>
<td>5.2. Addressing Aim1</td>
<td>179</td>
</tr>
<tr>
<td>5.3: Addressing Aim 2</td>
<td>180</td>
</tr>
<tr>
<td>5.4: Future Directions</td>
<td>183</td>
</tr>
</tbody>
</table>

## References

| References               | 185  |

## Appendix

| Appendix                 | 205  |
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CAFs</td>
<td>cancer-associated fibroblasts</td>
</tr>
<tr>
<td>COX-1, 2</td>
<td>cyclooxygenase 1 and 2</td>
</tr>
<tr>
<td>CPTC</td>
<td>conventional papillary thyroid carcinomas</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ETs</td>
<td>endothelins</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>ECE</td>
<td>endothelin-converting enzymes</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDN1</td>
<td>ET-1 gene</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ET(_A)R</td>
<td>endothelin A receptor</td>
</tr>
<tr>
<td>ET(_B)R</td>
<td>endothelin B receptor</td>
</tr>
<tr>
<td>FVPTC</td>
<td>follicular variant of papillary thyroid carcinomas</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed, paraffin-embedded</td>
</tr>
<tr>
<td>HCC</td>
<td>human hepatocellular carcinoma</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HIF-1(\alpha)</td>
<td>hypoxia-inducible factor-1(\alpha)</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
</tbody>
</table>
IHC  immunohistochemistry
INF-γ  interferon-γ
MIP-1β  macrophage inflammatory protein-1b
MMPs  matrix metalloproteinase
MMP-1  matrix metalloproteinase 1
MMP-2  matrix metalloproteinase-2
miRNAs  MicroRNAs
NO  nitric oxide
OSCC  oral squamous cell carcinoma
PBS  phosphate-buffered saline
PDGF  platelet-derived growth factor
PMNs  polymorphonuclear leukocytes
PSC  Pancreatic stellate cells
PTC  Papillary Thyroid Cancer
PTEN  phosphate and tensin homolog
TAM  tumour-associated macrophages
3’ UTR  3’ untranslated region
TGF-β  transforming growth factor-β
T4  thyroxine
T3  triiodothyronine
TNF-α  tumour necrosis factor-α
TNM  tumour-node-metastasis
UICC  International Union against Cancer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHRR</td>
<td>Universal Human Reference RNA</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase type plasminogen activator</td>
</tr>
<tr>
<td>VECs</td>
<td>vascular endothelial cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Low power view of papillary thyroid carcinoma</td>
<td>12</td>
</tr>
<tr>
<td>2.2</td>
<td>Higher power of papillary thyroid carcinoma</td>
<td>13</td>
</tr>
<tr>
<td>3.1</td>
<td>The purity of RNA</td>
<td>52</td>
</tr>
<tr>
<td>3.2</td>
<td>The purity of cDNA</td>
<td>54</td>
</tr>
<tr>
<td>3.3</td>
<td>IQ Thermal Cycler</td>
<td>57</td>
</tr>
<tr>
<td>3.4</td>
<td>Melting Curves</td>
<td>59</td>
</tr>
<tr>
<td>3.5</td>
<td>Amplification Curves</td>
<td>60</td>
</tr>
<tr>
<td>3.6</td>
<td>Preparation process of a serial dilution of UHRR</td>
<td>62</td>
</tr>
<tr>
<td>3.7</td>
<td>Standard Curve for PCR Efficiency</td>
<td>63</td>
</tr>
<tr>
<td>3.8</td>
<td>The desired bands of ET-1 primers product with 121 base pairs</td>
<td>66</td>
</tr>
<tr>
<td>3.9</td>
<td>The desired bands for ETAR primers product with 76 base pairs</td>
<td>66</td>
</tr>
<tr>
<td>3.10</td>
<td>Tissue Microarrayer Model TMA Galileo CK3500</td>
<td>67</td>
</tr>
<tr>
<td>3.11</td>
<td>A Tissue microarray recipient block</td>
<td>68</td>
</tr>
<tr>
<td>3.12</td>
<td>Rapid Multifunctional Microwave Tissue Processor Model KOS</td>
<td>71</td>
</tr>
<tr>
<td>3.13</td>
<td>IntelliPATH FLX autostainer</td>
<td></td>
</tr>
<tr>
<td>3.14</td>
<td>High magnification shows cytoplasmic ET-1 protein expression in control</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>tissue (placenta)</td>
<td></td>
</tr>
<tr>
<td>3.15</td>
<td>High magnification shows cell membrane ETAR protein expression in control</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>tissue (placenta)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.16: High magnification shows cytoplasmic ET-1 protein expression in normal thyroid tissue  

Figure 3.17: High magnification of ET\textsubscript{A}R protein expression in normal thyroid tissue  

Figure 3.18: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.19: Low and High magnifications, ET\textsubscript{A}R Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.20: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.21: Low and High magnifications, ET\textsubscript{A}R Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.22: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.23: Low and High magnifications, ET\textsubscript{A}R Immunostain of Papillary Thyroid Cancer Tissue  

Figure 3.24: Low magnification shows ET-1 protein expression in Metastatic Papillary Thyroid Carcinoma in the lymph node  

Figure 3.25: Low magnification shows ET\textsubscript{A}R protein expression in Metastatic Papillary Thyroid Carcinoma in the lymph node  

Figure 3.26: ET-1 expression levels in different gender groups  

Figure 3.27: ET-1 expression level in different age groups
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.28</td>
<td>ET-1 expression level in different tumour sizes</td>
<td>96</td>
</tr>
<tr>
<td>3.29</td>
<td>ET-1 expression level in different T staging</td>
<td>97</td>
</tr>
<tr>
<td>3.30</td>
<td>ET-1 expression level and its correlation with metastasis to regional lymph nodes</td>
<td>98</td>
</tr>
<tr>
<td>3.31</td>
<td>ET-1 expression level in different TNM staging</td>
<td>99</td>
</tr>
<tr>
<td>3.32</td>
<td>ET-1 expression level in different Pathological Variants</td>
<td>100</td>
</tr>
<tr>
<td>3.33</td>
<td>ET-1 expression level regarding Psammoma body</td>
<td>101</td>
</tr>
<tr>
<td>3.34</td>
<td>ET-1 expression level regarding Calcification in stroma</td>
<td>102</td>
</tr>
<tr>
<td>3.35</td>
<td>ET-1 expression level in the presence of Osseous metaplasia in stroma</td>
<td>103</td>
</tr>
<tr>
<td>3.36</td>
<td>ET-1 expression level regarding Lymphocytic thyroiditis</td>
<td>104</td>
</tr>
<tr>
<td>3.37</td>
<td>ET$_A$R expression levels in different gender groups</td>
<td>107</td>
</tr>
<tr>
<td>3.38</td>
<td>ET$_A$R expression level in different age groups</td>
<td>108</td>
</tr>
<tr>
<td>3.39</td>
<td>ET$_A$R expression level in different tumour sizes</td>
<td>109</td>
</tr>
<tr>
<td>3.40</td>
<td>ET$_A$R expression level in different T staging</td>
<td>110</td>
</tr>
<tr>
<td>3.41</td>
<td>ET$_A$R expression level and its correlation with regional lymph node metastasis</td>
<td>111</td>
</tr>
<tr>
<td>3.42</td>
<td>ET$_A$R expression level in different TNM staging</td>
<td>112</td>
</tr>
<tr>
<td>3.43</td>
<td>ET$_A$R expression level in different Pathological Variants</td>
<td>113</td>
</tr>
<tr>
<td>3.44</td>
<td>ET$_A$R expression level regarding Psammoma body</td>
<td>114</td>
</tr>
<tr>
<td>3.45</td>
<td>ET$_A$R expression level regarding Calcification in stroma</td>
<td>115</td>
</tr>
<tr>
<td>3.46</td>
<td>ET$_A$R expression level regarding Osseous metaplasia in stroma</td>
<td>116</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.46</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt;R expression level regarding Lymphocytic thyroiditis</td>
<td>117</td>
</tr>
<tr>
<td>3.48</td>
<td>Expression of ET-1 in thyroid cancer with lymph node metastasis and</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>without lymph node metastasis and in metastatic lymph nodes</td>
<td></td>
</tr>
<tr>
<td>3.49</td>
<td>Expression of ET&lt;sub&gt;A&lt;/sub&gt;R in thyroid cancer with lymph node metastasis and</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>without lymph node metastasis and in metastatic lymph nodes</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Nthy-ori3-1 cell line with about 60% confluency</td>
<td>132</td>
</tr>
<tr>
<td>4.2</td>
<td>B-CPAP cell line with about 60% confluency</td>
<td>132</td>
</tr>
<tr>
<td>4.3</td>
<td>Standard Curve for BSA serial dilution</td>
<td>141</td>
</tr>
<tr>
<td>4.4</td>
<td>Staining with Ponceau S solution</td>
<td>145</td>
</tr>
<tr>
<td>4.5</td>
<td>Coomassie staining of the gel</td>
<td>147</td>
</tr>
<tr>
<td>4.5</td>
<td>miR-195 expression levels in gender groups</td>
<td>153</td>
</tr>
<tr>
<td>4.6</td>
<td>miR-195 expression level in different age groups</td>
<td>154</td>
</tr>
<tr>
<td>4.7</td>
<td>miR-195 expression levels in different tumor size</td>
<td>155</td>
</tr>
<tr>
<td>4.8</td>
<td>miR-195 expression level in different T staging</td>
<td>156</td>
</tr>
<tr>
<td>4.9</td>
<td>miR-195 expression level and its correlation with metastasis to regional lymph nodes</td>
<td>157</td>
</tr>
<tr>
<td>4.10</td>
<td>miR-195 expression levels in different TNM staging</td>
<td>158</td>
</tr>
<tr>
<td>4.11</td>
<td>miR-195 expression level in different Pathological Variants</td>
<td>159</td>
</tr>
<tr>
<td>4.12</td>
<td>miR-195 expression level regarding Psammoma body</td>
<td>160</td>
</tr>
<tr>
<td>4.13</td>
<td>miR-195 expression level regarding the presence of Calcification in stroma</td>
<td>161</td>
</tr>
<tr>
<td>4.14</td>
<td>miR-195 expression level regarding Osseous metaplasia in stroma</td>
<td>162</td>
</tr>
<tr>
<td>Figure 4.15: miR-195 expression level regarding Lymphocytic thyroiditis</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>Figure 4.16: miR-195 expression levels in thyroid cancers without metastasis and metastatic lymph nodes</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Figure 4.17: VEGF-A Immunofluorescent staining</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Figure A: 1: VEGF-A Western Blot.</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Figure A.2: Bcl-2 Western Blot</td>
<td>206</td>
<td></td>
</tr>
<tr>
<td>Figure A: 3: p53 Western Blot</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td>Figure A.4: ET-1 Western Blot</td>
<td>208</td>
<td></td>
</tr>
<tr>
<td>Figure A:5: ETAR Western Blot</td>
<td>209</td>
<td></td>
</tr>
<tr>
<td>Figure A. 6: HIF-1α Western Blot</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Figure A.7: GAPDH Western Blot</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Figure A.8: β-Actin Western Blot</td>
<td>212</td>
<td></td>
</tr>
</tbody>
</table>
List of Tables

Table 2.1: Summary of ET receptors expression levels in different cancers .......................................................... 16
Table 2.2: Results of different types of ET antagonists in human cancer cell lines ............................................. 31
Table 2.3: The effects of endothelin system antagonists on xenograft models .................................................. 33
Table 3.1: PCR primer pairs for detection of ET-1 axis expression levels ......................................................... 56
Table 3.2: Details of ET-1 expression levels ......................................................................................................... 88
Table 3.3: Details of ETAR expression levels ..................................................................................................... 89
Table 3.4: The relationships between ET-1 mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas ................................................................. 92
Table 3.5: The correlation between ETAR mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas ................................................................................. 105
Table 4.1: The relationships between miR-195 expression and clinicopathological characteristics of 123 papillary thyroid carcinoma ...................................................................................... 151
Publications Arising from This Thesis


Publications Tangential to This Thesis


Conference Presentations

1. Neck masses: A retrospective study of 1208 cases. **Soussan Irani**, Vinod Gopalan, Robert Smith, Alfred Lam. Poster presentation in 2011 Gold Coast Health and Medical Research Conference, Australia

2. The correlation between ET-1 axis and miRNA-195 expression levels with clinicopathological characteristics of Papillary Thyroid Carcinoma. **Soussan Irani**, Ali Salajegheh, Robert Smith, Alfred Lam. Poster presentation in 2012 Gold Coast Health and Medical Research Conference, Australia


Chapter 1:

Principles: Aims
1.1: Aims

The endothelins (ETs) are a family of genes which affect vascular tone and angiogenesis. ET-1, the most common circulating form of endothelin, is produced by many epithelial tumours. It can stimulate proto-oncogene expression in vascular and non-vascular cells. ET-1 induces vascular endothelial growth factor (VEGF) expression by increasing hypoxia-inducible factor-1α (HIF-1α) stimulation.

In this study, we undertook interrogation of endothelin-1 (ET-1), its primary angiogenic receptor, endothelin A receptor (ET\textsubscript{A}R) and miRNA-195 at the RNA and protein levels in thyroid cancer.

This study was intended to provide a better understanding on the implications of these mechanisms for angiogenesis in cancer lesions as they are considered potential therapeutic targets for cancer treatment.

Also, we investigated the expression levels of ET-1, its receptor and miRNA-195 in metastatic lymph nodes. This was intended to provide insight for the degree of utilisation of the endothelin pathway in metastatic tissues.

These aspects were studied using a variety of molecular methods. PCR based procedures were the main methods to identify ET-1, its receptor and miRNA-195 expression in primary and metastatic tumours. Immunohistochemistry was used to confirm the presence of endothelin axis products and their expression levels in both primary and metastatic sites.
The main aims of this study were:

1. To determine the expression of endothelin 1, its receptor and miRNA-195 in a large population of thyroid cancer in both primary sites and metastatic lymph nodes.

2. To determine how the pattern of expression of these genes is related to the clinicopathological features of thyroid cancer, in particular cancer markers like aggression and metastasis.
1.2: Introduction

Papillary Thyroid Cancer (PTC) is the most commonly diagnosed thyroid cancer accounting for 80% of all cases. The overall prognosis is favourable with 5-year survival rates of 95%, but in some cases the tumour behaves in an aggressive manner characterized by local recurrence and/or metastasis (1, 2). Thyroid tumours represent a good model for study as they comprise a range of lesions with different degrees of malignancy (3, 4).

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is an essential but complex physiologic activity involved in normal embryo development, corpus luteum formation and wound healing. In addition, several pathologic conditions are associated with neovascularisation, for example, in cardiac failure (5, 6). Tumours beyond 1-2 mm in diameter require angiogenesis to grow (6). During carcinogenesis, due to more cell demand for oxygen and nutrients, tumour cells secrete pro-angiogenic factors to initiate angiogenesis. Angiogenesis also causes the mobilization of endothelial cells from pre-existing vessels. As a result of this influence on cell mobility, in addition to its capacity to improve nutrient and oxygen delivery to tumours, angiogenesis has a key role in tumour growth and metastasis (7-9). During local invasion, the tumour cells must attach to the basement membrane of the blood and lymphatic system and degrade it in order to migrate through the endothelium and disseminate. Following this, the tumour cells are typically transported by the lymphatic system to the regional lymph node. Inside the lymph nodes, tumour cells produce proteolytic enzymes which degrade the local basement membrane and underlying connective tissue, which facilitates the infiltration of metastatic tumour cells into surrounding tissues. In the new environment, tumour cells need to survive and proliferate, and may subsequently disseminate to other organs.
Neovascularisation has a critical role in the development of metastasis, delivering the resources necessary to enable localised proliferation (10). Studies have shown that VEGF has a pivotal role in the control of angiogenesis and impacts biological aggressiveness in thyroid cancers, though other pathways are also involved (11-15).

The endothelins (ETs) are a family of genes which induce DNA synthesis and cellular growth in different cells, affecting vascular tone and angiogenesis (16). ET-1 is the most common circulating form of endothelin and is produced by many epithelial tumours (17). It can stimulate proto-oncogene expression in vascular and non-vascular cells (18). ET-1 appears to have a direct effect on neoplastic cells by inducing cellular proliferation, migration as well as invasion and inhibition of apoptosis (19). ET-1 induces VEGF expression by increasing HIF-1α stimulation (20).

MicroRNAs (miRNAs) are endogenous small non-coding RNAs. miRNAs can regulate gene expression in the post–transcriptional stage by interacting with the 3′ untranslated region (3′ UTR) of target mRNA. miRNAs are involved in cell proliferation, development, differentiation and metabolism (21). A single miRNA can potentially target hundreds of mRNA transcripts, modulating the expression of multiple genes simultaneously (22). It is suggested that up-regulation of oncogenically associated miRNAs decreases tumour suppressor gene expression and similarly that down-regulation of other miRNAs allows increased oncogene translation. The role of miRNAs in the development of metastases has also been shown in a variety of cancers (23). About half of human miRNAs are located at chromosomal breakpoints and are therefore susceptible to dys-regulation in human cancer (24).
Some miRNAs function as an angiogenic switch in quiescent endothelial cells. Like the potential involvement of miRNAs in oncogenesis and tumour suppression, there are both pro-angiogenic miRNAs and anti-angiogenic miRNAs. Specific miRNAs have been shown to affect endothelial cell functions (25). In human umbilical vein endothelial cells, 27 miRNAs are highly expressed, most of which modulate expression of receptors for angiogenesis-regulating factors (26).

miRNA expression patterns depend on tissue and cell type (27). For example, in normal thyroid tissue the majority of miRNAs are expressed, but in thyroid tumours 32% of miRNAs are up-regulated, and 38% are down-regulated with more than a 2-fold change compared to normal tissue (28).

Previous investigations indicated that deregulation of specific miRNAs can cause human diseases such as cancer (29). miRNAs are divided into two main groups: if overexpression of a miRNA results in down-regulation of tumour suppressor genes, miRNAs are now commonly termed oncomirs, or if under-expression of a miRNA results in up-regulation of oncogenes, they are termed tumour suppressor miRNAs (30). The role of miRNAs in the development of metastases has also been shown in a variety of cancers (23). In addition, the prognostic value of miRNAs expression in different cancers has been demonstrated; for example, let-7 is correlated with prognosis in lung cancer and miR-10b is related to metastasis in breast cancer (31, 32). There is increasing evidence to show that such prognostically linked miRNAs function as metastatic suppressors, such as the let-7 family, miR-100, miR-126, miR-218, miR-335 (33). Similar functions relating to progression are also controlled by miRNAs. For example, the let-7 family are involved in chemo- and radio-sensitivity and the
development of poorly differentiated and aggressive cancers, in addition to their role in metastasis (34, 35).

Specific miRNAs are also involved in endothelial cells, where they function as an angiogenic switch in quiescent endothelial cells, an important process in cancer development (36, 37).

The miR-15/16 family are a group of miRNAs with the same seed sequence, including as follows: miR-15, -16, -103, -107, -195, -424, -497, -503 and -646. miR-15 and miR-16 are considered to be tumour suppressor miRNAs due to their targeting Bcl-2 (38). MiR-15 and miR-16 induce apoptosis of leukemic cells through Bcl-2 (39). On the other hand, miR-15b and miR-16 might be involved in the control of angiogenesis because both can control VEGF expression (40). The potential role of these miRNAs in angiogenesis is further indicated due to the observation that hypoxia reduces miR-15b and miR-16 expression levels, and resultant increases in VEGF expression level may be attributed to this miRNA loss. MicroRNA-195 has a pivotal role in tumourigenesis as a tumour suppressor. It is suggested that it promotes apoptosis, mainly through targeting Bcl-2 expression (38). In a study, miRNA-195 was found to be down-regulated in both colorectal cancer tissue and cell lines. The authors suggested that miR-195 plays a key role in the progression of colorectal cancer. It was also suggested that down-regulation of miR-195 may disrupt cell cycle control which promotes cell proliferation and progress of cancer (41). In another study, miR-195 was down-regulated in both human hepatocellular carcinoma (HCC) tissues and cell lines to suppress tumour growth (42). Mattie et al. determined that miR-195 was up-regulated in HER-2/neu breast cancer s (43). Recently, the regulatory role of miR-195 in angiogenesis and specifically its direct effect on VEGF-A has
been described (44). Despite few promising angiogenic regulatory potential in miRNA 195, the role of this miRNA is not clear in most malignancies including thyroid cancer.

As there is an established connection between angiogenesis and cancer development, the identification of the role played by ET-1 and its receptor and miRNA-195 in angiogenesis and tumour cell survival is an important approach in cancer therapy. It is essential to examine the ways in which this angiogenic pathway alters in cancer development, so that they can be explored for potential to serve as a target for manipulation or blockage in cancer patients. Improving our understanding of the role of miRNA-195 also presents a significant opportunity as they are potential diagnostic and therapeutic targets (45).
Chapter 2:

Literature Review
2.1: Thyroid Gland

2.1.1: Thyroid Gland Anatomy and Biology

The thyroid gland is an endocrine gland that secretes thyroid hormones, thyroxine (T4) and triiodothyronine (T3), which are essential for the development of different organs such as central nervous system, skeleton, skeletal muscle, intestine, heart and sensory organs. Both hormones are synthesised and secreted by the follicular cells (46). The human thyroid is a bilobed structure; the lobes are connected by an isthmus in the midline, just in front of the proximal trachea. The left and right lobes are close to the carotid arteries which are important for the positioning of the gland during the embryological development (47). The thyroid gland derives its blood supply mostly from the superior and inferior thyroid arteries (48). The lymphatic channels are paralleled by the veins and drain into the upper deep nodes of the cervical chain.

2.1.2: Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy, accounting 1.9 % of all newly diagnosed cancer cases. The aetiology of thyroid cancer is still poorly understood but some potential risk factors have been identified. These include radiation, smoking, alcohol, certain benign thyroid conditions and iodine deficiency (49). Thyroid cancer represents a good model for study of angiogenesis and cancer mechanisms as thyroid cancers are vascular and
comprise a range of lesions with different degrees of malignancy. There are a number of different subtypes of the disease with different morphology and behaviour, but papillary thyroid cancer is the most commonly diagnosed thyroid cancer, accounting for 80% of all cases (4). The overall prognosis is favourable with 10-year survival rates of over 90%, but in some cases (particularly in the rarer subtypes such as anaplastic thyroid cancer) the cancer behaves in an aggressive manner characterized by local recurrence and metastasis (2).

2.1.3: Clinical Features of Papillary Thyroid Cancer

Thyroid cancer comprises a variety of neoplasms with different clinical and pathological features. Thyroid cancer is more common in women (3:1 ratio) with a median age of diagnosis being 46 years (49).

Papillary Thyroid Cancer (PTC) is the most common histologic type. This tumour has a distinct propensity for multifocal involvement of the thyroid gland and regional lymph node metastases but most cases are associated with a good prognosis (50). Parapharyngeal metastasis is recognised as a pattern of dissemination of thyroid cancer (51).
2.1.4: Pathological Features of Papillary Thyroid Cancer

Papillary carcinoma is a well differentiated thyroid cancer, derived from follicular cells and characterized by presenting papillary and follicular structures and formation of papillae, overlapping cell nuclei that have a ground glass appearance and longitudinal grooves (50). An example of papillary thyroid cancer histology can be seen in Figures 2.1 & 2.2.

![Figure 2.1: Low power view of papillary thyroid carcinoma. This H&E stained slide shows the papillary appearance typical of these tumours (from Prof. Lam’s archive).]
Figure 2.2: High power view of papillary thyroid carcinoma. *This H&E stained slide shows enlarged nuclei, nuclear overlapping, and nuclear clearing (from Prof. Lam’s archive).*
2.2: Endothelin and its Role in Cancer

2.2.1: Endothelin Types

In 1980, Furchgott and Zawadzki discovered that endothelial cells can modulate vascular tone by releasing a vasodilator factor (52). This factor was identified as nitric oxide (53). In 1985, a new protein operating as a vasoconstrictor was discovered, thus identifying nitric oxide’s counterpart in the maintenance of vascular tone. This substance was isolated by Yanagisawa et al. and was called endothelin-1 (ET-1). Following initial isolation, the sequences of the gene and the peptide for endothelin were discovered (54). ET-1 is one of a family of peptides known as endothelins (ETs), composed of three 21 amino acid proteins, including ET-1, endothelin-2 (ET-2), and endothelin-3 (ET-3) (55). ETs derive from precursor proteins after cleavage by membrane bound metalloproteinase endothelin-converting enzymes (ECE) (56). Endothelin-1 (ET-1) is the predominant isoform in the family, with the other isoforms ET-2 and ET-3 being rarer (19, 57). In addition, ET-1 is the most common circulating form of ETs which are produced by some normal cells, including endothelial cells, vascular smooth muscle cells as well as many epithelial tumours (58, 59). ET-2 is also a vasoconstrictor (60) and can protect cells from hypoxia via the endothelin B receptor (ET_BR) (61). ET-3 has a weak constriction function, and promotes angiogenesis. ET-1 and ET-3 increase the expression level of VEGF (62, 63).
2.2.2: Endothelin Receptors

Endothelins are able to work in both paracrine and autocrine fashions, by binding to cell surface receptors, namely endothelin A receptor (ET\textsubscript{A}R) and endothelin B receptor (ET\textsubscript{B}R) (59). ET\textsubscript{A}R has more affinity for ET-1 and ET-2 than for ET-3 but ET\textsubscript{B}R has equal affinity to all three ET isoforms (64). Both receptors are included in the G-protein–coupled receptor super-family (65). ET\textsubscript{A}R is found on vascular smooth muscle cells while ET\textsubscript{B}R is expressed on both endothelial cells and vascular smooth muscle cells (66).

In cancers, there are different expression profiles of the endothelin receptors when compared with normal tissues. ET\textsubscript{A}R/ET\textsubscript{B}R expression ratios differ between cancer types. Regarding the expression level of endothelin receptors, cancers are divided into three categories: (1) cancers that express predominantly ET\textsubscript{A}R such as nasopharyngeal, thyroid, prostate, colon, pancreatic, gastric, renal and breast cancers (67-74); (2) cancers predominately expressing ET\textsubscript{B}R such as melanoma and brain tumours glioblastoma and astrocytoma) (75-77) and (3) cancers which express both ET\textsubscript{A}R and ET\textsubscript{B}R such as oral, lung, bladder, vulvar and ovarian cancers (78-82) (Table 2.1). ET-1 mediates mitogenic effects in a variety of epithelial tumours via ET\textsubscript{A}R (83) and in non-epithelial tumours such as melanoma by ET\textsubscript{B}R (73).
Table 2.1: Summary of ET receptors expression levels in different cancers

<table>
<thead>
<tr>
<th>Cancer type (Reference)</th>
<th>ET&lt;sub&gt;AR&lt;/sub&gt; expression</th>
<th>ET&lt;sub&gt;B&lt;/sub&gt;R expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasopharyngeal cancer (67)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Thyroid cancer (68)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Prostate cancer (83)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Colon cancer (70)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Pancreatic cancer (71)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Gastric cancer (72)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Renal cancer (73)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Breast cancer (74)</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Melanoma (75)</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>Glioblastoma (76)</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>Astrocytoma (77)</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>Oral cancer (78)</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Lung cancer (79)</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Bladder cancer (80)</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Vulvar cancer (81)</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Ovarian cancer (82)</td>
<td>increased</td>
<td>increased</td>
</tr>
</tbody>
</table>
2.2.3: Molecular Biology Related to ET-1

ET-1 activation is mostly associated with an increase in intracellular calcium concentration, which has a key role in vascular contraction and vascular smooth muscle cell proliferation (58, 84). It is suggested that the majority of ET-1 in the body is synthesized by endothelial cells but ET-1 is also synthesized and released by airway epithelial cells, macrophages and pulmonary vascular endothelial cells (85, 86). Tissue levels of ET-1 are higher than plasma levels and plasma ET-1 levels below the picomolar range may cause contraction (87). In addition, ET-1 has a short half-life in blood because it is rapidly taken up by pulmonary vessels (88, 89). ET-2 is mainly produced by intestine and the kidneys (90). ET-3 is found in neural tissue, intestinal and renal tubular epithelial cells (91).

ET production can be stimulated by hypoxia, shear stress, growth factors and cytokines with prostacycline and nitric oxide (NO) acting as inhibitors (92). Endothelins also induce the expression of cytokines in different human tissues (93, 94). Induction of ET-1 gene (EDN1) transcription by β-catenin has been reported in colon cancer. Endothelial cells highly express miR-125a and miR-125b, which are known to suppress ET-1 expression through targeting 3'-UTR region of pre-pro-ET-1 mRNA in vascular endothelial cells (VECs) (95). Signaling from the ET-1/ET_AR axis participates in several signal transduction pathways such as MAPK and PI3K dependent Akt to promote cell proliferation, escape from apoptosis, angiogenesis, EMT, invasion and metastasis (96). When balanced with stimulation of ET_BR, a variety of effects are generated in vessel walls.
Mature endothelin is produced after cleavage of the pre-pro-endothelin precursor resulting in big endothelin which is then converted to mature endothelin by the action of an endothelin-converting enzyme (ECE) (54). Three isoforms of ECE have been recognized, namely ECE-1, ECE-2 and ECE-3 (97). Among those, ECE-1 and ECE-2 are considered the most important (98). ECE-1 is localized in the plasma cell membrane; it processes pre-ETs both intracellularly and on the cell surface (99). ECE-2 is localized to the trans Golgi network and is expressed in neural tissues and endothelial cells (89, 98).

2.2.4: ET-1 Functions

ET-1 has many different functions in normal tissues as well as disease conditions which will be explained in detail in the following sections.

2.2.4.1: Endothelin’s Role in Carcinogenesis

ET-1 regulates multiple cancer related processes, and different mechanisms in both tumours and surrounding stromal cells (100). It acts as a mitogen through ET\textsubscript{A}R by stimulating DNA synthesis (101). ET-1 also stimulates endothelial cell growth, induces vascular smooth muscle cell and pericyte mitogenesis (102). In addition, stimulation of ET\textsubscript{A}R results in the mitogenic signalling in transfected cells (103, 104). The mitogenic activity of ETs is amplified by co-stimulation of other growth factors like basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF),
transforming growth factor-β (TGF-β) and IL-6 (105). The ET-1/ETAR axis also evidently has a role in cancer progression as its overexpression correlates with the advanced stages of cancers such as ovarian cancer (106-108).

2.2.4.2: Endothelin’s Role in Angiogenesis in Cancer

The tumour microenvironment is different from that of normal tissue. An important difference is that within a cancer, most regions are hypoxic (109). In addition, the blood supply in cancer has a different structure from the normal vessels (110). For example, vascular channels in cancer are often lined by cancer cells, mimicking a normal vessel, a state called “mimicry”. Alternatively, vessels may be lined by a mix of cancer cells and endothelial cells, which is termed “mosaicism” (111, 112). The cancer blood vessels are also highly permeable and prone to leaking (113). Cancers displaying vasculogenic mimicry proliferate rapidly and are more aggressive with higher risk of metastasis than those displaying mosaicism (114, 115). HIF-1α expression around cancer cell-lined vessels is common and may show that hypoxia is involved in inducing cancer cell-lined vessel formation (116, 117). HIF-1α also regulates vascular endothelial growth factor (VEGF) which has been indicated as an enhancer for formation of cancer cell lined vessels in vitro (117). In oral cancers, HIF-1α expression level was shown to be positively correlated with the aggressiveness of the cancer (118).

ET-1 activates the hypoxia-responsive pathway by inducing stabilization of HIF-1α under normoxic conditions. Then, HIF-1α up-regulates VEGF transcription and VEGF production can thus be amplified by ET-1 under normoxic or hypoxic conditions (20). Tumour
blood vessels then develop, though in a different way from normal vasculature, beyond simply the identity of the cells lining them. For example, these vessels do not have any smooth muscle layer or any innervation, consisting of a single layer of endothelial cells (119-121). The second function of ET-1 related to the process of angiogenesis, is mediated by induction of the mitogenesis of endothelial cells via ET\textsubscript{B}R and of vascular smooth muscle cells, fibroblasts and pericytes via ET\textsubscript{A}R. This leads to increasing VEGF expression in tumour cells via HIF-1\textalpha and cyclooxygenase 1 and 2 (COX-1, 2). In addition, ET-1 increases vascular permeability through VEGF in hypoxic states (122). ET-1 and VEGF have reciprocal stimulatory interactions to induce the proliferation of endothelial cells and vascular smooth muscle cells (103).

Endothelial cell proliferation, migration, invasion and tube formation modulate neovascularization in cancers (63,93, 123-126).

2.2.4.3: Endothelin’s Role in Lymph vessels

ET\textsubscript{A}R and ET\textsubscript{B}R are expressed in endothelium and on smooth muscle cells, respectively, to modulate lymphatic vascular tone (127). ET-1 stimulates lymphatic endothelial cells and vessels to grow and invade. ET-1 induces lymphatic endothelial cell emerging and migration via increasing of matrix metalloproteinase-2 (MMP-2) level. Lymphatic endothelial cells produce and secrete ET-1 which in turn, after binding to ET\textsubscript{B}R, induces lymphatic endothelial cell proliferation and invasion. ET-1 is thus a mitogen for lymph vessel endothelial cells via ET\textsubscript{B}R, so ET\textsubscript{B}R blockade impairs lymphangiogenesis. Similarly, as a result of this behaviour, ET\textsubscript{B}R expression in cancer is associated with lymphatic invasion (128).
2.2.4.4: Endothelin’s Role in Development

ET-1 plays an essential role in the development of the embryonic period, such as early trophoblast proliferation and invasion (129). High expression of $\text{ET}_A\text{R}$ has been found in the first trimester placenta which decreases progressively towards term (130). Endothelins have a variety of physiological functions including neural crest cell development and neurotransmission (106). ET-1 has a pivotal role in the differentiation and development of neural crest derived tissues. ET-1 knockout mice develop severe abnormalities in craniofacial tissues derived from the first pharyngeal arch (131). ET-1, $\text{ET}_A\text{R}$ and ECE-1 deficient animals get severe craniofacial malformations of neural crest-derived facial and throat structures, including cleft palate, microglossia and micrognatia and the abnormal fusion of hyoid bone to the base of the skull (132). $\text{ET}_B\text{R}$ is thought to participate in dilating the placental vascular bed to maintain feto-maternal blood flow (145). $\text{ET}_B\text{R}$ also has a critical role in the development of melanocytes in the embryonic period (133). $\text{ET}_B\text{R}$ activity results in the proliferation and migration of melanocytes precursors from the neural tube to their final locations (134). Additionally, human keratinocytes produce ETs which promote melanocytes activation as mitogens (135). Mutations in the $\text{ET}_B\text{R}$ gene give rise to Hirschsprung’s disease which is characterized by megacolon and abnormal skin pigmentation. During development, correct migration of the neural crest derived cells migration requires $\text{ET}_B\text{R}$ signalling (136, 137).
2.2.4.5: Endothelin’s Role in Apoptosis

The cell survival and resistance to apoptosis, is crucial for cancer cells to survive during the tumour growth and metastasis (138, 139). ET-1 inhibits apoptosis of endothelial cells via ET_BR as an autocrine/paracrine survival factor (140) whereas in vascular smooth muscle cells it suppresses apoptosis through ET_AR (141). ET-1 protects tumour cells from drug-induced apoptosis through a Bcl-2-dependant mechanism involving the activation of PI3K/Akt pathways (142).

2.2.4.6: Endothelin, EMT, Invasion and Metastasis

Epithelial mesenchymal transition (EMT), a cancer hallmark, involves losing epithelial markers such as E-cadherin, and gaining mesenchymal markers, like N-cadherin and vimentin (107). The blockage of signals and pathways involving in EMT development is critical for reverting EMT and the related biological effects such as drug sensitivity. The ET-1/ET_AR axis is one of these pathways, having several roles in cancer progression and its overexpression correlating with advanced cancer stages (106-108). As part of this association with advanced staging, the ET-1/ET_AR axis has also been implicated in promotion of EMT as well as cell proliferation, angiogenesis, escape from apoptosis, invasion and metastasis (96). For example, it has been shown that overexpression of ET_AR in nasopharyngeal carcinoma initiates tumour cells metastasis (67).

Low E-cadherin and high vimentin in primary head and neck squamous cell carcinoma was associated with a 100% metastasis rate compared to those primary carcinomas with
incomplete EMT markers, which were associated with a metastatic rate of 44% (143). ET-1 and ET-3 have been found to up-regulate N-cadherin in human melanoma cell lines, a factor which is commonly associated with loss of E-cadherin and EMT progression (59). The observed down-regulation of E-cadherin and associated catenin adhesion proteins in melanocytes and melanoma cells are likely connected to the same mechanisms.

In addition to their roles in vasculogenesis, both VEGF and HIF-1α are associated with EMT in some cancers. For example, this phenomenon was observed in cancer cell lines from tongue and hypopharynx (144). VEGF is also correlated with invasion depth and increased risk of metastasis to lymph nodes in patients with head and neck squamous cell carcinoma (145). Due to its role in stimulating both VEGF production and HIF-1α stabilization, this places the endothelin axis at a significant control point for advanced tumour characteristics. In addition, ET-1 induces invasion and metastasis via the action of matrix metalloproteinase (MMPs) and urokinase type plasminogen activator (uPA), as well as destruction of gap junctions and causing adherence of tumour cells to extracellular matrix (ECM) (63, 107, 125, 146). Overexpression of endothelins and their receptors in invasive cancers like breast cancer leads to cross-talk with cytokines, MMPs and tumour-associated macrophages (147, 148). MMPs and urokinase type plasminogen activator system are affected by ET-1/ET<sub>AR</sub> axis in metastasis (149).
2.2.4.7: Endothelin’s Role in Inflammation

ET-1 and ECE-1 are expressed on polymorphonuclear leukocytes (PMNs), macrophages and mast cells in human inflamed tissues (150, 151). These findings show that ET-1 participates in inflammatory reactions (152). Inflammatory cells and tumour-associated macrophages (TAM) play an essential role in tumour progression (153). For example, macrophages promote invasion in breast cancer through increasing both ET-1 and -2 and by overexpression of ET\textsubscript{A}R/ET\textsubscript{B}R (154). ET-1 activation leads to up-regulation of macrophage inflammatory protein-1b (MIP-1β) in monocytic cells (155). MIP-1β or CCL4 is a cytokine involved in regulation of signalling through PI3K, NADPH oxidase, p38 MAPK, JNK-1 and HIF-1α via ET\textsubscript{B}R (156). ET-1 also induces inflammatory reactions by leukocyte rolling and adherence to endothelial cells, by increasing adhesion molecule expression through ET\textsubscript{A}R. ET\textsubscript{B}R overexpression has also been found to decrease immune responses in tumours even in the presence of inflammation. This effect is likely realised by ET-1 suppressing T-cell adhesion to endothelial cells through ET\textsubscript{B}R even when the endothelial cells are exposed to TNF-α, an effect that is itself ablated by nitric oxide (79). Many previous studies have shown that a variety of human solid tumours are infiltrated by T-cells. Intraepithelial T-cells markedly improve survival (157). T-cells trafficking is controlled by endothelium related pathways. The vasculature of many tumours lacking intraepithelial T-cells show strong expression of ET\textsubscript{B}R, whereas the vasculature of many tumours with intraepithelial T-cells show weak or no ET\textsubscript{B}R expression (158). ET-2 is a chemoattractant for macrophages via ET\textsubscript{B}R \textit{in vitro} and \textit{in vivo} (148).
2.2.4.8: Endothelin’s Role in Wound Healing

There is documented evidence to focus on the importance of endothelins in wound healing (159). ET-1 has direct effects on cell growth, extracellular matrix protein synthesis and the inhibition of matrix degradation (160). In wound healing, transformation of fibroblasts to myofibroblasts which are a contractile phenotype of fibroblast, is a key process (161). ET-1 promotes the myofibroblast differentiation along with extracellular matrix (ECM) production and contraction (162).

2.2.4.9: Endothelin Role in Nociceptive Response and Pain

Pain is modulated by ET-1(163). ET-1 can alter the intensity of pain in oral squamous cell carcinoma (OSCC) and oral melanoma (164). ET-1 is also synthesized by neurons and glial cells in the central and peripheral nervous system, indicating that ET-1 mediates nociception in non-cancerous and cancerous conditions (165, 166) (167, 168). ET-1 causes pain in humans by activation and sensitization of C nociceptors (169, 170). Overexpression of ET-1 has been reported in inflammatory reactions due to tumour necrosis factor-α (TNF-α) and interferon-γ (INF-γ) (171). ET-1 activates the pro-inflammatory factors in human monocytes and stimulates inflammatory cytokine production (172). Most of the pro-nociceptive actions of endothelins are mediated by ET_{A}R, however, local cutaneous injection of ET-1 causes pain and excitation of nociceptors through ET_{B}R, so both receptors are involved in the process (173) (174). ET_{A}R is expressed on sensory neurons and vascular smooth muscle cells but ET_{B}R is expressed on non-myelinating Schwann cells, endothelial cells, smooth muscle cells and
macrophages, indicating tissue specific effects (64, 175-177). Administration of ET receptors antagonists can be effective in pain relief (168). ETₐR antagonists have been found to improve analgesia in patients with end stage cancers (178).

2.2.4.10: The Role of Stroma in Carcinogenesis Related to Endothelin

Cancer progression depends on the biological characteristics of the malignant cells and their interactions with the benign cells and components of the surrounding stroma (179). Alterations influence adjacent stroma to have altered environment providing factors for growth (180, 181). Solid tumours are mixture of variety of cells; including cancer cells and cancer-associated cells (fibroblasts, macrophages and endothelial cells). All these cells can overexpress ET-1 and its receptors (96, 182).

The endothelin axis, likewise, influences not only tumour biology, but also plays a role in the ongoing processes of the tumour stroma. ET-1 stimulates collagen synthesis and is known to reduce the expression of matrix metalloproteinase 1 (MMP-1), a matrix degradation enzyme, in fibroblasts (183, 184). Fibroblasts normally suppress pre-neoplastic epithelial proliferation (185). They are, however, the first cells that are recruited during early carcinogenesis and can be induced to secrete many factors that affect cancer cells, which can result in increased aggressiveness (186). When they accumulate within the cancer area, they are termed cancer-associated fibroblasts (CAFs), becoming recruited by cancer signalling and have different morphological and functional features from their normal counterparts (187). CAFs have higher proliferative rates and are resistant to apoptosis (188). CAFs are made up of
different cells from various sources and local stromal fibroblasts are the major source (189).

Once recruited, fibroblasts may differentiate to myofibroblasts. This process is called fibroblast to myofibroblast trans-differentiation, and is activated by several growth factors such as TGF-β, PDGF, and bFGF which are produced by cancer cells (190, 191). CAFs have an essential role in head and neck squamous cell carcinoma (HNSCC) progression (192). The presence of myofibroblasts in the tumour stroma is a negative prognostic factor as they increase invasion of cancer cells. These myofibroblasts have a contractile phenotype and increase the proliferative and migratory capacity in cancer. For example, in oral squamous cell carcinoma (OSCC), the presence of myofibroblasts in the tumour stroma is associated with a higher risk of invasion and poor prognosis (193). The behaviour of CAFs is also modulated in part by endothelins (194).

In the oral cavity, fibroblasts can amplify the proliferation and migration of cancer cells stimulated by ET-1. In this process, ET-1 stimulates the release of bioactive ligands such as ADAM17 from fibroblasts which bind to epidermal growth factor receptor (EGFR) on cancer cells and then trigger an increase in COX-2 expression (194). It was shown that in HNSCC, the expression of COX-2 and EGFR is correlated with a poor prognosis (195). These data suggest that different mitogenic peptides may contribute to HNSCC progression and this could be evidence for stromal-epithelial interaction (195, 196). EGFR was also noted to be overexpressed in HNSCC patients in another study (197). Additionally, it was shown that activation of EGFR increases cancer cell migration and invasion due to decreasing cell adhesion (198).
ET-1 stimulates oral cancer cell invasion via both of its receptors, which are expressed by oral fibroblasts (194, 199). Oral fibroblast contractile phenotype is mediated primarily by ETₐR, however ET-1 stimulates oral fibroblasts to promote invasion of oral cancer cells in a paracrine manner (193).

2.2.4.11: Endothelins and Bone Formation

ET-1 is a mitogen for osteoblasts which express ETₐR in high density to inhibit osteoclast activity (200). ET-1 stimulates new bone formation which can be blocked by ETₐR antagonists, but not ETₐR antagonists (201). In the late stage of prostate cancer and breast cancer, osteoblastic activity is stimulated by factors secreted by cancer cells resulting in osteoblastic metastases, leading to the development of drugs targeting this process (202). A study on prostate cancer cell lines showed that the presence of both ET-1 and an anti-ET-1 antibody blocked osteoclastic bone resorption (203). Osteoblasts have a high affinity for ETₐR and ET-1 (204, 205). In animal studies, selective ETₐR antagonist treatment decreased new bone formation (206, 207). Additionally, atrasentan, an ETₐR antagonist, prolonged the time to the progression threshold levels of bone alkaline phosphatase; a biomarker of disease progression, by nearly twice compared with placebo in prostate cancer patients (505 days versus 254 days), though it did not reduce overall risk of progression (200).
2.2.4.12: The ET-1 Axis in Chemo-resistance

Drug resistance is a major therapeutic barrier in cancer management. Several lines of evidence show an association between the EMT phenotype and chemo-resistance in cancer cells (96). ET-1/ET\(_A\)R signalling has a key role in promoting EMT through regulating the interactions of cancer and microenvironment (208). ET-1/ET\(_A\)R knockdown, for example, reverts EMT phenotype, inhibits invasive behaviour, and increases the effects of chemotherapeutic agents (208). Therefore, it is suggested that ET\(_A\)R mediated EMT signalling in cancer cells occurs during resistance development. In support of this hypothesis, immunohistochemical analysis of human ovarian cancer tissues showed ET\(_A\)R overexpression in the resistant tumours, suggesting ET\(_A\)R expression level as a predictor of chemo-resistance in cancer therapies. In addition, hypoxia has a critical role in acquisition of the EMT phenotype and chemo-resistance onset. It is hypothesized that the stimulation of the ET-1/ET\(_A\)R pathway may mimic a hypoxic environment via HIF-1\(\alpha\) activation. On the other hand, ET-1 is itself targeted by the HIF-1\(\alpha\) gene, suggesting the presence of a feedback loop in the system. Together, these evidences suggest that the ET-1 axis contributes in signalling pathways triggering EMT, resulting in increased chemo-resistance in cancer (209).

2.2.4.13: Endothelin and Cancer Therapeutics

Since endothelin mediated pathways contribute to cancer growth and progression, angiogenesis and metastasis in a variety of cancers, they are thus possible targets for cancer therapeutics. Several drugs are available, derived from both cancer research and the targeting
of the Endothelin axis for treatment of cardiovascular disease. The ET axis can be targeted by several approaches in cancer, such as endothelin-converting enzyme (ECE) inhibition and antagonism of ET\textsubscript{A} and ET\textsubscript{B} receptors (106). Due to endothelin based effects on bone growth, ET\textsubscript{A}R antagonists have been suggested for the treatment of metastatic tumours in bone as discussed above (210). Endothelin A receptor antagonists can reduce angiogenesis by inhibiting endothelial cell mitogenesis. They might also have anti-apoptotic effects for endothelial cells by preventing the production of MMPs by macrophages (8). In normal cells, ET\textsubscript{B}R regulates ET-1/ET\textsubscript{A}R axis activities via mechanisms like increasing the production of nitric oxide, promoting ET-1 clearance, triggering apoptosis and blocking cell growth, but the same antagonistic effects are not shown to occur in tumour cells (211). In some cancers, such as breast cancer, ET\textsubscript{B}R initiates invasion but in other cancers like prostate cancer, ET\textsubscript{B}R absence results in ET-1 increasing due to reduced endothelin clearance, thus implying that endothelin receptor targeting will need to be tailored to the needs of various cancer types (154, 212). Endothelin-converting enzyme (ECE) is another potential therapeutic target, since it is required for the generation of active ET-1 peptides but a significant consideration for the use of this approach is that different isoforms of ECE-1 might have opposing effects (213).

The effects of endothelin antagonists in human cancer cell lines have been tested in multiple studies, and a summary of these can be seen in Table 2.2.
Table 2.2: Results of different types of ET antagonists in human cancer cell lines

<table>
<thead>
<tr>
<th>Type of cell line (Reference)</th>
<th>Antagonist</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasopharyngeal carcinoma (67)</td>
<td>Atrasentan</td>
<td>growth inhibition by 38% and cell death</td>
</tr>
<tr>
<td>Breast adenocarcinoma (214)</td>
<td>Atrasentan</td>
<td>reduced cell invasion by 28%</td>
</tr>
<tr>
<td>Ovarian adenocarcinoma(215)</td>
<td>Atrasentan</td>
<td>significant increased apoptosis (15-17%)</td>
</tr>
<tr>
<td>Prostate adenocarcinoma(216)</td>
<td>Atrasentan</td>
<td>tumour cell death (18% - 60%)</td>
</tr>
<tr>
<td>Ovarian adenocarcinoma (217)</td>
<td>Zibotentan</td>
<td>significant increased apoptosis(13-39%)</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma (218)</td>
<td>BQ123</td>
<td>decreased cell proliferation</td>
</tr>
<tr>
<td>Lung adenocarcinoma (84)</td>
<td>BQ123</td>
<td>decreased cell proliferation</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma (78)</td>
<td>BQ123 and BQ78</td>
<td>decreased cell proliferation</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma (219)</td>
<td>BQ123 and BQ78</td>
<td>suppression of cell growth</td>
</tr>
<tr>
<td>Oesophageal SCC (219)</td>
<td>BQ123 and BQ788</td>
<td>suppression of cell growth</td>
</tr>
<tr>
<td>Kaposi’s sarcoma (220)</td>
<td>BQ123 and BQ788</td>
<td>blocking the conversion of MMPs to active form</td>
</tr>
<tr>
<td>Melanoma (221)</td>
<td>Bosentan</td>
<td>induced cell death</td>
</tr>
</tbody>
</table>
The most commonly used antagonist in such studies is atrasentan. Atrasentan (ABT-627) is an orally bioavailable non-peptide, small molecule competitive ET inhibitor acting via decreasing the binding affinity of ET\(_A\)R for ET-1 (84). Atrasentan inhibited cell proliferation and increased apoptosis in nasopharyngeal cancer cell lines (67). In addition, atrasentan was shown to reduce cell invasion in breast cancer cell lines (222). In ovarian cell lines, atrasentan was found to increase apoptosis and inhibit cell proliferation and VEGF expression (215). Furthermore, it was found to lead to cell death in prostatic cancer (216).

The second most widely used ET inhibitor used in oncology trials is zibotentan (ZD4054), an orally bioavailable ET\(_A\)R antagonist (209, 223). It is the only ET antagonist which targets ET\(_A\)R without decreasing ET\(_B\)R expression levels. Zibotentan has been found to inhibit cell proliferation and tumour growth in human ovarian carcinoma cell lines (224), but not in breast cancer cell lines (225). In addition, zibotentan was found to revert the EMT phenotype, inhibit invasiveness and restore drug sensitivity in ovarian cancer cells (96). It reduces angiogenesis and also can reduce ET-1-induced EGFR transactivation (226). Zibotentan combined with the anti-mitotic chemotherapy agents paclitaxel or docetaxel enhances apoptosis compared with either agent alone (227).

BQ123 is an ET\(_A\)R antagonist and BQ788 is selective ET\(_B\)R antagonist, which are often used in combination. Combination therapy with BQ123 and BQ788 were demonstrated to suppress growth of OSCC and oesophageal squamous cell carcinoma (219). In addition, these drugs in combination blocked the conversion of MMPs to active forms resulting in inhibition of cell invasiveness (220). On its own, BQ123 was found to inhibit the proliferation of colorectal and lung cancer cell lines (84, 218).
Bosentan is an endothelin A receptor antagonist, which inhibits ET-1 action through a combination of interference with both ET\textsubscript{A} and ET\textsubscript{B} receptors (228). It has been found to induce cell death in human melanoma cell lines (221).

These ET inhibitors have been shown to be effective treatments for cancers in several animal xenograft models, the results of which are summarised in Table 2.3.

### Table 2.3: The effects of endothelin system antagonists on xenograft models

<table>
<thead>
<tr>
<th>Type of cancer cell line</th>
<th>Antagonist</th>
<th>Effects in the animals with cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaposi’s sarcoma (220)</td>
<td>A-182086</td>
<td>Complete inhibition of cell growth</td>
</tr>
<tr>
<td>Ovarian carcinoma (215)</td>
<td>Atrasentan</td>
<td>65% inhibition of tumour growth</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma (67)</td>
<td>Atrasentan</td>
<td>58% decrease in tumour volume</td>
</tr>
<tr>
<td>Ovarian carcinoma (217)</td>
<td>Zibotentan</td>
<td>69% inhibition of tumour growth</td>
</tr>
<tr>
<td>Breast carcinoma (225)</td>
<td>Zibotentan</td>
<td>decreased tumour volume</td>
</tr>
<tr>
<td>Colon carcinoma (229)</td>
<td>BQ123</td>
<td>significant reduction in tumour load</td>
</tr>
</tbody>
</table>
In ovarian cancer, treatment with ET-1 antagonists such as atrasentan or zibotentan could inhibit 65% and 69% of the cancers in animals (215, 217). Mai et al. showed a 58% decrease in nasopharyngeal cancer volume by treating with atrasentan (67). Also, Asham et al. noted a significant reduction in large bowel cancer load after treatment with the ET system antagonist, BQ123 (229). In breast cancer, Smollich et al. showed that zibotentan significantly decreased tumour volume (225). In addition, Rosano et al. reported that they had achieved complete inhibition of the proliferation of Kaposi’s sarcoma xenografts by using A-182086, an ET₄/₅ receptor antagonist (220).

In some cancers, the combination of one endothelin A receptor antagonist with an anticancer drug has shown good results as a useful therapeutic strategy. For example, ET₄R antagonism can improve the efficacy of paclitaxel on breast cancer in rats (224). In in vitro studies, using ET₅R antagonists such as zibotentan combining with paclitaxel or the DNA crosslinking chemotherapy agent cisplatinum showed an increase in antitumour activity in ovarian cancer (227, 230). ET-1 may modulate drug resistance pathways during treatment with cytotoxic therapies via several mechanisms, though it is likely to be that abrogation of hypoxia is the main factor that can be blocked by ET₅R (222).

Despite the promising results in cell lines and animal studies, clinical trial results for endothelin based approaches have been disappointing. The bulk of clinical trials to date have taken place in prostate cancer. The 2 largest studies were based on 809 and 312 patients using atrasentan and zibotentan respectively (200, 231). In these studies, the treatment with endothelin antagonists showed no improvement in prognosis of patients with prostatic adenocarcinomas. A clinical trial was also performed in which bosentan was used to treat
metastatic melanoma. The result of this trial was also disappointing, with only 6 of the 32 patients with the disease being stabilized by the treatment (232). Thus, more clinical trials may be needed for assessing the usefulness of the endothelin based treatments.

Endothelin A receptor antagonists used in these trials have been noted to have some side effects. For example, liver toxicity was observed in 2-18% of the patients treated with bosentan (233). The most common side effects with atrasentan therapy were headache, rhinitis, and peripheral oedema (234).

The current anti-angiogenesis therapy focuses on the action of VEGF. The prognostic and regulatory roles of VEGF have been extensively studied (11-15). In fact, VEGF was shown to collaborate with ET-1 and ET_A_R in cancer angiogenesis (235-237). Combination therapies targeting both VEGF and ETs may be a potent control of angiogenesis.

2.2.5: Endothelin Presence in Different Tissues and Diseases

Endothelin systems can be found in many tissues both in normal and disease conditions as outlined in the following sections.

2.2.5.1: Salivary Glands

In a study on the effect of endothelin on submandibular glands in rats, it has been shown that ET_A_R is localized around the interlobular ducts and acini compatible with
myoepithelial cells location but ET\textsubscript{B}R can be found in capillaries. ET\textsubscript{A}R activation results in myoepithelial contraction so enhances cholinergic and adrenergic salivation (238).

\subsection*{2.2.5.2: Tooth}

ET-1 has effect on intracellular calcium concentrations by signalling ET\textsubscript{A}R. Calcium is required for morphogenesis of teeth. Expression of endothelin receptors at high concentrations has been shown during tooth development (239, 240). ET may be involved in the control of pulpal blood pressure and flow (240). ET-1 signalling in pulpal fibroblasts induces the formation of ECM and fibronectin. Fibronectin has an important role in the developing tooth root (241, 242).

\subsection*{2.2.5.3: Brain}

In the brain, endothelin modulates cardiorespiratory centres as well as contributing to the development of sympathetic neurons (106, 243). In brain tumours, ET-1 expression is associated with expression of transforming growth factor beta 1 (TGF-\beta1) and tumour vascularity (244).
2.2.5.4: Lung

In lungs, endothelin regulates bronchial tone (245). Endothelin also assists proliferation of pulmonary airway blood vessels and via this action, the endothelin system promotes the development of pulmonary hypertension (246).

2.2.5.5: Vascular System and Heart

ET-1 is synthesized by cardiomyocytes, cardiac fibroblasts, and heart endothelial cells. Both ET_A and ET_B receptors are found on cardiomyocytes and fibroblasts, but the density of ET_AR is more prominent in cardiomyocytes (247).

In the cardiovascular system, the activation of ET_AR results in vasoconstriction but ET_B receptor activation promotes vasodilatation (106). Endothelins regulate vasoconstriction by the activation of ET_AR but contribute to the development of vascular disease in the cases of hypertension and atherosclerosis (248, 249). ET-1 has direct vasoconstrictor effects on smooth muscle cells, and has indirect vasoconstrictor effects on other neurohormonal and endocrine factors. This mechanism is considered as part of the aetiology for essential hypertension (250). In the heart, endothelins contribute to the myocardial contractility (251).

During chronic congestive heart failure, endothelin has two roles. Firstly, ET-1 contracts pulmonary vessels and increase pulmonary vascular resistance. Secondly, ET-1 acts as a mitogen on vascular smooth muscle cells and fibroblasts to facilitate vascular remodelling.
The severity of symptoms in congestive heart failure is correlated with plasma levels of ET-1, so it appears to be a prognostic indicator in these patients (253, 254).

**2.2.5.6: Adrenal Glands**

Conversion of angiotensin I to angiotensin II in *in vitro* is induced by ET-1 which stimulates adrenal synthesis of epinephrine and aldosterone (255).

**2.2.5.7: Myometrium**

ET-1 stimulates the production of arachidonic acid resulting in the synthesis and releasing of prostaglandins which are myometrial contractile agonists (94). In addition, ET-1 promotes myometrial cell growth via ET₄R activation (256). Myometrial dysfunctions such as premature uterine contractions and uterine leiomyoma are associated with ET-1 axis stimulation (94).

**2.2.5.8: Autoimmune Diseases**

The immune system has a pivotal role in the pathogenesis of connective tissue diseases such as Sjögren syndrome, scleroderma and lupus erythematosus (257). In all these diseases, activation of endothelin system has been shown (258). Endothelin in this context is synthesized by immune system mediating cells like lymphocytes and leukocytes (259, 260).
2.2.5.9: Atherosclerosis

ET-1 plays a key role in the pathogenesis of atherosclerosis through pro-inflammatory, mitogenic and chemoattractant properties (261). ET-1 elevates locally in atherosclerotic plaques and in circulation and vascular tissue (262).

2.2.5.10: Pancreatic Disease

Endothelins have an essential role in fibrosis in different organs such as liver, heart, kidney and lung. They are also critical factors in fibrosis of pancreas. Pancreatic stellate cells (PSC) express ET-1. ET-1 stimulates contractility and migration of PSC which are two characteristic features of activated PSC. Also, stellate cells are the main sources of ECM proteins in diseases (263).

2.2.5.11: Diabetes

Elevated ET-1 has been found in the blood of diabetic patients. There is a positive correlation between plasma ET-1 level and micro-angiopathy in patients with type 2 diabetes (264).
2.2.5.12: Renal Disease

Endothelin system is expressed in kidney involving in the regulation of blood flow, water and sodium reabsorption, and acid-base balance (265). ET-1 activates the inflammatory NF-κB signalling cascade via the ET₄R in human proximal tubule epithelial cells (266).

2.2.6: Endothelin Conclusions

In conclusion, while the ET system has plays an important role in the control of angiogenesis, it is not the only pathway that is involved in the control of angiogenesis. As demonstrated by the modest results of clinical trials to date, additional strategies may be needed to properly utilise this target for cancer treatment. Moreover, endothelin A receptor antagonists may have different effects on different cells, depending on the interaction between the stroma and tumour cells in cancer progression, along with the potential action of cancer stem cells. The mechanism of action in the clinical setting may be different from those in xenograft models. In xenograft models, due to shortened duration of cancer development, it is possible that interactions between cancer cells and stroma and cancer stem cells may not occur. This might explain the better endothelin antagonist effect on cell lines and xenografts than in clinical trials. The association between ET-1/ET₄R axis in both cancer progression and resistance to chemotherapy or radiotherapy improves our understanding of the pathway and the mechanisms of drug resistance associated with it could be useful in cancer therapy. The combination of ET₄R antagonists with anti-cancer drugs has been shown to be relatively effective and can prevent EMT-related signaling. This allows both cancer cells and vasculature
to be targeted. This is an important consideration, as both are involved in cancer progression. The ability of cancer cells to find alternative growth signaling pathways also needs to be considered. Further refining of combined therapeutic strategies is necessary as we continue to explore the possibilities of the endothelin axis in cancer treatment.
MicroRNAs (miRNAs), endogenous small non-coding RNAs, can regulate gene expression in the post–transcriptional stage by interacting with the 3’ untranslated region (3’ UTR) of target mRNA (21). A single miRNA can target hundreds of mRNA simultaneously (22). The down-regulation of oncogenically associated miRNAs increases oncogene translation and similarly that up-regulation of other miRNAs allows tumour suppressor gene expression. miRNAs have a role in the development of metastases in a variety of cancers (23).

Previous studies showed that deregulation of specific miRNAs can cause human diseases such as cancer (29). miRNAs can be divided into two main groups: oncogenic miRNAs, oncomirs, and suppressor miRNAs (30). miRNA expression also can be used as a prognostic marker in different cancers; for example, let-7 is correlated with prognosis in lung cancer and miR-10b is related to metastasis in breast cancer (31, 32). miR-21 is one of the most frequently up-regulated miRNAs in cancer which promotes cell mortality and invasion as well as inducing metastasis by targeting phosphate and tensin homolog (PTEN), a tumour suppressor, via inhibiting cell invasion (267). In addition, miR-21 initiates invasion, intravasation, and metastasis by down-regulating Pdcd4 (268). Haung et al. also revealed that miR-373 and miR-520c promoted metastasis (269). The miR-127 expression levels were significantly up-regulated in lymph node metastasis of cervical cancers compared to normal cervical epithelium (270). In another study, the expression of miR-200 family was correlated with E-cadherin in ovarian cancer patients (271). During EMT, E-cadherin and cytokeratins
are down-regulated in apical and basolateral tight and adherens junctions and N-cadherin and vimentin, as mesenchymal molecules are up-regulated (272).

miRNAs can be used as a biomarker and diagnostic tools for cancer detection (273), as they distinguish cancer tissues from normal tissues and they can also classify poorly differentiated tumours (21, 273). miRNAs have a critical role in the growth of cancer cells \textit{in vitro} and \textit{in vivo} when overexpressed or inhibited. Therefore, it might be possible to regulate miRNA expression via exogenous miRNAs to regulate cancer formation. Additionally, it is suggested that miRNAs might be useful for cancer therapy (274). There are two possible approaches for miRNA use in cancer treatment; firstly through inhibition of oncogenic miRNAs, and secondly through replacement of under-expressed tumour suppressor miRNAs with either miRNA mimics or viral vector-encoded miRNAs (275).

Some miRNAs are involved in angiogenesis affecting quiescent endothelial cells. There are both pro-angiogenic miRNAs and anti-angiogenic miRNAs (25).

miR-15, and miR-16 function as tumour suppressor miRNAs by targeting Bcl-2 (38). In a previous study on lung cancer, it was found that the miR-15/16/195 family directly target cyclin D1 and CDK6 which induces cell cycle arrest (276). Additionally, miR-15b and miR-16 both can control VEGF expression showing a potential role in angiogenesis (40).

MicroRNA-195, a member of miR-15/16 family, has an essential role in tumourigenesis as a tumour suppressor by targeting Bcl-2 expression (38). On the other hand, miR-195 inhibits G1-cell transition, a function that Bcl-2 also performs (42, 277). In a study, it was shown that microRNA-195 played a tumour-suppressor role in human glioblastoma cells
by arresting cell cycle progression at the G1/S transition but did not induce apoptosis. In addition, CCND3 and E2F3 were significantly down-regulated by miR-195 in human glioblastoma cell line which regulated cellular proliferation and invasion. These findings indicate that miR-195 may function as a tumour suppressor in glioblastoma cells by modulating the signaling pathways controlling both cellular proliferation and invasion (38).

miR-195 has also been found to function as a tumour suppressor, inhibiting the migration and invasion of tumour cells in several other cancers such as osteosarcoma, gastric cancer, colorectal cancer and hepatocellular carcinoma (42, 278-280). MiR-195 may suppress the invasion of cancer cells by targeting CCND1 and/or RAF1 in breast cancer (281).

Additionally, the role of miR-195 has been identified in angiogenesis by targeting VEGF in hepatocellular carcinoma (44). As a result, miR-195 may modulate the ET-1 axis in cancer as the ET-1 axis has effects in both angiogenesis via VEGF and in proliferation through Bcl-2 (20, 142).

Previous studies have shown that miRNA-195 was down-regulated in colorectal cancer and HCC both in tissue and cell lines (41, 42). miR-195 was also down-regulated in nasopharyngeal carcinoma (282). Down-regulation of miR-195 in esophageal squamous cell carcinoma is associated with inhibition of tumour cell viability, migration, invasion and colony formation in vitro by targeting Cdc42 (283). Reduced expression of miR-195 is associated with poor prognosis of colon cancer (280). Despite the investigation of the RNA in several cancers, the role of miR-195 is not clear in thyroid cancer, particularly in PTC.
miRNAs are preserved in formalin–fixed paraffin embedded tissues probably due to their small size thus, can be easily detected in these samples (284-286). Therefore, in this study paraffin embedded tissues were used to assay miR-195 expression level in PTCs.
Chapter 3:
ET Axis Study
3.1: Methodology

To assess ET-1 axis’ role in papillary thyroid cancer, and its role in the development of clinicopathologic characteristics, first, ET-1 axis expression levels were observed in papillary thyroid cancer tissue samples by performing RT-PCR. Then, the results were compared with the ET-1 axis expression at the protein level by immunohistochemistry (IHC).

RT-PCR is a fast technique to detect a nucleic acid fragment and quantify the initial amounts in the reaction. In addition, as this technique can detect very small amounts of target nucleic acid, it offers sensitivity, along with specificity due to amplifying a specific sequence of nucleic acid (287). To do RT-PCR, the first step is the extraction of total RNA from formalin fixed paraffin embedded tissue samples.

3.1.1: Patients and Tissue Samples

Patients with papillary thyroid carcinomas were recruited from different collaborating hospitals in Australia. The histological sections from these carcinomas and their clinical/pathological data were reviewed by a pathologist (supervisor, Professor Alfred Lam) and original diagnoses were confirmed. The malignant thyroid tumours were classified with reference to the criteria defined by the World Health Organization classification of malignant tumours (288). The American Joint Committee on Cancer (AJCC)/International Union against Cancer (UICC) tumour-node-metastasis (TNM) staging system was used to stage the thyroid tumours (289).
In total, 123 matched thyroid papillary carcinomas including 79 conventional papillary thyroid carcinomas (CPTC) and 44 follicular variant of papillary thyroid carcinomas (FVPTC), 40 metastatic lymph nodes and 7 normal thyroid tissues were selected from archival formalin-fixed, paraffin-embedded (FFPE) tissue. Histologic sections (4 μm thick) were cut and stained with Haematoxylin and Eosin (H&E) and reviewed by the pathologist. All blocks containing over 90% tumour with < 10% stromal tissue contamination were selected.

3.1.2: Haematoxylin and Eosin (H&E) Staining Protocol

Haematoxylin and eosin staining of tissue sections was undertaken in order to identify tumour tissue and allow separation from surrounding non-tumour tissue. Staining was performed using the following protocol.

1. The paraffin blocks were cut 4 μm thick.

2. The sections were deparaffinised, using 2 washes of xylene, 10 minutes each.

3. Then, excess xylene was driven off from the sections in 2 washes of absolute alcohol, 5 minutes each.

4. Slides were then rehydrated in 95% alcohol for 2 minutes and 70% alcohol for 2 minutes, after which the sections were washed briefly in distilled water.

5. The sections were stained in haematoxylin solution for 8 minutes.
6. After washing in running tap water for 5 minutes, the sections were counter-stained in eosin solution for 30 seconds.

7. Dehydration of the sections was done through a wash of 95% alcohol, followed by 2 washes of absolute alcohol, 5 minutes each.

8. The sections were cleared in 2 washes of xylene, 5 minutes each, followed by mounting with xylene based mounting medium.

3.1.3: RNA Extraction

Total RNA was extracted from formalin fixed paraffin embedded tissue samples using Qiagen miRNeasy FFPE Kits (Qiagen Pty. Ltd., Hilden, NRW, Germany) as follows:

1. Tissue blocks sections were cut 6-10 μm (from each block 5-6 sections were typically enough). As the sample surface had been exposed to air, leading to oxidative degradation of RNA, the first 2–3 sections were discarded.

2. The sections immediately were placed in 2 ml micro-centrifuge tubes and were deparaffinised according to the manufacture protocol using deparaffinisation solution.

4. 240 μl Buffer PKD was added to the sections, and they were then mixed by vortexing, centrifuged for 1 min at 11,000 x g (10,000 rpm).

5. Then, 10 μl proteinase K was added and mixed gently by pipetting up and down.
6. After that, the mixture was incubated at 56°C for 15 min, then at 80°C for 15 min.

7. The lower, uncoloured phase was then transferred into a new 2 ml micro-centrifuge tube, incubated on ice for 3 min, and centrifuged for 15 min at 20,000 x g (13,500 rpm).

8. The supernatant was then transferred to a new micro-centrifuge tube as the pellet contained insoluble tissue debris, including cross-linked DNA.

9. Later, DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately 25 μl) and 10 μl DNase I stock solution were added, mixed by inverting the tube, and centrifuged briefly.

10. The mixture was then incubated at room temperature for 15 min.

11. Then, 500 μl Buffer RBC was added and mixed the lysate thoroughly.

12. In the next step, 1200 μl ethanol (100%) was added to the sample, and mixed well by pipetting.

13. Immediately, 700 μl of the sample, including any precipitate was transferred, to an miRNasy MinElute spin column placed in a 2 ml collection tube, and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). The flow-through was discarded.

14. Reusing the collection tube, the entire sample had been passed through the miRNasy MinElute spin column.

15. Then, 500 μl Buffer RPE was added to the miRNasy MinElute spin column, and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). The flow-through was discarded.
16. Reusing the collection tube, another 500 μl Buffer RPE was added to the miRNeasy MinElute spin column, and centrifuged for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The collection tube was discarded with the flow-through.

17. The miRNeasy MinElute spin column was then placed in a new 2 ml collection tube, and centrifuged at full speed with open lid for 5 min. The collection tube with the flow-through was discarded.

18. Finally, the miRNeasy MinElute spin column was placed in a new 1.5 ml collection tube, 30 μl RNase-free water was added directly to the spin column membrane, and centrifuged for 1 min at full speed to elute the RNA.

19. Total RNA quality was assessed by using a Bio-Rad Experion electrophoretogram instrument (Bio-Rad, Hercules, USA). The purity of all RNAs was then assessed by a nanodrop instrument (Bio-Rad, Hercules, CA, USA) (Figure 3.1).
Figure 3.1: The purity of RNA. This figure shows the absorption spectrum taken on a nanodrop spectrophotometer for a sample used in the research. The ratio of absorbance at 260 and 280 nm is higher than 2 and shows high purity of mRNA. The ratio of absorbance at 280 and 230 nm is a second measurement of mRNA purity which is higher than 2.2. Both ratios indicate that there was little contamination in this sample.
3.1.4: cDNA Preparation

RNA was converted to cDNA using miScript Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions as follows:

1. For each sample which was in a different concentration, a master mix was prepared to convert to cDNA. A master mix was prepared by adding 4µl miScript RT Buffer, 2 µl of miScript Nucleics Mix 2µl of miScript Reverse Transcriptase Mix and 1000 ng of template RNA. RNase –free water was added to make a total volume of 20µl. All steps were done on ice.

2. The mixture was then incubated in thermocycler as follows: first at 37ºC for 60 min and secondly at 95ºC for 5 min to inactivate the process.

Again, the purity of all cDNA samples was assessed by nanodrop instrument (Bio-Rad, Hercules, CA, USA). All mRNAs were diluted to get 30ng/µl to perform RT-PCR (Figure 3.2).
Figure 3.2: The purity of cDNA. This figure shows the absorption spectrum taken on a nanodrop spectrophotometer for a cDNA sample used in the research: The ratio of sample absorbance at 260 and 280 nm is higher than 2 showing the purity of cDNA. The ratio of 260 at 230 nm is also higher than 2 which represents the purity of cDNA.
Following production of cDNA from the target tissues, the expression of the genes in the endothelin pathway was interrogated using quantitative PCR. The protocol used for these PCRs is described in the following sections.

3.1.5: PCR Primer Design

Primers were designed for analysis of expression of ET-1, ET_A and GAPDH.

Primers were designed as follows:

1. Databases at the NCBI (National Center for Biotechnology Information) website were used to obtain information for primer design.

2. The Nucleotide database was used to obtain mRNA sequences for the target genes.

3. mRNA sequences for all isoforms were obtained. The information was checked to ensure that only sequences from Homo sapiens and the correct chromosome were selected.

4. Then the sequences of all isoforms were aligned using Clustal W2.

5. Aligned isoforms were selected to that the region used for primer design would amplify all known isoforms.

6. The pan-isoform region was selected and subject to a Human BLAT search to locate intron boundaries.
7. Then, primers were designed using Primer 3. To avoid detection of DNA contamination in the PCR, inclusion of an intron splice site in the amplicon was checked.

8. Finally, the most compatible sequence was selected. This was done using a BLAST search set to detect somewhat similar sequences, in order to eliminate primers likely to cause non-specific amplification.

Designed Primers for analysis of expression were ET-1 (GenBank accession number NM_001955.4), ETAR (GenBank accession number NM_001957.3) and GAPDH (GenBank accession number NM_002046) as the ubiquitous control gene (Table 3.1).

<table>
<thead>
<tr>
<th>Interested mRNA</th>
<th>Primers</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>Forward 5’-AGCCCTAGGTCCAAGAGAGC-3’ Reverse 5’-TTCTCTGCTTGCAAAAAATTC-3’</td>
<td>121 bp</td>
</tr>
<tr>
<td>ETAR</td>
<td>Forward 5’-TGGTGTGACTGCGATCTTC-3’ Reverse 5’-GCAATTCTCAAGCTGCAATC-3’</td>
<td>76 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5’-TGCACCACCAACTGCTTAGC-3’ Reverse 5’-GGCATGGACTGTGGTCATGAG-3’</td>
<td>87 bp</td>
</tr>
</tbody>
</table>
3.1.6: Real-time Polymerase Chain Reaction

1. The best temperature for annealing was determined (59°C) by running RT-PCR for ET-1, ETAR and GAPDH at different temperatures (between 58.5°C -60°C) and examining PCR products to ensure no non-specific bands were produced and that the maximum product was produced. This was determined by comparisons of 1% agarose gel electrophoresis for each set of PCR conditions. The best annealing temperature for all primers was 59.5°C.

2. RT-PCR was then performed for all 170 samples using ET-1, ETAR and GAPDH primers in an IQ Thermal Cycler (Bio-Rad, Hercules, CA, United States of America) (Figure 3.3). The total mixture contained iQ SYBR green supermix (Bio-Rad) and prepared amount of cDNA in addition to sets of each primer (forward and reverse), with the precise mixes outlined below. All samples were run in triplicate and accompanied by a non-template control. The annealing temperature for the primers was 59°C (Figures 3.4&3.5).

Figure 3.3: IQ Thermal Cycler.
Thermal cycling conditions included initial denaturation for 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 59°C, 15 seconds at 72°C and 30 seconds at 55°C.

For the ET-1 study, PCR was performed in a total volume of 20 μl reaction mixture containing 10 μl iQ SYBR green supermix (Bio-Rad), 2 μl of each 5 μmol/l primer, 6 μl of Template cDNA at 30ng/μl. In the last tube of each reaction, 6 μl of water was added as non-template control.

For the ETAR study, a total volume of 10μl reaction mixture was prepared for PCR as follow: 5μl of iQ SYBR green supermix (Bio-Rad), 1 μl of each 5 μmol/l primer, 2 μl of Template cDNA at 30ng/μl. In the last tube of each reaction, 2 μl of water was added as non-template control.

The GAPDH study for the ET-1 experiment, was performed in a total volume of 20 μl reaction mixture containing 10 μl iQ SYBR green supermix (Bio-Rad), 2 μl of each 5 μmol/l primer, 6 μl of Template cDNA at 30ng/μl. In the last tube of each reaction, 6 μl of water was added as non-template control.

For the ETAR study, the GAPDH study PCR was performed in a total volume of 10μl reaction mixture was prepared for PCR as follow: 5μl of iQ SYBR green supermix (Bio-Rad), 1 μl of each 5 μmol/l primer, 2 μl of Template cDNA at 30ng/μl. In the last tube of each reaction, 2 μl of water was added as non-template control.
Figure 3.4: Melting Curves: Clustering of ET-1 (top) and ET₃R (bottom) mRNA expression.

Melting curve was performed in IQ thermal cycler. Melting curves show a single peak at 77-78 °C with minimal non-specific amplification.
**Figure 3.5: Amplification Curve:** Amplification curves for ET-1 and ET\textsubscript{AR} mRNA expression.

Amplification curves show a broad range of ct values, illustrating the variety of expression status in tissues examined.
3.1.7: Generation of a Standard Curve to Assess the Quality of the RT-PCR

by Using Universal Human Reference RNA (UHRR)

Ct is a cycle at which fluorescence is statistically significant above background and is inversely proportional to the log of the initial copy number. It depends on the number of template at the initial phase, the more template, sand the fewer the number of cycles to reach the point where the fluorescence signal is detectable above background. Ct values are determined for a set of standard wells, containing known amounts of the template RNA, which generate a standard curve that can be used to assess the quality of the QRT-PCR assay.

Efficiency of PCR was evaluated by using a serial dilution of Universal Human Reference RNA (UHRR). UHRR which is composed of total RNA from 10 human cell lines is a reference for microarray gene-profiling experiments. The serial dilution was started from 128 ng/µl (Figure 5). The best working concentration was 30ng/µl.

This is how a set of standard template RNA was made and used in parallel to the RT-PCR reactions in this research. A known concentration of UHRR was purchased and for the ease of mathematical calculation, the concentration was adjusted to 128 ng/µl. Then, half the volume in the tube number one was transferred to the tube number 2 and diluted with equal amount of H2O (RNase free) to make a 64ng/ul. This process was continued until the 8th tube was made in 1ng/ul concentration (Figure 3.6).
Figure 3.6: Preparation process of a serial dilution of UHRR. In the first tube, the concentration was 128 ng/ul. After transferring of half of the first tube to the second tube, equal amount of H2O (RNase free) was added to make a 64ng/ul. This process was continued until the 8th tube was made in 1ng/ul concentration.

Figure 3.7 shows an example of the graphing of ct vs. [log] concentration data for the ET-1 PCR. The data shows reasonable linearity, with a correlation coefficient for the line of best fit of 0.918, indicating a consistent change over several concentrations. The efficiency of the PCR can be worked out using the line of fit from this graph by the formula $E = 10^{(-1/slope)}$, where a perfect efficiency is equal to 2, representing a doubling of material at each PCR cycle. All of the test PCRs showed reasonably comparable efficiencies, with values of 2.14, 2.6 and 2.66 for the ET-1, ETAR and GAPDH PCRs, respectively. The ETAR PCR was found to have a relatively narrow range of reliability however, operating linearly between 64 and 8ng/uL.
Figure 3.7: Standard Curve for PCR Efficiency. This graph shows the ct data obtained for PCR at different starting concentrations. Reasonable variability of the data can be seen in the correlation $R^2$ of 0.918. Efficiency of the reaction is determined by the slope of the line. Values for sample concentrations of 1ng/uL and 2ng/uL were not used, as these showed a failure of linearity, indicating that the PCR was less reliable in this region.
3.1.8: Agarose Electrophoresis

Furthermore, the final PCR products were separated on 2% agarose gels. This helped to identify and ensure the correct band had been amplified. Agarose gels were prepared using the following protocol.

To prepare 2% Agarose Gel:

1. One gram agarose was added to 50ml TAE buffer.

2. After gentle stirring, the mixture was placed in microwave for 45 seconds.

3. After complete melting, before coming to boil, the microwave was turned off and the agarose was allowed to cool.

4. For 50 ml of gel, 2.5 µl of Red Safe (Invitrogen, Melbourne, Australia) was added to make the bands visible.

5. Then, the Agarose was poured in the Gel tray.

6. A comb was placed in the gel. All bubbles were removed with a pipette tip.

7. After setting the gel at room temperature, the comb was removed carefully by pulling upward in a continuous motion.

8. On a piece of parafilm, 2 µl of loading dye (xylene cyanol and Bromide phenol) was placed for each sample. Then, 6µl of PCR mixture was added to each dye dot, and mixed by pipetting up and down.
9. All the mixtures were then loaded into the wells of gel. In the first well 10 µl of ladder was loaded.

10. Then, the gel was placed in the electrophoresis chamber, and TAE Buffer was added to cover the samples. The power supply was turned on and the gel was run at 90 volts for 25 minutes.

11. After that, power supply was turned off and the gel tray was removed from the electrophoresis tank.

12. Using a spatula, the gel was removed from the tray and placed on UV light plate.

13. The stained samples and ladder were seen under the UV light (Figures 3.8 & 3.9).
Figure 3.8: Endothelin PCR agarose gel. The desired bands of ET-1 primers product with 121 base pairs. The used ladder was 100 bp.

Figure 3.9: Endothelin receptor A PCR agarose gel. The desired bands for ETAR primers product with 76 base pairs. The used ladder was 50 bp.
3.1.9: Tissue Microarray

Tissue microarrays were constructed in order to allow rapid determination of gene expression at the protein level by assaying multiple tissue samples in a single staining experiment.

Tissue microarrays (TMA) were constructed using a Tissue Microarrayer Model TMA Galileo CK3500, (Integrated System Engineering Srl (ISE), Milano, Italy) (Figure 3.10).

Figure 3.10: Tissue Microarrayer Model TMA Galileo CK3500
1. All representative tumour donor blocks were cut for H & E staining to define the morphology and pathology of the representative regions.

2. From those regions, 3 cylindrical core tissue specimens from the tumour region (diameter=0.6 mm) were acquired and arrayed into a new recipient paraffin block (35 x 20mm $^2$) (Figure 3.11).

3. Then, sections of 4 µm were cut from the TMAs and processed for immunohistochemistry.

Figure 3.11: A Tissue microarray recipient block. This block was made using cores taken from 13 paraffin blocks. Every 3 dots represent 1 donor block.
3.1.10: Immunohistochemistry Staining for ET-1 and ET\textsubscript{A}R

Immunohistochemistry (IHC) is a diagnostic or research process to detect antigens such as proteins in cells of a tissue section by binding antibodies to antigens (290).

After optimisation, IHC was done as follows:

1. The sections were deparaffinised in 2 washes of xylene, 5 minutes each

2. Then, the sections were rehydrated in 2 washes of 100% ethanol for 3 minutes each, 95% and 70% alcohol for 3 minutes each and rinsed in distilled water.

3. In the next step, 0.01 M citrate buffer (pH=6) was prepared and pre-heated in microwave up to 50°C.

4. After immersing slides in the pre-heated citrate buffer the container was kept in a Rapid Multifunctional Microwave Tissue Processor Model KOS (Milestone, Sorisole, Bergamo, Italy)(Figure 3.12) to heat up to 80°C and was kept at this temperature for 20 minutes.
Figure 3.12: Rapid Multifunctional Microwave Tissue Processor Model KOS
5. After cooling and washing in phosphate-buffered saline (PBS), immunohistochemistry for ET-1 and ET$_A$R was performed in an IntelliPATH FLX autostainer (Biocare Medical, Concord, CA, USA)(Figure 3.13), providing more standardized results than manual staining techniques.

Figure 3.13: IntelliPATH FLX autostainer
For ET-1, slides were incubated with anti ET-1 mouse monoclonal antibody (ab2786) by Abcam (Cambridge, UK) at 1:180 dilution for one hour at room temperature. Anti ET\textsubscript{A}R rabbit polyclonal antibody (ab76259) by Abcam was used at a 1:180 dilution for one hour at room temperature. The positive control used was placenta tissue known to express ET-1 and ET\textsubscript{A}R (Figures 3.14 & 3.15). The omission of primary antibodies served as negative control.

6. After washing with PBS, the slides were incubated with secondary antibodies for 1 hour.

7. Followed by washing with PBS, the slides were incubated with 5\% DAB for 5 minutes.

8. Then, the slides were incubated with copper sulphate for 10 minutes.

9. After washing with PBS, the slides were incubated with Haematoxylin for 5 minutes.

10. The slides were then incubated with Scott's Bluing solution for 5 minutes, followed by washing with PBS.

11. Finally, the slides were mounted immediately using a xylene based mounting medium.

Normal thyroid tissue was also stained with both ET-1 and ET\textsubscript{A}R antibodies as another references (Figures 3.16& 3.17).
Figure 3.14: High magnification shows cytoplasmic ET-1 protein expression in control tissue (placenta). The placenta tissue is composed of villi cuts. Each vilus is covered by a trophoectoderm shell. Inner cells are called cytotrophoblasts and outer cells are named syncytiotrophoblasts (291). Medium staining (score +2) was observed in the cytoplasm of trophectoderm shells and endothelial cells.
Figure 3.15 High magnification shows cell membrane ET_{AR} protein expression in control tissue (placenta). This figure illustrates shows medium staining (score +2). The cell membranes of trophectoderm shells and cytotrophoblasts were stained.
Figure 3.16: High magnification shows cytoplasmic ET-1 protein expression in normal thyroid tissue. The cytoplasms of all normal thyroid follicle lining cells showed heavy staining (score +3). Some nuclei also showed staining as can be seen through brown overstaining instead of a blue haemotoxylin counterstain.
Figure 3.17: High magnification of ETₐR protein expression in normal thyroid tissue.

This slide illustrates the cell membranes of a few cells lining normal thyroid follicles developing light staining (score +1).
The cytoplasmic and nuclear (ET-1) and cell membrane (ET<sub>A</sub>R) immunostaining intensity of the tumour cells was categorised into 3 groups: weakly positive staining (score 1+) (Figures 3.18 & 3.19), moderately positive staining (score 2+) (Figures 3.20 & 3.21), and strongly positive staining (score 3+) (Figures 3.22 & 3.23). The final score was designated as low or high: score 1+ indicated low and score 2+ - 3+ indicated high. In addition, the metastatic thyroid cancer in lymph node was stained as well (Figures 3.24 & 3.25). Whole sections from randomly selected thyroid carcinomas were stained for immunohistochemistry to check the validity of the results obtained in TMA.
Figure 3.18: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue. This figure shows ET-1 cytoplasmic immunostaining with a weak positive staining (score +1).
Figure 3.19: Low and High magnifications, ET\textsubscript{A}R Immunostain of Papillary Thyroid Cancer Tissue. The figure shows cell membrane ET\textsubscript{A}R protein immunostaining with a weak positive staining (score +1).
Figure 3.20: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue. This figure shows ET-1 cytoplasmic immunostaining with a moderate positive staining (score +2).
Figure 3.21: Low and High magnifications, ET₄R Immunostain of Papillary Thyroid Cancer Tissue. The figure shows cell membrane ET₄R protein immunostaining with a moderate positive staining (score +2).
Figure 3.22: Low and High magnifications, ET-1 Immunostain of Papillary Thyroid Cancer Tissue. *This figure shows ET-1 cytoplasmic immunostaining with a strong positive staining (score +3).*
Figure 3.23: Low and High magnifications, ETAR Immunostain of Papillary Thyroid Cancer Tissue. The figure shows cell membrane ETAR protein immunostaining with a strong positive staining (score +3).
Figure 3.24: Low magnification shows ET-1 protein expression in Metastatic Papillary Thyroid Carcinoma in the lymph node. This figure illustrates the general staining pattern for endothelin in a papillary thyroid carcinoma. ET-1 cytoplasmic immunostaining with a strong positive staining (score +3) in all cancer cells are seen.
Figure 3.25: Low magnification shows $\text{ET}_A \text{R}$ protein expression in Metastatic Papillary Thyroid Carcinoma in the lymph node. This figure illustrates the general staining pattern for endothelin receptor A in a metastatic papillary thyroid carcinoma. All cancer cell membranes show $\text{ET}_A \text{R}$ protein immunostaining with a strong positive staining (score +3).
3.1.11: **Data Analysis**

Mean values for each triplicate in the PCR study were calculated. Expression of ET-1 and ET\(_A\)R was normalized in each sample using the ΔCt method as follows:

\[ \Delta C_t = C_{ET-1, \text{or} \, ET_{A}R} \text{[sample]} - C_{GAPDH} \text{[sample]} \]

After obtaining ΔCt values for ET-1 and ET\(_A\)R the fold changes in the target genes were calculated for each sample group using the 2-ΔΔCt method.

\[ \Delta \Delta C_t = (C_{ET-1} - C_{GAPDH}) \text{CANCER} - (C_{ET-1} - C_{GAPDH}) \text{NORMAL} \]

\[ \Delta \Delta C_t = (C_{ETAR} - C_{GAPDH}) \text{CANCER} - (C_{ETAR} - C_{GAPDH}) \text{NORMAL} \]

Ct ratios (=Mean Ct ET-1[sample]/Ct GAPDH [sample]) were also calculated to display results and for further analysis. In order to reorient changes of expression for intuitive results graphing, inverse rations (1/Ratio) were calculated which reflected actual behaviour of ET-1 and ET\(_A\)R expression.

Normalised final data was analysed using one-way (ANOVA) to determine if there was a significant difference of mRNA expression between thyroid tissue samples both in primary and metastatic sites.

All fold changes <0.5 were considered as low expression levels, all fold changes >0.5 <2 were considered as normal expression levels, and all fold changes >2 were considered as high expression levels (11, 14, 15).
Additional comparisons were made to determine whether there were significant differences in ET-1 and ET AR expression levels in thyroid cancer subgroups, including follicular variants, patient gender and age, tumour size, T category, staging and other clinicopathological characteristics. Correlation analysis was also done to determine whether the expression of ET-1 and ET AR mRNA was related to these kinds of variable.

All statistical analysis was performed using SPSS version 21.0. Significance threshold was taken at p<0.05.
3.2: Results

3.2.1: Detection of ET axis mRNAs by RT-PCR in Primary Thyroid Carcinomas

The mRNA expression of ET-1 and its receptor (ET$_A$R) was determined in all primary and metastatic papillary thyroid carcinomas as well as in non-cancer thyroid tissue. ET-1 mRNA expression level was elevated in 27% of the primary carcinomas (n=33) compared to the control tissues. The expression was low in 48% (n=39) and normal in 25% (n=31). ET$_A$R mRNA expression was increased in 21% (n=26) of the primary papillary thyroid carcinomas. The expression was low in 48% (n=59) and normal in 31% (n=38). Tables 3.2 and 3.3 show all details about ET-1 and ET$_A$R expression levels in all samples.

Table 3.2: Details of ET-1 Expression Levels

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>High expression level</th>
<th>Normal expression level</th>
<th>Low expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid carcinomas without lymph node metastasis</td>
<td>28 (33.7%)</td>
<td>11 (11.3%)</td>
<td>44 (53%)</td>
</tr>
<tr>
<td>Thyroid carcinomas with lymph node metastasis</td>
<td>14 (35%)</td>
<td>0</td>
<td>26 (65%)</td>
</tr>
<tr>
<td>Metastatic lymph nodes</td>
<td>26 (65%)</td>
<td>1 (2.5%)</td>
<td>13 (32.5%)</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>0</td>
<td>7 (100%)</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.3: Details of ETAR expression levels

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>High expression level</th>
<th>Normal expression level</th>
<th>Low expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid carcinomas without lymph node metastasis</td>
<td>16 (19.3%)</td>
<td>14 (16.9%)</td>
<td>53 (63.9%)</td>
</tr>
<tr>
<td>Thyroid carcinomas with lymph node metastasis</td>
<td>7 (17.5%)</td>
<td>0</td>
<td>33 (82.5%)</td>
</tr>
<tr>
<td>Metastatic lymph nodes</td>
<td>7 (17.5%)</td>
<td>15 (37.5%)</td>
<td>18 (45%)</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>0</td>
<td>4 (57.1%)</td>
<td>3 (42.9%)</td>
</tr>
</tbody>
</table>

The relationship between the expression of ET-1 and ETAR with the clinicopathological features of papillary thyroid carcinomas are shown in Tables 3.4 and 3.5.

A significant difference between ET-1 mRNA expression level and gender was detected (p=0.040), with higher expression levels mostly found in males (37% vs. 22%) (Figure 3.26). ET-1 mRNA was more often overexpressed in those thyroid carcinomas with lymph node metastases compared to thyroid carcinomas without lymph node metastases (34% vs. 20%, p=0.0001) (Figure 3.30). This difference was also detected by ANOVA analysis of the $2^{-\Delta\Delta Ct}$ data (p=0.0001). There was an additional sub population of metastatic thyroid carcinomas that showed reduced expression of ET-1. Also, stromal calcification was often noted in papillary thyroid carcinoma with higher ET-1 mRNA expression (70% vs. 43%, p=0.018) (Figure 3.34).
Other than these, ET-1 mRNA expression did not correlate with the size, histological subtype, presence of psammoma bodies, osseous metaplasia, pathological stage of thyroid cancer as well as the presence of co-existing lymphocytic thyroiditis (p> 0.05).

Papillary thyroid carcinoma of larger size (diameter > 40mm) had significantly higher levels of ETAR mRNA expression than those with smaller size (46% vs. 18%, p = 0.003) (Figure 3.39). Other than this, the ETAR mRNA expression did not correlate with gender of the patients, histological subtype, presence of psammoma bodies, calcification, osseous metaplasia, pathological stage of thyroid cancer as well as the presence of co-existing lymphocytic thyroiditis (p> 0.05).

Pearson correlation testing showed a strong and significant positive correlation between ET-1 and ETAR mRNA expression in primary thyroid carcinomas (Pearson’s r=0.522, p<0.001).
3.2.2: Comparison Between the Expression in Primary Cancer and the Matched Lymph Node with Metastatic Cancer

For ET-1, high mRNA expression was detected in 30% (12/40) of the metastatic thyroid cancer in lymph node. The other 55% (22/40) of cancers in the lymph node showed low expression or 15% (6/40) normal expression of ET-1 mRNA. Of these 40 matched cancers, 53% (21/40) showed the same level of mRNA expression between the primary cancer and the cancer in the lymph node. For the other cancers, 28% (11/40) had a higher level of mRNA in the lymph node metastases than the primary thyroid cancer whereas 20% (8/40) had a lower level of mRNA expression.

For ET$_A$R, high mRNA expression was detected in 25% (10/40) of the metastatic thyroid cancers in lymph nodes. The other 63% (21/40) of the cancers in the lymph node showed low expression or 7% (9/40) showed normal expression of ET-1 mRNA. Of these 40 matched cancers, 40% (16/40) showed the same level of mRNA expression between the primary cancer and the cancer in the lymph node. For the other cancers, 35% (14/40) had a higher level of mRNA in the lymph node metastases than the primary thyroid cancer whereas 25% (10/40) had a lower level of mRNA expression.
Table 3.4: The relationships between *ET-1* mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas

<table>
<thead>
<tr>
<th>Clinical &amp; pathological variables</th>
<th>High</th>
<th>Low</th>
<th>Normal</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=41)</td>
<td>15 (37%)</td>
<td>21 (51%)</td>
<td>5 (12%)</td>
<td>0.040*</td>
</tr>
<tr>
<td>Female (n=82)</td>
<td>18 (22%)</td>
<td>38 (46%)</td>
<td>26 (32%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 (n=67)</td>
<td>16 (24%)</td>
<td>32 (48%)</td>
<td>19 (28%)</td>
<td>0.587</td>
</tr>
<tr>
<td>≥45 (n=56)</td>
<td>17 (30%)</td>
<td>27 (48%)</td>
<td>12 (22%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour size (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40 mm (n=110)</td>
<td>27 (25%)</td>
<td>56 (50%)</td>
<td>27 (25%)</td>
<td>0.126</td>
</tr>
<tr>
<td>&gt;40mm (n=13)</td>
<td>6 (46%)</td>
<td>3 (23%)</td>
<td>4 (31%)</td>
<td></td>
</tr>
<tr>
<td><strong>T staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 or T2 (n=78)</td>
<td>18 (23%)</td>
<td>38 (49%)</td>
<td>22 (28%)</td>
<td>0.386</td>
</tr>
<tr>
<td>T3 (n=45)</td>
<td>15 (33%)</td>
<td>21 (47%)</td>
<td>9 (20%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n=58)</td>
<td>20 (34%)</td>
<td>33 (57%)</td>
<td>5 (9%)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Negative (n=65)</td>
<td>13 (20%)</td>
<td>26 (40%)</td>
<td>26 (40%)</td>
<td></td>
</tr>
<tr>
<td><strong>TNM staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages1 or II (n=87)</td>
<td>20 (23%)</td>
<td>42 (48%)</td>
<td>25 (29%)</td>
<td>0.426</td>
</tr>
<tr>
<td>Stage III (n=36)</td>
<td>13 (36%)</td>
<td>17 (47%)</td>
<td>6 (17%)</td>
<td></td>
</tr>
<tr>
<td>Clinical &amp; pathological variables</td>
<td>High</td>
<td>Low</td>
<td>Normal</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------</td>
<td>-----</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>Pathological variant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional (n=79)</td>
<td>24 (30%)</td>
<td>38 (48%)</td>
<td>17 (22%)</td>
<td>0.327</td>
</tr>
<tr>
<td>Follicular (n=44)</td>
<td>9 (20%)</td>
<td>21 (48%)</td>
<td>14 (32%)</td>
<td></td>
</tr>
<tr>
<td>Psammoma body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=52)</td>
<td>18 (35%)</td>
<td>26 (50%)</td>
<td>8 (15%)</td>
<td>0.057</td>
</tr>
<tr>
<td>Absent (n=71)</td>
<td>15 (21%)</td>
<td>33 (47%)</td>
<td>23 (32%)</td>
<td></td>
</tr>
<tr>
<td>Calcification in stroma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=62)</td>
<td>23 (37%)</td>
<td>28 (45%)</td>
<td>11 (18%)</td>
<td>0.018*</td>
</tr>
<tr>
<td>Absent (n=61)</td>
<td>10 (16%)</td>
<td>31 (51%)</td>
<td>20 (33%)</td>
<td></td>
</tr>
<tr>
<td>Osseous metaplasia in stoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=6)</td>
<td>2 (33%)</td>
<td>4 (67%)</td>
<td>0 (0%)</td>
<td>0.165</td>
</tr>
<tr>
<td>Absent (n=117)</td>
<td>31 (26%)</td>
<td>55 (48%)</td>
<td>31 (26%)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytic thyroiditis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=40)</td>
<td>14 (35%)</td>
<td>16 (40%)</td>
<td>10 (25%)</td>
<td>0.547</td>
</tr>
<tr>
<td>Absent (n=83)</td>
<td>19 (23%)</td>
<td>43 (52%)</td>
<td>21 (25%)</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant
Figure 3.26: ET-1 expression levels in different gender groups. There was a significant difference between ET-1 expression levels in examined groups. Higher expression levels were mostly found in females ($p=0.040$).
Figure 3.27: ET-1 expression level in different age groups. Although high expression levels were mostly detected in the patients older than 45 years, the difference did not reach significance ($p=0.587$).
Figure 3.28: ET-1 expression level in different tumour sizes. PTCs smaller than 40mm were more likely to show low expression than tumours > 40mm, however, the difference did not reach a statistically significant level ($p=0.126$).
Figure 3.29: ET-1 expression level in different T staging. Although high expression levels were more common in the T3 tumours, there was no significant difference between different T stages ($p=0.386$).
Figure 3.30: ET-1 expression level and its correlation with metastasis to regional lymph nodes. There was a significant difference between PTCs with metastasis to lymph nodes and PTCs without metastasis to lymph nodes. High expression levels were mostly detected in PTCs with metastasis to lymph nodes ($p=0.0001$).
Figure 3.31: ET-1 expression level in different TNM staging. Although high expression levels were mostly detected in Stage III cancers, the difference failed to attain statistical significance ($p=0.426$).
Figure 3.32: ET-1 expression level in different Pathological Variants. High expression levels were mostly noticed in Conventional PTCs, but the difference did not reach a statistically significant level ($p=0.327$).
Figure 3.33: ET-1 expression level regarding Psammoma body. High expression level was more common in PTCs with Psammoma body, however, the difference did not reach statistical significance (p=0.057).
Figure 3.34: ET-1 expression level regarding Calcification in stroma. There was a significant difference between the groups examined. PTCs with Calcification in stroma showed significantly higher expression levels of ET-1 ($p=0.018$).
Figure 3.35: ET-1 expression level in the presence of Osseous metaplasia in stroma. High expression level was more frequent in PTCs with osseous metaplasia in stroma, but this did not reach significance (p=0.165). Additionally, the low numbers of osseous metaplasia were likely to hamper accurate analysis.
Figure 3.36: ET-1 expression level regarding Lymphocytic thyroiditis. High expression level was mostly detected in PTCs with Lymphocytic thyroiditis, but the difference was not significant (p=0.547).
Table 3.5: The correlation between $ET_A R$ mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas

<table>
<thead>
<tr>
<th>Clinical &amp; pathological variables</th>
<th>High</th>
<th>Low</th>
<th>Normal</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n=41)</td>
<td>9 (22%)</td>
<td>17 (41%)</td>
<td>15 (37%)</td>
<td>0.548</td>
</tr>
<tr>
<td>Female (n=82)</td>
<td>17 (21%)</td>
<td>42 (51%)</td>
<td>23 (28%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 (n=67)</td>
<td>15 (22%)</td>
<td>32 (48%)</td>
<td>20 (30%)</td>
<td>0.922</td>
</tr>
<tr>
<td>≥45 (n=56)</td>
<td>11 (20%)</td>
<td>27 (48%)</td>
<td>18 (32%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour size (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40 mm (n=110)</td>
<td>20 (18%)</td>
<td>58 (53%)</td>
<td>32 (29%)</td>
<td>0.003*</td>
</tr>
<tr>
<td>&gt;40 mm (n=13)</td>
<td>6 (46%)</td>
<td>1 (8%)</td>
<td>6 (46%)</td>
<td></td>
</tr>
<tr>
<td><strong>T staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 or T2 (n=78)</td>
<td>15 (19%)</td>
<td>39 (50%)</td>
<td>24 (31%)</td>
<td>0.761</td>
</tr>
<tr>
<td>T3 (n=45)</td>
<td>11 (25%)</td>
<td>20 (44%)</td>
<td>14 (31%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n=58)</td>
<td>12 (21%)</td>
<td>31 (53%)</td>
<td>15 (26%)</td>
<td>0.448</td>
</tr>
<tr>
<td>Negative (n=65)</td>
<td>14 (22%)</td>
<td>28 (43%)</td>
<td>23 (35%)</td>
<td></td>
</tr>
<tr>
<td><strong>TNM staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages1 or II (n=87)</td>
<td>18 (21%)</td>
<td>43 (49%)</td>
<td>26 (30%)</td>
<td>0.879</td>
</tr>
<tr>
<td>Stage III (n=36)</td>
<td>8 (22%)</td>
<td>16 (45%)</td>
<td>12 (33%)</td>
<td></td>
</tr>
</tbody>
</table>
**$ET_A R$ expression**

<table>
<thead>
<tr>
<th>Clinical &amp; pathological variables</th>
<th>High</th>
<th>Low</th>
<th>Normal</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathological variant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional (n=79)</td>
<td>15 (19%)</td>
<td>39 (49%)</td>
<td>25 (32%)</td>
<td>0.739</td>
</tr>
<tr>
<td>Follicular (n=44)</td>
<td>11 (25%)</td>
<td>20 (45%)</td>
<td>13 (30%)</td>
<td></td>
</tr>
<tr>
<td>Psammoma body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=52)</td>
<td>9 (17%)</td>
<td>30 (58%)</td>
<td>13 (25%)</td>
<td>0.180</td>
</tr>
<tr>
<td>Absent (n=71)</td>
<td>17 (24%)</td>
<td>29 (41%)</td>
<td>25 (35%)</td>
<td></td>
</tr>
<tr>
<td>Calcification in stroma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=62)</td>
<td>13 (21%)</td>
<td>30 (48%)</td>
<td>19 (31%)</td>
<td>0.996</td>
</tr>
<tr>
<td>Absent (n=61)</td>
<td>13 (21%)</td>
<td>29 (48%)</td>
<td>19 (31%)</td>
<td></td>
</tr>
<tr>
<td>Osseous metaplasia in stoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=6)</td>
<td>0 (0%)</td>
<td>4 (67%)</td>
<td>2 (33%)</td>
<td>0.220</td>
</tr>
<tr>
<td>Absent (n=117)</td>
<td>26 (22%)</td>
<td>55 (47%)</td>
<td>36 (31%)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytic thyroiditis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=40)</td>
<td>12 (30%)</td>
<td>19 (47%)</td>
<td>9 (23%)</td>
<td>0.362</td>
</tr>
<tr>
<td>Absent (n=83)</td>
<td>14 (17%)</td>
<td>40 (48%)</td>
<td>29 (35%)</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant
Figure 3.37: ET₄R expression levels in different gender groups. Although high expression levels were mostly detected in males, the difference did not reach significance ($p=0.548$).
Figure 3.38: ET_{AR} expression level in different age groups. Although high expression levels were mostly noticed in the patients younger than 45 years old, the difference did not reach significance (0.922).
Figure 3.39: ETAR expression level in different tumour sizes. There was a significant difference between examined groups. High expression levels were mostly found in PTCs larger than 40 mm (p=0.003).
Figure 3.40: ETAR expression level in different T staging. Although high expression levels were mostly detected in the T3 category, there was no significant difference between different T stages (p=0.761).
Figure 3.41: ET₄R expression level and its correlation with regional lymph node metastasis. High expression levels were mostly found in PTCs without metastasis to lymph nodes, however, the difference did not reach a statistically significant level (p=0.448).
Figure 3.42: ET₄R expression level in different TNM staging. Although high expression levels were mostly detected in Stage III the difference failed to attain statistical significance \((p=0.879)\).
Figure 3.43: ET₄R expression level in different Pathological Variants. High expression levels were mostly noticed in follicular variant PTCs, but the difference did not reach a statistically significant level (p=0.739).
Figure 3.44: ET\(_A\)R expression level regarding Psammoma body. High expression level was more common in PTCs without Psammoma body; however, the difference did not reach statistical significance (0.180).
Figure 3.45: ET₄R expression level regarding Calcification in stroma. There was no effective difference in distribution of expression in PTCs with Calcification in stroma ($p=0.996$).
Figure 3.46: ET₄R expression level regarding Osseous metaplasia in stroma. High expression levels were more frequent in PTCs without Osseous metaplasia in stroma, but not reaching significance (p=0.220). Again, low numbers in this category would hamper analysis.
Figure 3.47: ET₄R expression level regarding Lymphocytic thyroiditis. High expression levels were mostly detected in PTCs with Lymphocytic thyroiditis, but the difference was not significant (p=0.362).
Figure 3.48: Expression of ET-1 in thyroid cancer with lymph node metastasis and without lymph node metastasis and in metastatic lymph nodes. ET-1 was significantly under expressed in thyroid cancers with lymph node metastasis compared to in thyroid cancers without lymph node metastasis (p=0.0001).
Figure 3.49: Expression of ET\(_A\)R in thyroid cancer with lymph node metastasis and without lymph node metastasis and in metastatic lymph nodes. There was no significant difference between thyroid cancers with lymph node metastasis and thyroid cancers without lymph node metastasis.
3.2.3: Immunohistochemical Analysis of ET-1, and ET<sub>A</sub>R Protein Expression

ET-1 protein expression was mainly nuclear in thyroid cancers. The adjacent non-tumour thyroid often showed weaker nuclear staining. The expression was noted in the primary papillary thyroid carcinomas as well as metastatic thyroid carcinoma in lymph node. ET<sub>A</sub>R protein expression was mainly membranous in papillary thyroid carcinoma. The adjacent non-tumour thyroid often showed weaker membranous staining. The protein expression was noted in the primary papillary thyroid carcinomas as well as metastatic thyroid carcinoma in lymph node.

ET-1 protein expression was tested in 112 of the 123 papillary thyroid carcinomas. ET-1 protein was expressed at low levels in 6% (n=7) of the papillary thyroid carcinomas whereas a high expression level was seen in 94% (n=105). Of the 31 cases with high expression of ET-1 mRNA, 29 (94%) revealed a high level of ET-1 protein.

ET<sub>A</sub>R protein expression was studied in 74 of the 123 papillary thyroid carcinomas. ET<sub>A</sub>R was expressed at high level in 95% (n=70) and at low level in 5% (n=4) of the carcinomas. All the 18 cases with high expression of ET<sub>A</sub>R mRNA showed high expression of ET<sub>A</sub>R.

There was no significant difference between ET-1 and ET<sub>A</sub>R protein expression levels in papillary thyroid carcinomas with lymph node metastases, those without lymph node metastases and metastatic carcinomas in the lymph nodes. Also, no correlation of the ET-1/ET<sub>A</sub>R immunohistochemical stain with clinicopathological features was demonstrated.
3.3: Discussion

In papillary thyroid carcinoma, ET-1 production and antagonism of $ET_A R$ have been demonstrated in human thyroid cancer cells lines (292). ET-1 or $ET_A R$ have been only investigated at the tissue level for thyroid cancer in two research groups. Lenziardi et al. studied the ET-1 in 27 patients with papillary thyroid carcinoma by immunohistochemistry and noted 90% of them expressed the protein (293). Donckier et al. have shown higher expression of ET-1 and $ET_A R$ mRNA in 12 patients with papillary thyroid carcinomas than non-cancer thyroid tissues with immunohistochemistry and RT-PCR. In another study, they noted that ET-1 mRNA expression in thyroid cancers correlated with nitric oxide pathway activation (294). The current study was done on a large cohort of patients with papillary thyroid carcinoma (n=123). The results showed variable expression of ET axis (ET-1 or $ET_A R$) in all papillary thyroid carcinoma, metastatic carcinoma in lymph nodes and non-cancer thyroid tissues which correlates with the clinical and pathological parameters. Also, 40 matched primary and metastatic cancers from the same patient were studied to confirm the difference in expression profiles between them.

In the current study, a significant difference was observed between ET-1 expression levels and gender, in the primary thyroid carcinomas (Figure 3.25). To determine if this result was due to violation of the chi-square assumptions because of higher number of patients in female group, we performed Monte Carlo style analysis. The expression difference of ET-1 in population of the study was again established with this test (p=0.044). There is no immediately obvious reason for this expression difference, but may be related to the fact that thyroid cancers
are more common in women (74.9%) and the gender difference in clinical behavior in thyroid cancers, with males more often showing aggressive cancer.

There was a significant difference between \( ET_A R \) expression level and cancer size, as larger cancers expressed higher levels of \( ET_A R \) (Figure 3.38). This would make sense in terms of increased sensitivity to angiogenic signals for larger cancers with higher oxygen demands, and although the difference was not significant, our data also shows a trend of increased \( ET-1 \) expression in larger tumours. It has been shown that \( ET_A R \) overexpression is associated with aggressive cancer behavior and a worse prognosis in ovarian cancer (295). Indeed in our study, we also found increased expression of \( ET-1 \) in metastatic thyroid cancer (Figure 3.29). Therefore, it can be hypothesized that \( ET_A R \) or \( ET-1 \) overexpression enhances cancer progression or establishment of early growth of tumour masses. There was also a significant sub-population of metastatic thyroid cancers that showed low \( ET-1 \) expression. Whether this group represents a biologically distinct group or whether the reduced expression is due to a relative difference in the vasculature present is not clear, but may bear investigation in future. It is also worth noting that higher \( ET-1 \) expression was noted in tumour with calcified stroma (Figure 3.33). The reason is not clear. It can be hypothesized that the larger cancers with high \( ET-1 \) expression were often take more time to grow and have more time to form calcification. Endothelin as a vasoconstrictor (60) can protect cells from hypoxia via its receptors (61) and its production and expression can be stimulated by hypoxia (92). Therefore, as the finding of this research presented, the expression of \( ET-1 \) was noticed in larger tumours and higher T stage and significantly observed in carcinomas with calcification.
There are conflicting studies on ET axis and its relationship with clinicopathological parameters in various cancers. Endothelin axis expression in the tissue and in blood has been associated with advanced cancer characteristics in colorectal, breast and prostate cancer, while showing no linkage to clinicopathological parameters in bladder cancer (296-299). It is thus clear that activation of the ET-1 axis is likely highly cancer specific, perhaps representing differences in tissue organisation and the ability of primary tissue to utilise endothelins. In the present study, there was a significant positive correlation between $ET-1$ and $ET_A R$ expression levels in the primary thyroid carcinomas and also between the primary thyroid carcinomas and metastatic carcinoma in lymph nodes. These findings show that ET-1/ $ET_A R$ autocrine pathway is implicated in papillary thyroid cancer progression, though its linkage to the most severe forms has not been established. Notwithstanding, this pathway acts through different cancer relevant processes, such as proliferation, angiogenesis, inhibition of apoptosis, migration, invasion and metastasis (300). $ET-1$ is also known to stimulate multiple cancer related processes, including epithelial-mesenchymal transition (EMT) through $ET_A R$ (125). During EMT, cancer cells lose epithelial cell junction proteins to acquire a mesenchymal cell phenotype which gives them the ability to invade extracellular matrix, and become motile. Our results indicate that these factors are likely at work in large and metastatic thyroid lesions.

In our study, elevated both $ET-1$ and $ET_A R$ expression levels were commonly detected in thyroid carcinomas with lymph node metastases and metastatic cancer in lymph nodes. In patients with both primary cancer and metastatic cancer to compare, the expression of the latter was found to increase in many patients, though the trend was not universal and the average showed a reduction in expression for ET-1. Our finding concurs on some levels with Hagemann et al. who found that $ET-1$ was up-regulated in the serum of breast cancer patients.
with lymph node metastases compared to patients without lymph node metastasis. The authors suggested that highly vascularized metastatic lymph nodes may produce endothelins (301). Our findings support the hypothesis of an autocrine role of ET-1/ET_{A}R pathway in thyroid cancers. The ET-1/ET_{A}R axis was noted to enhance metastasis. Therefore, these findings identify ET_{A}R as a potential therapeutic target for thyroid cancers, but also show the necessity of identifying tissue specific effects before general use of ET axis modifiers.

However, it is also worth noting that a significant proportion of the tissues assayed showed reduced expression of both ET-1 and ET_{A}R, with ET-1 showing an average reduction in expression in primary thyroid cancers with lymph nodes. As a papillary thyroid carcinoma can grow for a long time before becoming metastatic or before being identified and diagnosed as a metastatic lesion, it is expected to have a larger variety in the population of metastatic lesions in papillary thyroid carcinoma. This also shows that regulation of the pathway is complex and might depend significantly on external influences and factors. These may also represent different molecular subtypes of thyroid cancer that could have significantly different biological behavior. This is also worth further study.

In our investigation, ET-1 immunostaining showed high expression in 94% of cases and ET_{A}R staining showed high expression in 95% of cases. The results confirmed the findings of high expression of proteins in two previous studies on papillary thyroid carcinomas which examined smaller numbers of thyroid cancers (68, 293). The high expressions of these proteins in situ reflected the findings of their corresponding mRNAs in some cases, though the loss of mRNA expression seen in some cases, despite the overwhelming protein overexpression would indicate that cancers showing low mRNA levels may be using unusual mechanisms to maintain protein production from the gene. These findings confirm the importance of ET-1/ET_{A}R
expression in thyroid cancer. In addition, we demonstrated that the proteins, ET-1 and ET\textsubscript{A}R, were located in specific cellular locations in thyroid tissue no matter whether they are in benign, malignant or even metastatic tumours.

The expression of ET-1/ ET\textsubscript{A}R proteins appears to be different in various cancers. Wülfing \textit{et al.} observed staining of ET-1 in 26.8% of bladder cancer tissue samples and in 58.8% in ET\textsubscript{A}R of the same cases (80). In another study, Eltze \textit{et al.} identified ET-1 and ET\textsubscript{A}R staining in 62% and 93% of bladder cancer samples, respectively (81). Differences in ET-1 and ET\textsubscript{A}R immunostaining in these and our studies indicate that ET axis expression levels are different in various cancer types, and may depend on the propensity of the originating cell type to utilise certain vascular triggers, rather than being an intrinsic response of the vasculature to cancer.

3.4. Conclusions for ET-1 Study

In conclusion, elevated ET-1/ ET\textsubscript{A}R expression levels seen in our research were produced by significantly different proportions of primary thyroid cancers or metastases compared to primary cancers, and may also be produced by endothelial cells within highly vascularized primary tumours and metastases. These results suggest that ET axis expression could be used as a possible indicator to predict the aggression levels of papillary thyroid carcinomas, identifying which carcinomas have metastasised as well as helping to locate the presence of new and developing metastases in lymph nodes and other locations, due to the role of the axis in establishing tumour vasculature. Although we have identified a link between ET axis expression and cancer aggression, more research is needed to determine whether ET axis levels in thyroid cancer tissues could serve as prognostic markers, or an indicator of response to therapy.
Chapter 4:

miRNA-195 Study
After assessing the role of ET-1 axis in PTC progress, we next examined the role of miR-195 in PTC progress, especially regarding its impact on clinicopathological parameters.

To do this, we determined the miR-195 expression levels in PTC tissue samples and PTC cell lines. Lastly, we examined the correlation between ET-1 axis and miR-195 expression in PTC tissue samples and cell lines.

4.1: Methodology

With the same tissue samples and extracted total RNA and cDNA, RT-PCR was also performed for miR-195 study.

4.1.1: miR-195 Primer Design

Primer design for miRNAs is significantly different from design for coding genes or large RNAs. Because a mature miRNA is only around 20 base pairs long (the length of a standard primer) a normal two primer system cannot be used. Instead, the sequence of the mature miRNA is used as one primer, with the other being a universal reverse primer which was provided by Qiagen, in conjunction with a standard sequence appended to all RNAs during the miScript cDNA synthesis. Thus, in order to obtain the sequence for the forward primer, the miR-195 sequence was searched in miRBase.
hsa- miR-195-5p (MIMAT0000461.UAGCAGCACAGAAUAAUUGC) was selected as the primer and was ordered.

The PCR primer for miR-195 was TAGCAGCACAGAAATATTGGC (Qiagen Pty. Ltd., Hilden, NRW, Germany).

**4.1.2: RT-PCR**

10X miScript primer Assay from Qiagen was used for RT-PCR. It was lyophilized at Room Temperature. Briefly, was centrifuged, added 550 μl TE Buffer, pH 8.0. Then the mixture was mixed by vortexing the vial 4-6 times. The mixture provided sufficient primer for 100x 50μl reactions.

A total volume of 20 μl reaction mixture containing 10 μl QuantiTect SYBR Green, 2 μl of each primer (miScript Universal Primer and miR-195), 2 μl of RNase-free water, 4 μl of Template cDNA at 1.5 ng/μl was performed. Human RNU6B (RNU6-2) miScript Primer from Qiagen was used as a ubiquitous control gene, with its PCR performed with identical conditions. In the last tube of each experimental run, 4 μl of water was added instead of miRNA as a non-template control. All samples were run in triplicate. Thermal cycling conditions included initial denaturation in 1 cycle of 10 minutes at 95°C. Following this was 40 cycles of 30 seconds at 59.5°C, 30 seconds at 55°C and 30 seconds at 70°C.
4.1.3: Data Analysis

The same method was used to evaluate miR-195 expression as for ET-1 and ETAR expression, as outlined in Chapter 3.

\[ \Delta Ct = Ct_{miR-195} [\text{mean sample}] - Ct_{RNU6B3} [\text{mean sample}] \]

After obtaining \( \Delta Ct \) for miR-195 for each sample, the fold change in miR-195 was calculated for each sample using the 2\( - \Delta \Delta Ct \) method.

\[ \Delta \Delta Ct = (Ct_{miR-195} - Ct_{RNU6B3}) \text{CANCER} - (Ct_{miR-195} - Ct_{RNU6B3}) \text{MEAN NORMAL} \]

Ratios (=Mean \( Ct_{miR-195}[\text{sample}] / Ct_{RNU6B3} [\text{sample}] \)) were also calculated to improve the display of results. In order to reorient changes of expression for intuitive results graphing, inverse ratios (1/Ratio) were calculated which reflect actual behaviour of miR-195 expression.

Normalised final data was analysed using one-way (ANOVA) to determine if there were significant differences of miRNA-195 expression between thyroid tissue samples both in primary and metastatic sites. Additional comparisons were made to determine whether there were significant differences in other thyroid cancer subgroups, including follicular variants, patient gender, grades and staging and similar clinicopathological characteristics.

All statistical analysis was performed using SPSS version 21.0. Significance level was taken at \( p < 0.05 \).
4.1.4: Non-PCR Based Methods

4.1.4.1: Cell Culture

The cell lines used in this research were the K1 thyroid cancer cell line (derived from human papillary thyroid carcinoma), B-CPAP (derived from human metastasizing papillary thyroid carcinoma) and an immortalized normal thyroid cell line (Nthy-ori3-1, derived from human thyroid follicular epithelium). K1 and Nthy-ori3-1 cell lines were purchased from American Type Cell Culture (ATCC) and B-CPAP was purchased from DSMZ. B-CPAP and Nthy-ori3-1 cells were cultured in flasks at 37ºC in a humidified atmosphere of 5% CO2 in culture medium containing 90% RPMI 1640 (Invitrogen, Grand Island, NY) and 10% FBS (Life Technologies, Pty Ltd.) with 100 U/ml penicillin and 100 U/ml streptomycin (Per Strep, Invitrogen, Grand Island, NY). The K1 cell line was cultured in DMEM (Dulbecco’s modified Eagle’s medium, (Life Technologies, Carlsbad, CA, USA) Ham’s F12 (Life Technologies, Carlsbad, CA, USA), MCDB (Sigma-Aldrich, St. Louis, MO, USA), 10% foetal calf serum, glutamine and 100 units/ml penicillin (Per Strep, Invitrogen, Grand Island, NY) and 100 μg/ml streptomycin (Per Strep, Invitrogen, Grand Island, NY).
4.1.4.2: Cell Reviving Protocol

1. A Cryo Vial (Corning, NY, USA) was taken from liquid nitrogen and kept in a water bath for few seconds at 37°C.

2. The cell line was thawed by adding warm (37°C) culture media.

3. Thawed cells were transferred to a 15 ml tube (Corning, NY, USA), and centrifuged for 3-4 min at 1300 rpm.

4. The media was discarded; warm PBS (2ml) was added for washing and the tube centrifuged for 3-4 min at 1300rpm.

5. Warm media (4-5 ml) was added to the cells and mixed thoroughly with a pipette.

6. Then the cells were transferred into a culture flask (Corning, NY, USA) (7 ml of media for a 25cm² flask and 14 ml for a 75² flask was added).

7. The cells were observed under a microscope and incubated for 24 hours

8. After 24 hours the cells were checked to make sure they adhered to the bottom of the flask.

9. The media was changed every day until the cells reached 80% confluence (Figures 4.1& 4.2).
Figure 4.1: Nthy-ori3-1 cell line with about 60% confluence. Normal thyroid cells are anchorage dependant cells and grow at the bottom of cell culture flask.

Figure 4.2: B-CPAP cell line with about 60% confluence. The cells are arranged in follicular pattern to form follicular pattern of PTC tissue sample.
**4.1.4.3: Cell Splitting Protocol**

1. First the media was discarded.

2. Then the cells were washed with warm PBS twice.

3. Two microliters Trypsin-EDTA 0.25% (Invitrogen, Grand Island, NY) was added.

4. Cells were kept in an incubator for 2-3 minutes. Once the cells were detached completely, complete media was added to the flask (3-4 ml depending on the size of flask) to inactivate trypsin.

5. After transferring the cells to a 15ml tube, they were centrifuged for 3-4 min at 1300 rpm.

6. After discarding the media containing trypsin, warm PBS was added for washing.

7. Then PBS was discarded followed by adding media, mixing thoroughly and transferring to 4-6 25cm$^2$ flasks or 2-3 75cm$^2$ flasks. More media was added to each flask.

**4.1.4.4: Protocol for Freezing the Cells**

1. All media in the flasks was discarded and 2-3 ml of Trypsin-EDTA 25% was then added, and incubated for 2-3 minutes. The cells were observed under a microscope. If the cells were not detached, gentle shaking and agitation was used for cells detachment.

2. After adding 2-3 ml of media the cells were centrifuged at 1300 rpm.
3. Then the supernatant was discarded and the cells were washed with warm PBS.

4. 3-4 ml of media was added and the cells were counted by using trypan blue (10μl of media containing cells and 10μl of trypan blue) and a hemocytometer counting chamber, (Neubauer improved hemocytometer). The cells were counted in 4 squares. After obtaining the mean number of cells per square, it was multiplied by 20,000 to determine the number of cells in 1 ml.

5. According to the number of cells, a mixture of the freezing media (media plus 10% FBS) and 10% DMSO was made. For 1,000,000 cells, 1 ml of the media was enough.

6. After discarding the old media from cells the freezing mixture was added to the cells.

7. The mixture of cells and freezing media was allocated into cryo vials.

8. The vials were transferred to a freezing box immediately, and kept in -80°C freezer for 24 hours, after which they were transferred to the liquid nitrogen tank.

4.1.5: mRNA and miRNA Extraction From Cell Lines

mRNA and miRNA were extracted from cell lines using NucleoSpin® miRNA Kits (MACHEREY-NAGEL GmbH & Co. KG.,Germany). Extraction was carried out using manufacturer’s instructions as follows:

1. First $10^7$ cultured cells were collected by centrifugation and lysed by adding 300 μl Buffer ML. They were mixed by pipetting up and down > 5 times.
2. After placing a NucleoSpin® Filter (violet ring) into a collection tube (2 ml, lid) the mixture was loaded and centrifuged for 1 minute at 11,000 x g to homogenise the lysate, reduce viscosity and clear from undissolved debris.

3. Then the supernatant was transferred to a new 1.5 ml microcentrifuge tube without disturbing the pellet.

4. To adjust binding conditions for large RNA, the NucleoSpin® Filter was discarded, and exactly 150 μl 96–100 % ethanol was added to 300 μl homogenized cell lysate, the lid was closed, and vortexed immediately for 5 s, following by incubation for 5 min at room temperature (18–25 °C).

5. To bind large RNA and allow separation of small RNA, a NucleoSpin® RNA Column (blue ring) was placed in a collection tube (2 ml, lid) and the sample was loaded including any precipitate onto the column, and centrifuged for 1 min at 14,000 x g.

6. The NucleoSpin® RNA Column (blue ring) was transferred into a new collection tube (2 ml) for further processing in Step 9. The flowthrough from this centrifugation was retained and continued to be treated in the following steps.

7. The flowthrough containing the small RNA had 300 μl Buffer MP added to it and was vortexed for 5 seconds to precipitate protein. The solution was then centrifuged for three minutes at 11,000 x g for three minutes to pellet the protein. The supernatant was then loaded into a NucleoSpin® Protein Removal Column (white ring) and centrifuged for three minutes at 11,000 g.
8. The flowthrough containing the small RNA had 800 μl Buffer MX added to it and was vortexed for 5 seconds to prepare the solution for final purification in step 11.

9. The silica membrane of the NucleoSpin® RNA Column containing the large RNA was desalted by adding 350 μl Buffer MDB to the NucleoSpin® RNA Column (blue ring) and centrifuged for 1 min at 11,000 x g. The column was then placed on a new collection tube.

10. In the next step, DNA was digested by adding 100 μl rDNase directly onto the silica membrane of the NucleoSpin® RNA column (blue ring), and incubated at room temperature (18–25 °C) with open lid at least 15 mins.

11. Purification of small RNA and large RNA in one fraction was then done by placing the NucleoSpin® miRNA Column (blue ring) containing large RNA in a collection tube (2 ml) and loaded with the solution containing small RNA from Step 8. The column was then centrifuged for 30 s at 11,000 x g. The flow-through was discarded and placed the column back into the collection tube.

12. To wash and dry the silica membrane, first 600 μL Buffer MW1 was added to the NucleoSpin® RNA / miRNA column, centrifuged for 30 s at 11,000 x g. The flow-through was discarded and placed the column back into the collection tube, followed by second wash by adding 700 μL Buffer MW2 to the NucleoSpin® RNA / miRNA column, centrifuged for 30 s at 11,000 x g. The flow-through was discarded and placed the column back into the collection tube.

13. Finally, mRNA and miRNA were eluted by placing the NucleoSpin® RNA / miRNA column in a new collection Tube (1.5 ml), followed by adding 50 μl RNase free H2O to the
column, incubated for 1 min at room temperature (18–25 °C) with open lid. Then the lid was closed and centrifuged for 30 s at 11,000 x g. This step was repeated twice to obtain maximum yield of mRNA and miRNA.

4.1.6: micro RNA Transfection

Transfection is a method for the deliberate introduction of nucleic acids into cells. The transfection experiments for this research consisted of the transfection of an artificial miR-195 mimic to evaluate its effects on the expression of the genes of interest (HIF-1 α and VEGF-A, ET-1 and ETAR, and Bcl2 and p53). GAPDH and β-actin were used as controls. Following the transfection of cells with miRNA mimic, the angiogenic capacity, and proliferation capacity of these cells were compared to untransfected (vehicle exposed) and untreated control cells. Transfection was done using a miR-195 mimic (Qiagen Pty. Ltd., Hilden, NRW, Germany). The most effective concentration for transfection was determined by testing different concentrations of miRNA and transfection reagent using B-CPAP cells in a 24-well plate. Transfection was then carried out as follows:

4.1.6.1: Cell Transfection Protocol

1. Just before transfection, 6 x 10⁵ cells were seeded in 10 cm² dishes in 5 ml of culture medium containing serum and antibiotics.
2. Diluting 1.5 μl miRNA mimic (20 μM stock) in 1000 μl culture medium without serum gave a final miRNA mimic concentration of 5 nM. Then 45 μl HiPerFect Transfection Reagent was added to the diluted miRNA mimic, and mixed by vortexing.

3. Mixed mimic and Transfection reagent was incubated for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes.

4. Incubated the complexes were then added drop-wise onto the cells for a total of 1 ml, with gentle swirling of the dish to ensure uniform distribution of the transfection complexes.

5. Finally, the cells were incubated with the transfection complexes under normal growth conditions. After 24 hours, the transfection was stopped by replacing the transfection media with culture media without transfection reagents. The media was changed every day thereafter, or as required. After 72 hours cells were trypsniised to extract protein.

### 4.1.6.2: Protein Extraction

All cell lines (normal thyroid, untransfected B-CPAP and transfected B-CPAP) were harvested and collected for protein extraction using NP40 Cell Lysis Buffer (Invitrogen, Grand Island, NY). Using the following protocol.

1. First, the cells were collected in PBS by centrifugation at 1200 rpm for 4 minutes.

2. The cells were then washed twice with cold PBS.

3. The supernatant was discarded and the cell pellet was collected.
4. Cell lysis buffer was prepared containing NP40 (100 µl/10^7 cells), PMSF (1:100) and PIC (protease inhibitor cocktail) (1:10). For 10^8 cells, 1 ml of Lysis Buffer was required. Then, the cell pellet was lysed in Cell Lysis Buffer for 30 minutes, on ice, with vortexing at 10 minute intervals.

5. The extract was then transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 10 minutes at 4°C.

6. Finally, the clear lysate was aliquoted into tubes (30 µl each tube) and stored at -80°C.

**4.1.6.3: Protein Assay**

The extracted protein quantity was assessed using a Protein Quantification Assay kit (MACHEREY-NAGEL GmbH & Co. KG., Germany) and was measured by microplate reader.

1. First, a BSA dilution series was prepared. Tube Number 1 contained only BSA solution (1x) (reference protein) and Tube # 7 contained only 50 µl PSB (protein solving buffer). To tubes # 2- #7, 50 µl PSB was added and BSA solution was added to tubes #2- #6. A serial dilution was done as follows: in #1 tube BSA concentration was 1µg/µl. In #2 tube BSA concentration was 0.5 µg/µl. In #3 tube BSA concentration was 0.25µg/µl. In #4 tube BSA concentration was 0.125 µg/µl. In #5 tube BSA concentration was 0.063µg/µl. In #6 tube BSA concentration was 0.031 µg/µl.

2. Then, 20 µl of each dilution series solution (#1- #7) was added into microplate wells.
3. In the next step, 20 µl of sample from cell lines was added to empty wells, followed by adding 40 µl PSB to each well (dilution series and protein samples) to make a final volume of 60 µl.

4. 40 µl Quantification Reagent (QR) was added to each well (dilution series and protein samples), followed by gentle shaking of the microplate (avoiding bubble formation as light scattering has an impact on the measurement) until a complete colour change from blue to yellow occurred.

5. The microplate was then incubated for 30 minutes.

6. The light extinction was then measured photometrically at 570 nm by a FLUOstar Omega Multi-mode microplate reader (BMG LABTECH GmbH, Allmendgruen Ortenberg, Germany).

7. Finally, protein concentration of samples was calculated in relation to the BSA dilution series (Figure 4.3).
Figure 4.3: Standard Curve for BSA serial dilution. The point on the right shows concentration of 1µg/µl, and the point on zero shows the concentration of 0.031µg/µl.

4.1.6: Western Blot Analysis

Twenty micrograms of protein was used for Western Blot Analysis. The gels and transmembrane blot were purchased from BIO-RAD. All the primary and secondary antibodies except anti-goat (Sigma-Aldrich, St. Louis, MO, USA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies were as follows:

**P53 (53 kDa):** p53 (Pab 1801): sc-98, a mouse monoclonal antibody, raised against amino acids 32-79 mapping near the N-terminus of p53 of human origin.

**Bcl2 (26 kDa):** Bcl-2 (9N-19): sc-492, a rabbit polyclonal affinity purified antibody, raised against a peptide mapping at the N-terminus of Bcl-2 of human origin.
GAPDH (37kDa): GAPDH (FL-335): sc-25778, a rabbit polyclonal antibody raised against amino acids 1-335 representing full length GAPDH of human origin.

β-Actin (43 kDa): β-Actin (C4): sc-47778, a mouse monoclonal antibody raised against gizzard Actin of avian origin.

HIF-1α (132kDa): HIF-1α (H-206): sc-10790, a rabbit polyclonal antibody raised against amino acids 575-780 of HIF-1α of human origin.

VEGF-A (42kDa): VEGF (A-20): sc-152, a rabbit polyclonal raised against a peptide mapping at the N-terminus of VEGF-A of human origin.

ET-1(24 kDa): ET-1 (N-8): sc-21625, a goat polyclonal antibody raised against a peptide mapping within an internal region of endothelin-1 of human origin.

ET₄R (69kDa): ETAR (H-60): sc-33535, a rabbit polyclonal antibody raised against amino acids 21-80 mapping within an N-terminal extracellular domain of ETAR of human origin.

The ladder used was a Precision plus protein TM Western C TM standard, (BIO-RAD) with an attendant secondary antibody, Precision-Protein TM, Strep T-actin-HRP conjugate (BIO-RAD). β-actin and GAPDH were used as positive controls. The intensity of the bands was measured by the image software (VersaDoc, BIO-RAD).
4.1.6.4.1: Protocol for Western Blot

After optimisation for each antibody, Western Blot analysis was performed as follows:

1. First, the protein concentration was calculated for each well. Final protein used in each well was 20µg of protein, and total volume in each well was 20µl. Each sample was prepared as follows: 5µl of Laemmli sample buffer (4x) was added to 1µl of β-mercaptoethanol, and 20 µg of protein. Final volume was adjusted to 20 µl by adding distilled water. Laemmli sample buffer contains Bromo phenol which makes the bands visible.

2. After preparing the samples, they were heated to 95ºC for 5 minutes.

3. Then, running Buffer was prepared. Running buffer was 10X Tris/Glycine/SDS Buffer (Bio RAD. Cat No. 161-0772). To prepare 1x running buffer, 120 ml buffer was added to 1080 ml distilled water and shaken rigorously.

4. A ready to use gel (Mini-Protean TGX Gels, 4-15%, 10 well comb, 30µl) was used. The comb was then removed (with wet gloves), and the gel was washed with running distilled water, then the strip at the bottom of the gel was removed.

5. In the next step, the gel was placed properly, followed by pouring running buffer in the tank. The gap between the gels was filled with running buffer.

6. After loading the samples (20 µl in each well), and 10 µl ladder (Precision Plus Protean, Dual Code Standards, BIO-RAD) the whole tank was filled with buffer.
7. The power supply was set to 100 volts, turned on and ran until bands reached to the bottom. As Laemmli sample buffer was a deep blue (containing Bromophenol blue), the bands were obvious.

8. After gel electrophoresis, the gel was released and placed in distilled water, then carefully transferred to the staining membrane (Trans-Blot Turbo Transfer System, BIO-RAD). During transfer gloves and all equipment were kept wet. Any air trapped between the pad and membrane was removed by using a blot roller. The transfer device was set to 2.5 A, 25 V for 10 minutes.

9. At the end of the run, the membrane was removed, and washed with PBS for 5 min on a shaker.

10. To make sure that the protein bands were transferred to the membrane, and for locating protein bands on western blots, the membrane was placed in Ponceau S solution (Sigma-Aldrich). After 5 min of manual shaking the Ponceau S solution was discarded. The membrane was washed with distilled water twice (Figure 4.4). To destain, the membrane was placed in 0.1M NaOH for 2-4 minutes until to get destained. After destaining, the membrane was washed with distilled water and then with PBS on the shaker.
Figure 4.4: Staining with Ponceau S solution. This figure shows proteins moving according to their weights. The first two columns are Nthy-ori3-1cell lines, and the second two columns are K1 cell lines. On the left side the standard ladder shows 250kD molecular weight on top and 10 kD molecular weight at the bottom.

11. To make sure that all protein bands had been transferred to the membrane properly and nothing had been left on the gel, the gel was stained with Coomassie staining.

12. The membrane was then incubated in cold low fat milk solution (5% milk in PBST) for 2 hours on the shaker to perform a protein block.
13. Protein block was followed by incubation with primary antibody overnight at 4° on the shaker. All primary antibodies were diluted in cold milk solution (5% non-fat dried milk in 0.1% Tween 20 in PBS). P53, Bcl2, ET-1, ET\textsubscript{A}R, HIF-1\textalpha were diluted at 1:100, VEGF-A was diluted at 1:300, GAPDH diluted at 1:500 and β-actin was diluted at 1:1000.

14. After incubation, the membrane was washed 2-6 times in PBST each for 5 min, and the membrane was then incubated with secondary antibody diluted in cold milk solution (5% in PBST). The secondary antibodies were as follows:

Goat Anti-mouse IgG-HRP, Goat Anti-rabbit IgG -HRP, and Anti-Goat IgG (whole molecule)-Peroxidase. The secondary antibodies for P53, Bcl2, ET-1, ET\textsubscript{A}R, and HIF-1\textalpha were diluted at 1:1000, for VEGF-A, GAPDH and β-actin secondary antibodies were diluted at 1:5000. The ladder secondary antibody (Precision Protean Strep T actin-HRP conjugate, BIO-RAD) was diluted at 1:10,000.

15. The membrane was then washed 3-6 times in PBST for 5 mins each. A final wash was with PBS removed any remaining Tween 20.

16. Finally, the membrane was incubated in chemiluminescence reagent (BIO-RAD) for 3-5 min. The reagent was a mixture of Luminol-Enhancer Solution and Peroxide Solution 1.5 ml each. Images were taken by a VersaDoc Imaging System (BIO-RAD).
4.1.6.4.2: Protocol for Preparing Coomassie Staining

1. First, to prevent protein diffusion, fixation buffer was prepared by adding 40% distilled water, 10% acetic acid, and 50% methanol. The gel was kept in this mixture for 30 minutes – 1 hour.

2. Then, to visualise the fixed proteins, the same mixture (point 1) was added to 0.25% Coomassie Brilliant Blue R-250. After filtering with Whatman No.1 filter paper, the gel was incubated in this mixture for 4 hours at room temperature on a shaker (Figure 4.5).

Figure 4.5: Coomassie staining of the gel. Presence of strong staining shows the transferral of the majority of proteins to the membrane.
4.1.6.4.3: Protocol for Destaining of the Gel (Coomassie staining)

1. First a mixture of 67.5% distilled water, 7.5% acetic acid, and 25% methanol was made.

2. The gel was then incubated in this mixture on the shaker till the stain was removed.

3. The gel was then stored in 5% acetic acid solution at 4°C until needed for immunostaining.

4.1.6.5: Immunofluorescence Staining

In addition to western blotting for angiogenic protein expression, normal thyroid (N-Thy-ori 3-1), untransfected B-CPAP and transfected B-CPAP cells were stained using immunofluorescence method for VEGF-A antibody as follows.

1. First the cells were grown in 6 well plates with a coverslip at the bottom.

After 72 hours the cells were fixed by incubation with 2% formaldehyde for 10 min at room temperature.

2. For permeabilization, the cells were rinsed 3 times in PBS, 2 min each, followed by incubation with PBS containing 0.25% Triton X-100 for 30 minutes. For membrane bound antigens this step was omitted to prevent membrane destruction and the cells were incubated directly with protein block.

3. After 3 washes with PBS the cells were incubated with 1% BSA in PBS for 30 min to block non-specific binding of the antibodies.
4. Then, the cells were incubated with the diluted primary antibody 1:50 (VEGF Mouse Monoclonal Antibody; clone VG1, Invitrogen, Life technologies, Carlsbad, CA, United States of America) in 1xPBS and 1%BSA at room temperature for 1-2 hours.

5. After washing with PBS three times the cells were incubated with secondary antibodies for 1 hour; FITC (Goat Anti-Mouse polyclonal antibody; abcam, Sapphire Bioscience Pty. Ltd.) at 1/1000 dilution. From this step to the end the slides were protected from the light by working in a dark room.

6. The cells were washed with PBS 5 times for 5 min each.

7. The coverslip was then taken out and mounted on a microscope slide with a drop of DAPI (fluoroshield with DAPI, Sigma Aldrich, St. Louis, Mo, USA) included to counterstain nuclei.
4.2: Results

4.2.1: Detection of miR-195 by RT-PCR in Primary Thyroid Carcinomas

The level of miRNA-195 expression was determined in all primary and metastatic papillary thyroid carcinomas as well as in non-cancer thyroid tissue. miR-195 expression level was elevated in 18.7% of the primary carcinomas (n=23) compared to the control tissues. The expression was low in 69.9% (n=86) and normal in 11.4% (n=14) of the primary carcinomas. Metastatic thyroid cancers in lymph node mostly showed low expression in 45% (n=18). The relationship between the expressions miRNA-195 with the clinicopathological features of papillary thyroid carcinomas are shown in Table 4.1.

There was a significant relationship between miR-195 expression level and tumour size (p=0.002) (Figure 4.7), as low expression levels were mostly detected in small tumours (72.7% vs 46.2%). A significant relationship was also detected between miR-195 expression level and T staging (p=0.020) (Figure 4.8). Low expression levels were mostly noted in the T1 and T2 category compared to T3 (74.4% vs 62.2%). There was a significant difference between miR-195 expression in thyroid cancers with lymph node metastasis and thyroid cancers without metastasis, as high expression levels were mostly detected in thyroid cancers without metastasis in 19.3% (n=16) (p=0.017) (Figure 4.9). Other than these characteristics, miR-195 expression did not correlate with the age, histological subtype, and presence of psammoma bodies, calcification, osseous metaplasia, and pathological stage of thyroid cancer or the presence of co-existing lymphocytic thyroiditis (p > 0.05).
Table 4.1: The relationships between miR-195 expression and clinicopathological characteristics of 123 papillary thyroid carcinoma

<table>
<thead>
<tr>
<th>Clinical &amp; pathological variables</th>
<th>miR-195 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male (n= 41)</td>
<td>7 (17.1%)</td>
</tr>
<tr>
<td>Female (n=82)</td>
<td>16 (19.5%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;45 (n=67)</td>
<td>11 (16.4%)</td>
</tr>
<tr>
<td>≥45 (n=56)</td>
<td>12 (21.4%)</td>
</tr>
<tr>
<td>Tumour size (mm)</td>
<td></td>
</tr>
<tr>
<td>≤40 mm (n=110)</td>
<td>16 (14.5%)</td>
</tr>
<tr>
<td>&gt;40 mm (n=13)</td>
<td>7 (53.8%)</td>
</tr>
<tr>
<td>T staging</td>
<td></td>
</tr>
<tr>
<td>T1 or T2 (n=78)</td>
<td>9 (11.5%)</td>
</tr>
<tr>
<td>T3 (n=45)</td>
<td>14 (31.1%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>Positive (n=40)</td>
<td>7 (17.5%)</td>
</tr>
<tr>
<td>Negative (n=83)</td>
<td>16 (19.3%)</td>
</tr>
<tr>
<td>TNM staging</td>
<td></td>
</tr>
<tr>
<td>Stages I or II (n=87)</td>
<td>13 (14.9%)</td>
</tr>
<tr>
<td>Stage III (n=36)</td>
<td>10 (27.8%)</td>
</tr>
<tr>
<td>Pathological variant</td>
<td></td>
</tr>
<tr>
<td>Conventional (n=79)</td>
<td>16 (20.3%)</td>
</tr>
<tr>
<td>Follicular (n=44)</td>
<td>7 (15.9%)</td>
</tr>
<tr>
<td>Psammoma body</td>
<td></td>
</tr>
<tr>
<td>Present (n=52)</td>
<td>9 (17.3%)</td>
</tr>
<tr>
<td>Absent (n=71)</td>
<td>14 (19.7%)</td>
</tr>
<tr>
<td>Calcification in stroma</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Present (n=62)</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>OSSEOUS METAPLASIA IN STOMA</td>
<td></td>
</tr>
<tr>
<td>Present (n=6)</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>Absent (n=117)</td>
<td>22 (18.8%)</td>
</tr>
<tr>
<td>LYMPHOCYTIC THYROIDITIS</td>
<td></td>
</tr>
<tr>
<td>Present (n=40)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>Absent (n=83)</td>
<td>14 (16.9%)</td>
</tr>
</tbody>
</table>

* Statistically significant
Figure 4.5: miR-195 expression levels in gender groups. Low miR-195 expression levels were mostly noticed in males; however, the difference did not reach to a significant point (p=0.228).
Figure 4.6: miR-195 expression level in different age groups. Although low miR-195 expression levels were mostly detected in patients younger than 45 years old, there was not any significant difference statistically speaking ($p=0.691$).
Figure 4.7: miR-195 expression levels in different tumor size. There was a significant difference between examined groups. Low miR-195 expression levels were mostly found in PTCs smaller than 40 mm (p=0.002).
Figure 4.8: miR-195 expression level in different T staging. There was a significant difference between examined groups. Low miR-195 expression levels were mostly found in PTCs with T1 and T2 categories (p=0.020).
Figure 4.9: miR-195 expression level and its correlation with metastasis to regional lymph nodes. The graph shows a significant difference between thyroid carcinomas with and without lymph node metastasis regarding miR-195 expression level. Low miR-195 expression levels were mostly detected in PTCs with metastasis to lymph nodes (p=0.017).
Figure 4.10: miR-195 expression levels in different TNM staging. Low miR-195 expression levels were mostly detected in Stages I or II, however, there was no significant difference between different stages (p=0.147).
Figure 4.11: miR-195 expression level in different Pathological Variants. Although low miR-195 expression levels were more common in follicular variant tumors, there was not any significant difference between pathological variants (p=0.355).
Figure 4.12: miR-195 expression level regarding Psammoma body. Diagram shows low miR-195 expression levels were more common in PTCs with Psammoma body, however, there was no significant difference between examined groups (p=0.791).
Figure 4.13: miR-195 expression level regarding the presence of Calcification in stroma.

Low miR-195 expression levels were more common in the absence of calcification in stroma, however, there was any significant difference between examined groups (p=0.494).
Figure 4.14: miR-195 expression level regarding Osseous metaplasia in stroma. Low miR-195 expression levels were mostly noticed in PTCs without osseous metaplasia in stroma, however, statistical analysis did not show any significant difference between examined groups (p=0.639).
Figure 4.15: miR-195 expression level regarding Lymphocytic thyroiditis. Diagram shows although low miR-195 expression levels were mostly detected in the presence of lymphocytic thyroiditis, but statistical analysis did not show any significant difference between examined groups (p=0.738).
Figure 4.16: miR-195 expression levels in thyroid cancers without metastasis and metastatic lymph nodes. Thyroid cancers without lymph node metastasis tended to have lower expression levels than thyroid cancers with lymph node metastasis. In addition, metastatic lymph nodes showed mostly lower expression levels than primary thyroid cancers that produced metastases.
4.2.2: Comparison Between the Expression in Primary Cancer and the Matched Lymph Node with Metastatic Cancer

There was a significant difference between primary cancers and metastatic thyroid cancers in the lymph node (p=0.001). Low expression was noted as being more common in primary thyroid cancers compared to lymph nodes (69.9% vs 45%). miR-195 was more often down-regulated in those thyroid carcinomas with lymph node metastases compared to thyroid carcinomas without lymph node metastases (82.5% vs 63.9%, p=0.017).

There was also a significant difference between miR-195 expression level in primary cancers with metastasis and metastatic tumours in lymph nodes (p<0.001). Lower expression levels were mostly detected in primary cancers with metastasis (82.5% vs 45%).

Similarly, a significant difference was detected between miR-195 expression level in thyroid carcinomas without lymph node metastases compared to metastatic thyroid cancer in lymph node (p=0.037). miR-195 was mostly down-regulated in thyroid carcinomas without lymph node metastases as compared to the lymph nodes themselves (63.9% vs 45%).
4.2.3: Correlation Between miR-195 and ET-1 Axis Expression Levels in PTC

Primary thyroid cancers showed the low expression level of both miR-195 and ET-1 mRNA in 83.1% (49/123) of cases.

Indeed, Pearson correlation testing showed a significant positive correlation between miR-195 and ET-1 expression in primary thyroid cancers (Pearson r =0.254, p<0.005).

4.2.4: Comparison Between the Expression in K1 and B-CPAP Cell Lines

Initially, we examined the expression level of miR-195 in K1 cell line (derived from a papillary thyroid carcinoma). There was no significant difference between miR-195 expression level in normal thyroid cell line (Nthy-ori3-1, derived from human thyroid follicular epithelium) and the K1 cell line. Then, miR-195 expression level was examined in the B-CPAP cell line (derived from a metastasizing papillary thyroid carcinoma). miR-195 expression level was down-regulated in B-CPAP cell line with a fold change of 43. 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 compared to thyroid carcinomas without lymph node metastases.
4.2.5: VEGF-A Immunofluorescent Staining Results

B-CPAP cells transfected with miR-195 showed fewer VEGF-A positive cells, as well as showing near complete loss of protein expression. This can be seen in Figure 4.17.

Figure 4.17: VEGF-A Immunofluorescent staining. Staining of B-CPAP cell, DAPI stained nuclei (blue) and expression of VEGF-A (green). Introduction of exogenous miR-195 decreases production of VEGF-A in the examined cells.
4.2.6: Western Blot Results

The effect of altered miR-195 expression level was compared in the Nthy-ori3-1 cell line, untransfected B-CPAP, and miR195 transfected B-CPAP cell lines. The used primary antibodies were ß-actin and GAPDH, VEGF-A and HIF-1α, ET-1 and ETₐR, and p53 and Bcl2. Due to difficulties in optimization, accurate Western Blots were not possible but general trends could be observed. These are discussed below, while pictures of the rough Western Blot results can be seen in Appendix A.

4.2.6.1: The Decreased Protein Levels

The protein level in transfected cells was decreased significantly for VEGF-A and Bcl2 compared to untransfected B-CPAP cells.

4.2.6.2: The Unchanged Protein Levels

ET-1 and ETₐR protein levels did not show any significant difference in B-CPAP cells transfected with miR-195 compared with untransfected B-CPAP cell lines and Nthy-ori3-1 cell lines.
4.2.6.3: Undetected Protein Levels

For HIF-1α no bands were detected in all cell lines examined, consistent with the high turnover of this gene in oxygenated conditions. However, for p53 a band was detected in the Nthy-ori3-1 cell line but not for the B-CPAP cell line, either untransfected or transfected.

4.2.6.4: VEGF-A and Bcl2 are direct targets of miR-195

The decreased protein level in miR-195 transfected cells for VEGF-A and Bcl2 indicates that VEGF-A and Bcl2 miR-195 negatively regulates their expressions in B-CPAP cells and that they are likely to be direct targets of miR-195 (Figure 4.17).
4.3: Discussion

miRNAs play important roles in a variety of cellular processes such as cell proliferation, differentiation, and apoptosis (21). Recently, miRNAs have become a focus of interest due to an increased number of studies demonstrating that deregulation of miRNAs commonly happens in cancer progress (25). There is accumulating data suggesting that miRNAs show altered expression in human thyroid carcinomas depending on their histological subtype (302, 303). Previous study on human thyroid cancer indicated that most miRNAs were significantly deregulated, and that most of them were up-regulated and that no miRNAs were down-regulated with a fold change higher than two (304). However, another study showed that 38% of miRNAs were down-regulated with more than a 2-fold change in thyroid cancer compared to 32% up-regulated miRNAs. In addition, it has been indicated that in all well differentiated thyroid cancers more miRNAs showed higher expression levels compared to less differentiated cancers. Some of these up-regulated miRNAs in PTC include miR-187, miR-221, miR-222, miR-181b, miR-146b, miR-155, and miR-122a (302).

Recent studies have demonstrated that miRNA expression alterations contribute to the initiation and progression of cancer (305). But the expression levels or functions of miRNAs in normal and tumour cells in different cancers, including thyroid cancer, have been incompletely characterised. In this research, we attempted to further understand the potential functional role of miR-195 in angiogenesis thyroid cancer cells.

In the present study, we assessed the miR-195 expression in a large series of PTCs, and correlated miR-195 expression with clinicopathological data. miR-195 expression level was low in 69.9% (n=86) of the cases. We found a significant decrease of miR-195 expression in
primary tumours compared to normal tissue, suggesting that miR-195 may play a role in the thyroid cancer development by suppressing oncogenic genes or pathways, such that loss of this repression increases transformation of cells.

Down-regulation of miR-195 has been identified in some other cancers such as hepatocellular carcinoma, esophageal carcinoma, and colorectal carcinoma (42, 44, 278, 280, 283, 306-313). This would indicate that similar oncogene repression may be occurring in these cancer types as well. The underlying mechanism for down-regulation of miR-195 in some cancers is not clear. However, miR-195 is located at chromosome 17p13.1 which has been reported to be deleted in human cancers, so allelic deletion may be a key mechanism to down-regulate it (42).

In the current study, there was not any significant difference between miR-195 expression levels in different genders. This finding is in agreement with other studies and indicates that the mechanisms it affects in thyroid cancer are not related to those effects that cause the disease to be more common in women than in men (44, 279, 311).

In our series, there was a significant difference between miR-195 expression level and tumour size (p=0.002), as lower expression levels were mostly detected in smaller tumours (n=80; 72.7%). In addition, there was a significantly different expression level between the patients with T1 or T2 tumours and patients with T3 tumours, lower expression levels were mostly indicated in T1 or T2 (n=58; 74.4%) (p=0.020). However, in a study on human tongue squamous cell carcinoma, miR-195 was mostly down-regulated in larger tumours (T3, T4) (311), and in Heneghan et al. study, circulating levels of miR-195 correlated significantly with tumour size in breast cancer, higher systemic mir-195 levels were observed in small tumours.
(T1 and T2) (314). Although these differences could be explained by the fact that miR-195 was up-regulated in breast cancer. Studies in gastric and colorectal cancer, however, did not show any significant difference between miR-195 expression level and T category (279, 280). This would indicate that these tumour size effects may be specific to the biology of thyroid cancer and do not represent effects on vasculature or angiogenesis that would regulate cancer size.

In our series, cancers with lymph node metastasis mostly expressed lower miR-195 expression levels compared to cancers without lymph node metastasis (82.5% vs 63.9%). This finding was confirmed by the cell culture study, as miR-195 expression level was down-regulated in B-CPAP cell line not in K1 cell line, reflecting their original metastatic status. Similar results were observed in a study on colorectal cancer, indicating the reduced miR-195 expression level was significantly related to lymph node positivity (44). In a study on esophageal squamous cell carcinoma, it was noted that down-regulated miRNAs, including miR-195 were related to invasion and lymph node metastasis (306).

In addition, down-regulation of miR-195 in thyroid cancers with lymph node metastasis compared to the thyroid cancers without lymph node metastasis clearly shows the role of miR-195 in invasion and metastasis. This finding was also shown in glioblastoma cells and hepatocellular carcinoma (38, 44). The metastatic thyroid cancers in lymph nodes also showed a lower expression level in 45% of cases which might show that even after metastasis cancer cells keep miR-195 at a low level to complete metastatic processes or to re-metastasize to new locations.

As the expression level of miR-195 in thyroid carcinogenesis has been clearly established, we can assume that the down-regulation of miR-195 might contribute to the
development of PTCs, and on into lymph node metastasis, though its role in angiogenesis, which is essential for metastasis, is perhaps less clear (315).

miR-195 has previously been implicated as a tumour suppressing miRNA, which our results would also indicate. The role of miR-195 in arresting the cell cycle in the G1/S via CCND1, and E2F3 has been demonstrated (38), but anti-angiogenic effects could be another pathway for its function as a tumour suppressor. Very recent work has reported that miR-195 has a role in angiogenesis in cancers by targeting VEGF, one of the most important pro-angiogenic factors secreted by cancer cells (44). Therefore, it seems likely that miR-195 has dual functions in some cancer cells; operating as a tumour suppressor by inhibiting both cell proliferation and invasion (21), and as an anti-angiogenic factor by reducing the capacity to secrete VEGF (38).

In our study, transfection with a miR-195 mimic inhibited the expression of VEGF-A, the most potent angiogenic growth factor (316), in the B-CPAP cell line. This finding confirms the results of Wang et al. who showed suppression of VEGF following application of miR-195 in hepatocellular carcinoma (44). This clearly shows the potential role of miR-195 in angiogenesis in thyroid cancer and the implications of the loss of expression of the miRNA. Angiogenesis is an essential factor in the development of a cancer, with VEGF and its receptor (VEGFRs) contributing significantly to tumour-associated angiogenesis (317).

In regards to HIF-1α in this study, no protein was detected in the examined cells in normoxic conditions, as was expected, as the protein is highly transient in normal conditions. HIF-1α mediates expression of VEGF to stimulate angiogenesis and vascular permeability in cancers (318). HIF-1α is also involved in cell proliferation and apoptosis, and its
overexpression is a negative prognostic factor and correlated with increased proliferation (319). The lack of increased HIF-1α expression following miR-195 knockdown indicates that loss of VEGF expression does not automatically result in an increased signalling for angiogenesis by compensatory activation of pseudohypoxic signals, and any feedback mechanisms in thyroid cancer likely require direct oxygen starvation not present in cultured cells.

There is some evidence that HIF-1α interacts on a feedback level with Bcl2, another miR-195 target, along with p53. In some cancers such as non-small cell lung cancer, HIF-1α expression is inversely associated with Bcl2 expression, and in breast cancer they have a strong positive correlation (320, 321). On the other hand, it has been proven that there is an inverse relationship between p53 and Bcl2 in cancers, while p53 is known to inhibit HIF-1α activity by targeting the HIF-1α subunit for ubiquitination, and the loss of p53 enhances HIF-1α levels (51, 54). VEGF expression is also connected with p53 overexpression or mutation (316). In addition, p53 is required for VEGF induction during the initial phases of hypoxia (322). Mutant p53 or p53 deficiency can up-regulate HIF-1α activity and VEGF overexpression in tumour cells under normoxic condition (323). p53 can regulate hypoxic signaling through some miRNAs such as miR-107 by suppressing the expression of HIF-1β, as well as VEGF expression (324). p53 locates at the same genomic locus on chromosomal band 17p13.1 as the microRNA cluster miR-195 suggesting that multiple tumour suppressor genes may exist on this chromosome and potentially be expressed or silenced by related mechanisms. Decreased p53 expression and down-regulation of miR-195 have been shown in primary peritoneal carcinoma (325).
Previous studies have indicated that Bcl2 is inversely correlated with miR-195 levels, which is linked to mediation of apoptosis in colorectal cancer (317-319). Therefore, we extended our studies to include Bcl2 as well as p53 to gain a more complete picture of how these mechanisms interact with miR-195 transfection. In the present study, normal cell lines showed a strong band for p53 but both untransfected and miR-195 transfected B-CPAP cells did not show any bands. It can be explained by the presence of a mutation in p53 in B-CPAP cells resulting in silencing of expression, or rendering the protein undetectable to the antibody used (326). ET-1 and its receptor ET\(_A\)R have an essential role in carcinogenesis. The ET-1 axis activates angiogenic pathways by inducing stabilization of HIF-1\(\alpha\) which up-regulates VEGF transcription (95, 101). Next the role of ET-1 axis in miR-195 induced angiogenic function was examined. Normal thyroid cells, untransfected B-CPAP cells and miR-195 transfected B-CPAP cells showed bands for both ET-1 and ET\(_A\)R, unaffected by miR-195 transfection. This would indicate that miR-195’s effects on cell proliferation, invasion, metastasis and angiogenesis are not mediated through the ET-1 pathway, at least directly in thyroid cancer cells.

In conclusion, our results have revealed that the levels of miR-195 are much lower in PTC cell lines compared to normal thyroid cells. In addition, our finding suggest that miR-195 in PTC functions as an anti-angiogenesis and tumour suppressing factor in cancer through knockdown of VEGF and Bcl2, but does not exert effects via HIF-1\(\alpha\) or p53. miR-195 also appears to play a role in invasion and metastasis. Additionally, it could be said that miR-195 as a tumour suppressor gene is not related to manipulation of the ET-1 axis directly in cancer cells, though it may in cells that produce endogenous ET-1 by affecting pathways that ET-1 also affects.
The identification of miRNAs as anti-angiogenetic or tumour suppressor factors might help in finding new diagnostic markers for human cancers and possibly for anticancer therapy.
Chapter 5:

Conclusions and Further Studies
5.1: Conclusion

Angiogenesis has a crucial role in cancer development. Previous studies on thyroid cancer have indicated the role of angiogenesis in thyroid cancer (327, 328). Many factors have been known as angiogenic factor, however, VEGF is the most potent factor involved in cancer development (329).

The endothelins (ETs) induce DNA synthesis and cellular growth in different cells, affecting vascular tone and angiogenesis (16). ET-1, the most common circulating form of endothelin, can stimulate proto-oncogene expression in vascular and non-vascular cells (18). ET-1 induces VEGF expression by increasing hypoxia-inducible factor-1α (HIF-1α) stimulation (20).

On the other hand, miRNAs are involved in cell proliferation, development, differentiation and metabolism (21). Some miRNAs function as an angiogenic factor in quiescent endothelial cells. There are both pro-angiogenic miRNAs and anti-angiogenic miRNAs. (25). The role of miRNAs in the development of metastases has also been shown in a variety of cancers (23).

The miR-15/16 family are considered to be tumour suppressor miRNAs due to their targeting Bcl-2 (38). miR-15b and miR-16 might be involved in the control of angiogenesis because both can control VEGF expression (40). MicroRNA-195 has a pivotal role in tumourigenesis as a tumour suppressor. It is suggested that miRNA-195 promotes apoptosis, mainly through targeting Bcl-2 expression (38). The regulatory role of miR-195 in angiogenesis and specifically its direct effect on VEGF-A has been described (44).
5.2: Addressing Aim 1

The majority of previous studies on ET-1 axis have been performed on cancer cell lines. In addition, there is only one study on thyroid cancer, especially PTC, and the ET-1 axis. In this study, we determined the levels of expression for members of the endothelin axis and miR-195 in a large population of thyroid cancer tissues.

There were difficulties in the conduction of the experiment, and one of the first of these, which will have added to the degree of error in the research, is the difficulty in obtaining good quality RNA from formalin fixed tissue. RNA can degrade prior to formalin fixation. In addition, due to chemical modifications in formalin-fixed paraffin-embedded (FFPE) tissue, RNA degradation continues over time. Freezing of fresh tissue samples preserves good quality RNA for gene expression studies; however, this procedure is not routinely performed in most hospitals which makes obtaining such tissues difficult. In addition to RNA degradation concerns, formalin fixation creates cross-linking between nucleic acids and proteins (330). All these facts explain the difficulties of extracting mRNA from FFPE and the need to examine the possibility of obtaining fresh frozen tissue.

Additionally, despite the relatively large number of thyroid tissues we were able to obtain, during our analysis the numbers for certain pathological subcategories were very low. This means that some of the later analyses performed have a high degree of uncertainty about their accuracy. In the future, more specific collections may need to be made to determine whether or not members of the endothelin axis affect the development of such characteristics.
Several miRNAs are involved in angiogenesis in cancers. In this study only one of them was studied. miRNA-195 down-regulation in PTCs may influence the risk of cancer development and regional lymph node metastasis through VEGF-1 and Bcl2. Down-regulation of miRNA-195 in metastatic cell line (B-CPAP) compared to less aggressive cell line (K1) may confirm its role in metastasis.

5.3: Addressing Aim 2

Expression levels of different mRNAs in different cancers and their correlation with clinicopathologic characteristics can give a lot of information about cancer development. There are very few previous studies on ET-1 mRNA expression levels in tissues and its correlation with clinicopathologic characteristics in cancers, especially in thyroid cancer. A few studies have indicated ET-1 axis expression by using IHC. There is only one study on thyroid cancer tissue regarding ET-1 expression level. This study was performed on 12 surgical human thyroid samples (68). Therefore, it was impossible to compare the clinicopathological characteristic results with other studies. On the other hand, all previous studies on ET-1 expression levels were done at protein level by using IHC (296, 297, 299, 331) and one study was done on blood samples (301). Despite the risk of mRNA degradation as discussed above, the size of the study population helps to smooth out the error caused by this factor and improves the accuracy of our observations.
Elevated ET-1 and ET₄R expression levels were more common in males; however, PTC is more common in females (49). This finding may indicate that PTC in males is more aggressive when it develops.

Both ET-1 and ET₄R were up-regulated in larger tumors, which might be due to higher oxygen demands in PTC, and ET-1 axis role in angiogenesis.

Regarding T category and staging, both ET-1 and ET₄R were elevated in the T3 category, suggesting ET-1 axis has a role in EMT, and invasion. In addition, both markers were up-regulated in Stage III, indicating the role of ET-1 axis in metastasis.

Several attempts to classify PTCs using molecular markers have been made. In the current study, ET-1 was elevated in conventional variant and ET₄R was up-regulated in follicular variant, however the differences did not reach to a significant level. Therefore, ET-1 axis expression levels cannot be used for PTC classification.

The presence of psammoma body, calcification in stroma, and osseous metaplasia in stroma in PTCs with higher ET-1 expression levels might suggest that PTCs with higher expression levels are older lesions and have more time to form psammoma body, calcification in stroma and to develop osseous metaplasia.

Although the difference was not significant, low miR-195 expression levels were more common in males. High ET-1 axis expression levels were also more frequent in males, which again points to the possibility that PTCs are more aggressive in males.
ET-1 high expression levels were more common in patients older than 45 years old, on the other hand, low miR-195 levels were more common in the patients younger than 45 years. In addition, High ET-1 axis expression levels were more frequent in larger tumors, however, low miR-195 expression levels were more frequent in smaller tumors. These findings make a certain amount of sense, given the ability for miR-195 to interfere in angiogenic mechanisms which may explain why ET-1 axis and miR-195 expressions are not correlated to each other.

Low miR-195 expression levels were more frequent in T1 or T2 category cancers. In addition, low expression levels were more common in PTCs with positive lymph node metastasis, besides in stages I or II. All these findings may indicate that miR-195 negatively control the invasion of PTC as well as the metastasis to the regional lymph nodes.
5.4: Future Directions

Elevated expression of ET axis in our research was produced by significantly different proportions of primary thyroid cancers or metastases compared to primary cancers. The possibility of the production of these members of ET family by endothelial cells within highly vascularized primary tumors and metastases should be investigated more in detail. The use of markers such as CD34 and Lymphatic Vessel Endothelial Hyaluronan (HA) Receptor-1 (LYVE-1) in further research could serve as a tool to recognize specific changes in the vasculatures in primary and metastatic tumors.

The results in this research suggest that ET axis expression could be used as a possible indicator to predict the aggression levels of papillary thyroid carcinomas, identifying which carcinomas have metastasised as well as helping to locate the presence of new and developing metastases in lymph nodes and other locations, due to the role of the axis in establishing tumour vasculature. The identification of the missing link between ET axis expression and cancer aggression is needed to determine whether ET axis levels in thyroid cancer tissues could serve as prognostic markers, or an indicator of response to therapy. Our results have also shown, however, that this relationship is complex, and further research should be done to determine why some tumours show increased RNA expression and why others show reduced expression. Despite both forms of tumours showing high protein expression, the differential RNA levels may represent important physiological differences between these tumour subgroups. Since RNA is a more precise molecule in terms of the measuring tools available, understanding the significance of these RNA differences is important if the endothelin axis is to be used as a diagnostic tool in future.
Expression levels of different miRNAs are different in malignant tumors, benign tumors, non-neoplastic lesions and normal tissues. Therefore, miRNAs can differentiate malignant tumors from benign conditions and normal tissue. Due to the potential role of miR-195 in this study, in particular its link with invasion and metastasis, further investigation should be performed to confirm the exact role of miR-195 in angiogenesis, invasion and metastasis in other cancers.

Although we expected to observe a stronger correlation between angiogenic genes in thyroid cancer and miR-195, this study only noticed a particularly strong effect on VEGF-A expression. Further investigation should run broader genetic assays to clearly present the interaction of this miRNA and angiogenic gens such as ET axis. Determining the interaction between angiogenic pathways and how miRNAs mediate such interactions is another area that should be pursued in order to maximise our ability to manipulate this system in future cancer therapies.
References:


187


301. Hagemann T, Binder C, Binder L, Pukrop T, Trumper L, Grimshaw MJ. Expression of endothelins and their receptors promotes an invasive phenotype of breast tumor cells but is insufficient to induce invasion in benign cells. DNA Cell Biol. 2005;24:766-76.


Appendix A

Figure A.1: VEGF-A Western Blot. VEGF-A (42 kDa): First column is standard ladder, the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of the band for miR-195 transfected B-CPAP is stronger than the Normal thyroid and miR-195 untransfected B-CPAP bands.
Figure A.2: Bcl-2 Western Blot. Bcl-2 (26 kDa): First column is standard ladder, the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of the band for miR-195 transfected B-CPAP is stronger than the Normal thyroid and miR-195 untransfected B-CPAP bands.
Figure A. 3: p53 Western Blot. p53 (53 kDa): First column is standard ladder; the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. A strong band can be seen for normal thyroid cells, B-CPAP shows weak staining in both cases.
Figure A.4: ET-1 Western Blot. ET-1 (24 kDa): First column is standard ladder; the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of band for all samples examined (Normal Thyroid, miR-195 transfected B-CPAP, and miR-195 untransfected B-CPAP is the same, with weak intensity.
Figure A. 5: ETAR Western Blot. ETAR(68 kDa): First column is standard ladder, the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of band for all samples examined (Normal Thyroid, miR-195 transfected B-CPAP, and miR-195 untransfected B-CPAP is same with weak intensity.
Figure A. 6: HIF-1α Western Blot. HIF-1α (132 kDa): First column is standard ladder; the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The is no band for all samples examined Normal Thyroid, miR-195 transfected B-CPAP, and miR-195 untransfected B-CPAP.
Figure A.7: GAPDH Western Blot. GAPDH (37kDa): First column is standard ladder; the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of band for all samples examined (Normal Thyroid, miR-195 transfected B-CPAP, and miR-195 untransfected B-CPAP is same with medium intensity.
Figure A.8: β-Actin Western Blot. β-Actin (43 kDa): First column is standard ladder, the other columns are Normal Thyroid, miR-195 untransfected B-CPAP and miR-195 transfected B-CPAP, respectively. The intensity of band for all samples examined (Normal Thyroid, miR-195 transfected B-CPAP, and miR-195 untransfected B-CPAP) is same, with strong intensity.