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Expression profile of endothelin 1 (ET-1) and its receptor endothelin receptor A (ET_AR) in papillary thyroid carcinoma and their correlations with clinicopathological characteristics

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Running title: **endothelins in thyroid carcinoma**

Abstract:

The endothelin axis is a group of signalling molecular and their receptors which has been implicated in vascularisation of cancers and its expression has been observed to change in different cancer types. In this research, we examined the expression of endothelin 1 and the endothelin receptor A at the protein and mRNA levels in 123 papillary thyroid carcinomas and 40 matched lymph nodes with metastatic papillary thyroid carcinomas. We found altered endothelin axis mRNA expression in several clinicopathological parameters with increased endothelin 1 expression in thyroid papillary carcinoma showing stromal calcification, cancers in males and primary cancers with lymph node metastases. Increased endothelin receptor A mRNA expression was noted in the larger cancers. There is a significant correlation between expression of endothelin receptor A and endothelin 1 in papillary thyroid carcinoma. Both endothelin receptor A and endothelin 1 mRNA expressions were significantly higher in metastatic carcinoma in the lymph node than in primary thyroid cancer. The metastatic carcinoma in the lymph node had increased expression compared to matched primary thyroid carcinoma. Expression of endothelin 1 and endothelin receptor A were also documented as being high at the protein level. Our results indicate that in thyroid cancer, endothelin 1 and the endothelin receptor A are involved in the establishment of tumour vasculature for growth in advanced stages and lymph node metastases. Targeting the endothelin axis may be useful in planning angiogenesis therapy for thyroid cancer.

Keywords: endothelin 1, ET-1, endothelin receptor A, ET_AR, papillary thyroid carcinoma.

Introduction

Thyroid cancer represents a good model for study of angiogenesis and cancer mechanisms as they are vascular and comprise a range of lesions with different degrees of malignancy (1). Papillary thyroid cancer is the most commonly diagnosed thyroid cancer, accounting for 80% of all cases (1). The overall prognosis is favourable with 10-year survival rates of over 90%, but in some cases the cancer behaves in an aggressive manner characterized by local recurrence and metastasis (2). Studies have shown that vascular endothelial growth factor (VEGF) has a pivotal role in the control of angiogenesis and impacts biological aggressiveness in thyroid cancers (3-7). However, VEGF is not the only factor involved in angiogenesis in cancer. Endothelins (ETs) are another group of factors that contribute to the control of angiogenesis and growth of cancer.

The endothelins (*ETs*) are a family of genes which induce DNA synthesis and cellular growth in different tissues, primarily affecting vascular tone and angiogenesis (8). The endothelin axis is composed of three 21 amino acid proteins, including endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3) paired with two G-protein coupled receptors, endothelin receptor A (ET_AR) and endothelin receptor B (ET_BR) (9). In cancer, they have roles in the control of numerous factors in cancer development and progression, including angiogenesis, stromal reaction, epithelial mesenchymal transitions, apoptosis, invasion, metastases and drug resistance (10).

ET-1 appears to have direct effects on neoplastic cells by inducing cellular proliferation, angiogenesis, migration, invasion and inhibition of apoptosis (10). ET-1 mediates mitogenic effects in some epithelial tumours such as colorectal, ovarian, and prostate cancers (11-13). The ET-1/ ET_AR autocrine pathway is crucial in carcinogenesis and metastasis in certain cancers (14-15). Increased expression of ET-1 or ET_AR has been

associated with advanced pathological stages and poorer prognosis in different cancers (16-17).

VEGF was shown to be related to ET-1 in angiogenesis. ET-1, through ET_AR, up-regulates vascular endothelial growth factor (VEGF) which in turn stabilizes the VEGF regulatory protein HIF-1α (18). Previous studies have demonstrated that ET-1 expression is strongly correlated with neovascularization and VEGF expression via ET_AR (19-20). In papillary thyroid cancer, the role of endothelins has seldom been studied (21-24). In this study, we would like to improve the understanding of angiogenesis in thyroid cancer by investigating the roles of ET-1 and its receptor ET_AR in large cohort of patients with papillary thyroid carcinoma.

Materials and Method

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 the author (AL). The malignant thyroid tumours were classified with reference to the criteria defined by World Health Organization classification of malignant tumours (25). 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 (26). For each patient selected for the study, one of the paraffin blocks of the primary cancer was chosen. Also, if the patient had lymph node metastases, one block of the lymph node with metastatic cancer was also chosen. The selected archival blocks contained over 90% tumour with < 10% stromal tissue contamination. In addition, blocks from 7 non-cancer thyroid tissues (resected for benign thyroid diseases) were selected as controls

In total, 123 papillary thyroid carcinomas from 82 women and 41 men were selected for the study. The age of patients ranged between 18 and 87 years, with an average of 45.5 years. Among the patients, 46% of the patients were < 45 years and 54% of the patients were ≥ 45 years. The carcinomas included 79 conventional papillary thyroid carcinomas, 44 follicular variant of papillary thyroid carcinomas. In 40 patients with papillary thyroid carcinoma, at least one lymph node with metastatic carcinoma was noted and chosen for the study.

RNA extraction

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

RNA was assessed using an Experion electrophoresis instrument (Bio-Rad, Hercules, CA, USA). RNA was converted to cDNA using miScript Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. The preparation and evaluation of these samples have been described previously (3).

Real-time polymerase chain reaction (RT-PCR)

Primers were designed for analysis of expression of ET-1 (GenBank accession number NM_001955.4), ET_AR (GenBank accession number NM_001957.3) and GAPDH (GenBank accession number NM_002046) as the ubiquitous control gene. PCR primers for ET-1 were forward 5'-AGCCCTAGGTCCAAGAGAGC-3' and 5'-TTCCTGCTTGGCAAAAATTC-3' for reverse. The ET_AR primers were 5'-TGGTGTGCACTGCGATCTTC-3' as forward and 5'-GCAATTCTCAAGCTGCCATTC-3' as reverse. The GAPDH primers used were 5'-TGCACCACCAACTGCTTAGC-3 'for forward primer and 5'-GCATGCACTGTGGTCATGAG-3' for reverse primer.

RT-PCR was performed for all 170 samples (including matched lymph node sample and controls) using ET-1, ET_AR and GAPDH primers in an IQ Thermal Cycler (Bio-Rad) PCR machine. 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 mRNA at 30ng/µl. In the last tube of each reaction, 6 µl of water was added as non-template control. For ET_AR study, 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 mRNA at 30ng/µl. In the last tube of each reaction, 2 µl of water was added as non-template control.

All samples were run in triplicate and accompanied by a non-template control. The annealing temperature for the primers was 59.5°C. Thermal cycling conditions included

initial denaturation for 10 minutes at 95˚C followed by 40 cycles of 30 seconds at 95°C, 15 seconds at 59.5°C and 30 seconds at 72°C. Known concentrations of cDNA from universal human reference RNA (Stratagene, Cedar Creek, TX, USA) were used to build the standard curve for the determination of PCR efficiency. Final products were separated on 1.5% agarose gels to ensure the correct band had been amplified.

The mean values of cycle thresholds (ΔCt) for each triplicate in the PCR were calculated. Expression of ET-1 and ET_AR were normalized in each sample using the ΔCt of the target gene and ubiquitous control gene. The fold changes in the target genes were calculated for each sample group using the $2^{-\Delta\Delta Ct}$ method with the average ΔCt of the normal tissues as the reference point. A fold change of <0.5 was considered as low expression, a fold change of >0.5 and <2 was considered as normal expression, and a fold change of >2 was considered as high expression (19).

Immunohistochemistry staining for ET-1 and ET_AR

A tissue microarray (TMA) was constructed using a Model TMA Galileo CK3500 tissue microarrayer (Integrated System Engineering, Milano, Italy). Briefly, all representative tumour donor blocks were cut for haematoxylin & eosin staining to define the pathology of the representative regions. From those regions, 3 cylindrical core tissue specimens (diameter=0.6 mm) were acquired and arrayed into a new recipient paraffin block (35 x 20mm ^{2}). Then, sections of 4 μm were cut from the TMAs and processed for immunohistochemistry.

The histological slides were deparaffinised and rehydrated in xylene and ethanol. Slides were heated in 0.01 M citrate buffer for 20 minutes in a Rapid Multifunctional Microwave Tissue Processor Model KOS (Milestone, Sorisole, Bergamo, Italy). After cooling and washing in phosphate-buffered saline (PBS), immunohistochemistry for ET-1

and ET_AR was performed in an IntelliPATH FLX autostainer (Biocare Medical, Concord, CA, USA), providing more standardized results than manual staining techniques. 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_AR rabbit polyclonal antibody (ab76259) by Abcam was used at a 1:180 dilution for one hour at room temperature. The positive controls used were placenta and colon adenocarcinoma tissue known to express ET-1 and ET_AR. The omission of primary antibodies served as negative control. The cytoplasmic (ET-1) and cell membrane (ET_AR) immunostaining intensity of the tumour cells was categorised into groups: weakly positive staining (score 1+), moderately positive staining (score 2+), and strongly positive staining (score 3+). The final score was designated as low or high: score 1+ indicated low and score 2+ - 3+ indicated high. Whole sections from randomly selected thyroid carcinomas were stained for immunohistochemistry to check the validity of the results obtained in TMA.

Data analysis

All mRNA and protein expressions and the clinical/pathological data of the thyroid cancers were entered into statistical analysis software, Statistical Package for Social Sciences version 21.0 (SPSS Inc., New York, USA) for analysis. Significance level was taken at $p < 0.05$.

Results

Detection of ET axis mRNAs by RT-PCR in primary thyroid carcinomas

The mRNA expression of *ET-1* and its receptor (ET_AR) in all primary and metastatic papillary thyroid carcinomas as well as in non-cancer thyroid tissue was detected. *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_AR 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). The relationship between the expressions of *ET-1* and ET_AR with the clinicopathological features of papillary thyroid carcinomas are shown in Tables 1 and 2.

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). 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). 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 $r=0.522$, $p<0.001$).

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 cancers in the lymph node showed 55% (22/40) 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 higher level of mRNA in the lymph node metastases than the primary thyroid cancer whereas 20% (8/40) had lower level of mRNA expression.

For ET_AR, high mRNA expression was detected in 25% (10/40) of the metastatic thyroid cancer in lymph node. The other cancers in the lymph node showed 63% (21/40) low expression or 7% (9/40) 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 higher level of mRNA in the lymph node metastases than the primary thyroid cancer whereas 25% (10/40) had lower level of mRNA expression.

Immunohistochemical analysis of ET-1, and ET_AR 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 (Figure 1). ET_AR 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 (Figure 2).

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_AR protein expression was studied in 74 of the 123 papillary thyroid carcinomas. ET_AR 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_AR mRNA showed high expression of ET_AR.

There was no significant difference between ET-1 and ET_AR 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_AR immunohistochemical stain with clinicopathological features was demonstrated.

Discussion

In papillary thyroid carcinoma, ET-1 production and antagonism of ET_AR have been demonstrated in human thyroid cancer cells lines (22). ET-1 or ET_AR have been only investigated at tissue levels 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 (21). Donckier *et al*. have shown higher expression of ET-1 and ET_AR mRNA in 12 patients with papillary thyroid carcinomas than non-cancer thyroid tissues with immunohistochemistry and RT-PCR (24). In another study, they noted that ET-1 mRNA expression in thyroid cancers correlated with nitric oxide pathway activation (24). 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_AR) 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. 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 behaviour in thyroid cancers, with males more often showing aggressive cancer.

There was a significant difference between ET_AR expression level and cancer size, as larger cancers expressed higher levels of ET_AR. 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_AR overexpression is associated with aggressive cancer behaviour and a worse prognosis in ovarian cancer (27). Indeed in our study, we also found increased expression of *ET-1* in metastatic thyroid cancer. Therefore, it can be hypothesized that ET_AR 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 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. 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.

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 (28-31). 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_AR 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_AR autocrine pathway is implicated in papillary thyroid cancer progression. This pathway acts through different cancer relevant processes, such as proliferation, angiogenesis, inhibition of apoptosis, migration, invasion and metastasis (10). ET-1 stimulates multiple

cancer related processes, including epithelial-mesenchymal transition (EMT) through ET_AR (15). 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_AR expression levels were mostly 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. Our finding concurs 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 (32). Our findings support the hypothesis of an autocrine role of ET-1/ ET_AR pathway in thyroid cancers. The ET-1/ ET_AR axis was noted to enhance metastasis. Therefore, these findings identify ET_AR 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.

In our investigation, ET-1 immunostaining showed high expression in 94% of cases and ET_AR 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 (21, 23). The high expressions of these proteins *in situ* reflected the findings of their corresponding mRNAs. These findings confirm the importance of ET-1/ ET_AR expression in thyroid cancer. In addition, we demonstrated that the proteins, ET-1 and ET_AR, were located in specific cellular locations in thyroid tissue no matter whether they are in benign, malignant or even metastatic tumours.

The expressions of ET-1/ ET_AR proteins appear to be different in various cancers. Wülfing *et al*. observed staining of ET-1 in 26.8% of bladder cancer tissue samples and in 58.8% in ET_AR of the same cases (33). In another study, Eltze *et al*. identified ET-1 and ET_AR staining in 62% and 93% of bladder cancer samples, respectively (34). Differences in ET-1 and ET_AR 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.

In conclusion, 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 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.

Disclosure/Conflict of interest

The authors wish to state that they have no conflicts of interest or disclosures to make.

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References

1. Lam AKY, Lo CY, Lam KSL. Papillary carcinoma of thyroid: a 30-year clinicopathological review of the histological variants. Endocr Pathol 2005; 16: 323-330.
2. Lam KY, Lo CY, Chan KW, Wan KY. Insular and anaplastic carcinoma of the thyroid: A 45-year comparative study at a single institution and a review of the significance of p53 and p21. *Ann Surg* 2000; 231: 329-38.
3. Salajegheh A, Smith RA, Kasem K, Gopalan V, Nassiri MR, William R, Lam AK. Single nucleotide polymorphisms and mRNA expression of VEGF-A in papillary thyroid carcinoma: potential markers for aggressive phenotypes. Eur J Surg Oncol. 2011; 37: 93-9
4. Yu XM, Lo CY, Lam AK, Lang BH, Leung P, Luk JM. The potential clinical relevance of serum vascular endothelial growth factor (VEGF) and VEGF-C inrecurrent papillary thyroid carcinoma. Surgery. 2008;144: 934-40;
5. Yu XM, Lo CY, Lam AK, Leung P, Luk JM. Serum vascular endothelial growth factor C correlates with lymph node metastases and high-risk tumor profiles in papillary thyroid carcinoma. Ann Surg. 2008; 247: 483-9.
6. Yu XM, Lo CY, Chan WF, Lam KY, Leung P, Luk JM. Increased expression of vascular endothelial growth factor C in papillary thyroid carcinoma correlates with cervical lymph node metastases. Clin Cancer Res. 2005; 11: 8063-9.
7. Salajegheh A, Pakneshan S, Rahman A, Dolan-Evans E, Zhang S, Kwong E, et al. Co-regulatory potential of VEGF-A and VEGF-C in thyroid carcinoma. Hum Pathol 2014 (in press).

8. Motte S, McEntee K, Naeije R. Endothelin receptor antagonists. Pharmacol Ther 2006; 110: 386-414.
9. Bagnato A, Rosanò L. The endothelin axis in cancer. Int J Biochem Cell Biol. 2008; 40:1443-51.
10. Irani S, Salajegheh A, Smith RA, Lam AK. A review of the profile of endothelin axis in cancer and its management. Crit Rev Oncol Hematol 2014 (in press)
11. Peduto Eberl L, Bovey R, Juillerat-Jeanneret L. Endothelin-receptor antagonists are proapoptotic and antiproliferative in human colon cancer cells.Br J Cancer 2003; 88:788-95.
12. Rosanò L, Spinella F, Salani D, et al. Therapeutic targeting of the endothelin a receptor in human ovarian carcinoma. Cancer Res 2003; 63:2447-53.
13. Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS, Simons JW. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res1996; 56:663-8.
14. Rosanò L, Spinella F, Bagnato A. The importance of endothelin axis in initiation, progression,and therapy of ovarian cancer. Am J Physiol Regul Integr Comp Physiol. 2010; 299: R395-404.
15. Bagnato A, Spinella F, Rosanò L. Emerging role of the endothelin axis in ovarian tumor progression. Endocr Relat Cancer. 2005; 12:761-72.
16. Bagnato A, Salani D, Di Castro V, et al. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer Res. 1999; 59:720-7.
17. Spinella F, Rosanò L, Di Castro V, Natali PG, Bagnato A. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem. 2002; 277: 27850-5.

18. Boldrini L, Gisfredi S, Ursino S, et al. Expression of endothelin-1 is related to poor prognosis in non-small cell lung carcinoma. Eur J Cancer 2005; 41:2828-35.
19. Jiao W, Xu J, Zheng J, Shen Y, Lin L, Li J. Elevation of circulating big endothelin-1: an independent prognostic factor for tumor recurrence and survival in patients with esophageal squamous cell carcinoma. BMC Cancer. 2008; 8:334.
20. Wülfing P, Kersting C, Tio J, et al. Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clin Cancer Res.2004; 10:2393-400.
21. Lenziardi M, Viacava P, Fiorini I, et al. Presence of endothelin-1 in the normal and pathological human thyroid. J Endocrinol Invest. 1995; 18: 336-40.
22. Donckier JE, Mertens-Strijthagen J, Flamion B. Role of the endothelin axis in the proliferation of human thyroid cancer cells. Clin Endocrinol (Oxf). 2007; 67: 552-6.
23. Donckier JE, Michel L, Van Beneden R, Delos M, Havaux X. Increased expression of endothelin-1 and its mitogenic receptor ETA in human papillary thyroid carcinoma. Clin Endocrinol (Oxf). 2003;59: 354-60
24. Donckier JE, Michel L, Delos M, Havaux X, Van Beneden R. Interrelated overexpression of endothelial and inducible nitric oxide synthases, endothelin-1and angiogenic factors in human papillary thyroid carcinoma. Clin Endocrinol(Oxf). 2006; 64: 703-10.
25. DeLellis R, Lloyd R, Heitz P, Eng C: World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Endocrine Organs. Lyon: IARC (International Agency for Research on Cancer) Press; 2004.
26. Sobin L, Wittekind C: International Union against Cancer (UICC) TNM Classification of Malignant Tumours, 7th edn. Berlin: Springer Press; 2009.

27. Salani D, Di Castro V, Nicotra MR, et al. Role of endothelin-1 in neovascularization of ovarian carcinoma. Am J Pathol. 2000; 157: 1537-47.
28. Montironi R, Mazzucchelli R, Barbisan F, et al. Immunohistochemical expression of endothelin-1 and endothelin-A and endothelin-B receptors in high-grade prostatic intraepithelial neoplasia and prostate cancer. Eur Urol. 2007; 52:1682-9.
29. Alanen K, Deng DX, Chakrabarti S. Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma. Histopathology. 2000; 36:161-7.
30. Wülfing P, Diallo R, Kersting C, et al. Expression of endothelin-1, endothelin-A, and endothelin-B receptor in human breast cancer and correlation with long-term follow-up. Clin Cancer Res. 2003; 9:4125-31.
31. Gohji K, Kitazawa S, Tamada H, Katsuoka Y, Nakajima M. Expression of endothelin receptor a associated with prostate cancer progression. J Urol. 2001; 165:1033-6.
32. Hagemann T, Binder C, Binder L, Pukrop T, Trümper 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.
33. Wulfing C, Eltze E, Yamini J, Wulfing P, Bierer S, Bocker W, et al. Expression of the endothelin axis in bladder cancer: relationship to clinicopathologic parameters and long-term survival. Eur Urol. 2005;47:593-600.
34. Eltze E, Bertolin M, Korsching E, Wülfing P, Maggino T, Lellé R.Expression and prognostic relevance of endothelin-B receptor in vulvar cancer. Oncol Rep. 2007;18: 305-11.

Figure Legend

Figure 1
ET-1 protein expression was mainly located in the nuclei of the cells.
3 A. ET-1 protein expression in normal thyroid
3 B. ET-1 protein expression in papillary thyroid carcinoma.
3 C. ET-1 protein expression in metastatic papillary thyroid carcinoma in lymph node. (i) at low magnification; (ii) at high magnification.

Figure 2
ET_AR protein expression was mainly located in the membrane of the cells.
4 A. ET_AR protein expression in normal thyroid
4 B. ET_AR protein expression in papillary thyroid carcinoma.
4 C. ET_AR protein expression in metastatic papillary thyroid carcinoma in lymph node (i) at low magnification; (ii) at high magnification.

Table1. The relationships between *ET-1* mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas

Clinical & pathological variables	*ET-1* expression			p-value
	High	Low	Normal	
Gender				0.040*
Male (n=41)	15 (37%)	21 (51%)	5 (12%)	
Female (n=82)	18 (22%)	38 (46%)	26 (32%)	
Age				0.587
<45 (n=67)	16 (24%)	32 (48%)	19 (28%)	
≥45 (n=56)	17 (30%)	27 (48%)	12 (22%)	
Tumour size (mm)				0.126
≤40 mm (n=110)	27 (25%)	56 (50%)	27 (25%)	
>40mm (n=13)	6 (46%)	3 (23%)	4 (31%)	
T staging				0.386
T1 or T2 (n=78)	18 (23%)	38 (49%)	22 (28%)	
T3 (n=45)	15 (33%)	21 (47%)	9 (20%)	
Lymph node metastasis				0.0001*
Positive (n=58)	20 (34%)	33 (57%)	5 (9%)	
Negative (n=65)	13 (20%)	26 (40%)	26 (40%)	
TNM staging				0.426
Stages1 or II (n=87)	20 (23%)	42 (48%)	25 (29%)	
Stage III (n=36)	13 (36%)	17 (47%)	6 (17%)	
Pathological variant				0.327
Conventional (n=79)	24 (30%)	38 (48%)	17 (22%)	
Follicular (n=44)	9 (20%)	21 (48%)	14 (32%)	
Psammoma body				0.057
Present (n=52)	18 (35%)	26 (50%)	8 (15%)	
Absent (n=71)	15 (21%)	33 (47%)	23 (32%)	
Calcification in stroma				0.018*
Present (n=62)	23 (37%)	28 (45%)	11 (18%)	
Absent (n=61)	10 (16%)	31 (51%)	20 (33%)	
Osseous metaplasia in stoma				0.165
Present (n=6)	2 (33%)	4 (67%)	0 (0%)	
Absent (n=117)	31 (26%)	55 (48%)	31 (26%)	
Lymphocytic thyroiditis				0.547
Present (n=40)	14 (35%)	16 (40%)	10 (25%)	
Absent (n=83)	19 (23%)	43 (52%)	21 (25%)	

***** Statistically significant

Table2. The correlation between ET_AR mRNA expression and clinicopathological characteristics of 123 papillary thyroid carcinomas

Clinical & pathological variables	ET_AR expression			p-value
	High	Low	Normal	
Gender				0.548
Male (n=41)	9 (22%)	17 (41%)	15 (37%)	
Female (n=82)	17 (21%)	42 (51%)	23 (28%)	
Age				0.922
<45 (n=67)	15 (22%)	32 (48%)	20 (30%)	
≥45 (n=56)	11 (20%)	27 (48%)	18 (32%)	
Tumour size (mm)				0.003*
≤40 mm (n=110)	20 (18%)	58 (53%)	32 (29%)	
>40mm (n=13)	6 (46%)	1 (8%)	6 (46%)	
T staging				0.761
T1 or T2 (n=78)	15 (19%)	39 (50%)	24 (31%)	
T3 (n=45)	11 (25%)	20 (44%)	14 (31%)	
Lymph node metastasis				0.448
Positive (n=58)	12 (21%)	31 (53%)	15 (26%)	
Negative (n=65)	14 (22%)	28 (43%)	23 (35%)	
TNM staging				0.879
Stages1 or II (n=87)	18 (21%)	43 (49%)	26 (30%)	
Stage III (n=36)	8 (22%)	16 (45%)	12 (33%)	
Pathological variant				0.739
Conventional (n=79)	15 (19%)	39 (49%)	25 (32%)	
Follicular (n=44)	11 (25%)	20 (45%)	13 (30%)	
Psammoma body				0.180
Present (n=52)	9 (17%)	30 (58%)	13 (25%)	
Absent (n=71)	17 (24%)	29 (41%)	25 (35%)	
Calcification in stroma				0.996
Present (n=62)	13 (21%)	30 (48%)	19 (31%)	
Absent (n=61)	13 (22%)	29 (48%)	18 (30%)	
Osseous metaplasia in stoma				0.220
Present (n=6)	0 (0%)	4 (67%)	2 (33%)	
Absent (n=117)	26 (22%)	55 (47%)	36 (31%)	
Lymphocytic thyroiditis				0.362
Present (n=40)	12 (30%)	19 (47%)	9 (23%)	
Absent (n=83)	14 (17%)	40 (48%)	29 (35%)	

***** Statistically significant