**Quantitative analysis of the expression of transformation growth factor alpha
(TGF- α) and epidermal growth factor receptor (EGFR) in papillary thyroid
carcinoma: clinicopathological relevance**

Running title: **TGF- α and EGFR in papillary thyroid carcinoma**

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ABSTRACT:

Aims: There is no quantitative data on the mRNA expression of epidermal growth factor receptor (EGFR) and transformation growth factor alpha (TGF- α) in thyroid carcinoma. The aims of this study are to detect, quantify and analyse the clinicopathological correlations of the expression of these genes in a large cohort of patients with thyroid carcinoma.

Methods: EGFR and TGF- α expression were investigated using real time quantitative polymerase chain reaction and immunohistochemistry on 71 papillary thyroid carcinoma (PTC), 68 paired non-cancer thyroid tissue adjacent to the PTC and 20 benign thyroid lesions.

Results: TGF- α and EGFR mRNA increased in PTC when compared with benign thyroid lesions. In many PTC with high level of expression of TGF- α and EGFR mRNA, the morphological non-cancer tissue adjacent to the cancer also showed high level of expression of these mRNAs. The levels of expression of mRNA of TGF- α and EGFR correlated with each other and with level of protein expression. The level of expression of TGF- α mRNA was significantly related to lymphovascular permeation while the expression of EGFR mRNA was related to the pathological subtype of PTC and cancer recurrence.

Conclusions: TGF- α and EGFR were over-expressed, correlated with each other and

associated with the pathological parameters in papillary thyroid carcinoma. The results provide information for management of thyroid cancer in the era of gene targeting therapy.

Keywords: EGFR; TGF- α ; papillary thyroid carcinoma.

INTRODUCTION

Transforming growth factor alpha (TGF- α) is a 50-amino acid single chain polypeptide of 5.6 kDa [1]. This small polypeptide shares around 30% structural similarity with epidermal growth factor (EGF) but distinctly competes for the common receptor, epidermal growth factor receptor (EGFR) [2]. TGF- α is the most potent angiogenic factor among the ligands that bind to EGFR as well as a strong collaborator in promoting carcinogenesis by other oncogenes [3]. Over-expression of TGF- α has been found in various types of human cancers [4-7]. Moreover, TGF- α has been proved to stimulate invasion and growth of thyroid cancer cells in culture [8]. Taken together, TGF- α is highly related to carcinogenesis and aggressiveness.

EGFR is a transmembrane glycoprotein which consists of 1186 amino acids. Once ligands bind to EGFR, this kinase triggers different downstream signalling pathways and results in biological responses varying from proliferation, differentiation, migration and apoptosis [9]. EGFR is one of the receptors often found up-regulated in human carcinomas and often related to poor prognosis or advanced pathological stages [10-11].

Thyroid cancer is the most common endocrine malignancy and approximately 75% of patients with thyroid cancer have papillary thyroid carcinoma (PTC) [12]. In

papillary thyroid carcinoma, RET/PTC re-arrangement is a common genetic event [13]. Recent study suggests that RET/PTC-induced cell growth is mediated in part by EGFR [14]. Also, PTC stromal invasion may be regulated through EGFR-dependent activation of matrix metalloproteinase (MMP)-2/gelatinase A [15].

Gene therapy is believed to be important in the management of cancer [16]. EGFR-targeted therapy has emerged as a promising therapeutic approach in some cancer patients [17]. In thyroid cancer, mutational events and gene amplification may identify a minor portion of thyroid cancers that may benefit from EGFR inhibitors [18-19]. Also, it is likely that identification of the presence and level of TGF- α and EGFR expression is useful when using the gene therapy in human cancers.

To the best of our knowledge, there is no study to quantify the levels of mRNA expression of TGF- α and EGFR in papillary thyroid carcinoma. Also, there is no study comparing these genes' expressions in the cancer tissue and adjacent non-cancer thyroid tissue. In this study, we quantified the TGF- α and EGFR mRNA expression level in thyroid carcinomas and compared that with adjacent non-cancer thyroid carcinomas. The expression of these mRNA was also correlated with their protein expressions and the clinicopathological features in a large cohort of patients.

MATERIALS AND METHODS

Patients and tissue samples

Seventy-one patients (17 men, 54 women) with PTC who had a median age of 46.5 years (12-85 years) were recruited in this study. Informed consent was obtained from all patients to collect their tissues samples for conducting this study. The mean size of the tumours was 2.5 cm (1-9cm). For controls, 20 patients (5 men, 15 women) with benign thyroid disease (nodular goitre) were used. The PTCs were staged according to TNM (tumour, lymph node, metastases) and MACIS (metastases, age, completeness of resection, invasion, size) staging systems [20-21]. The TNM was used as it is the most commonly used staging system [20]. According to the TNM staging system, 31 were stage I, 7 were stage II, 32 were stage III and 1 was stage IV cancers. MACIS by Mayo Clinic was also adopted as it is the most predicting staging system in thyroid cancer [21]. Most patients fall into the low risk category (MAICS score <6) and are cured of the cancer at the time of surgery. On MACIS system, the cancers were divided into 4 risk groups. There were 42 group 1 (score <6) cancers, 9 group 2 (6-6.99) cancers, 13 group 3 (7-7.99) cancers and 7 group 4 (>8) cancers.

Management was by a pre-agreed standardized multidisciplinary protocol. The follow-up period was defined as the interval between the date of surgery for thyroid carcinoma and the date of death or closing date of the study. The actual survival rate

of the patients was calculated from the date of surgical resection of the thyroid cancers to the date of death or last follow-up. Only cancer-related death was counted as end point in the statistical analysis. The disease-free survival was calculated from the time of surgery to the date of first recurrence of the cancer. The mean follow up of the patients was 40 months (range 2 to 94 months). In the follow-up period, 11 patients had recurrent disease and with 2 died of PTC.

Tissue samples were obtained directly after surgery. For PTC, samples were taken both from the cancer and the non-cancer area in the contra-lateral lobe of thyroid. A part of the samples was snap-frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction. The rest was fixed in 10% buffered formalin for paraffin embedding. These paraffin blocks were used in immunohistochemistry and haematoxylin and eosin (H&E) staining. H&E staining was used to confirm the pathological diagnosis by the author who is a pathologist (AKYL.) before starting this research. Eighty per cent (57 of 71) of PTC were conventional and 13% (9 of 71) were papillary micro-carcinomas. The other histological variants were 4% (n=3) follicular variant and 3% (n=2) tall cell variant. Also, the clinical and pathological parameters of the thyroid cancers were reviewed and entered into database for analysis. In addition, 20 tissues samples obtained from patients with nodular goitre were used as non-cancer controls.

RNA extraction and reverse transcription

Total RNA was extracted from frozen thyroid tissues using TriZol reagent (Invitrogen Corporation, Carlsbad, CA, USA), and all samples were DNase I (Ambion, Austin, TX, USA) treated. The purity of the RNA was analysed by optical density at wavelengths of 260 and 280 nm using Lambda 35 spectrometer (PerkinElmer, Wellesley, MA, USA). The RNA quality was assessed by intact 18s and 28s ribosomal bands on the gel. The DNA digested RNA was then mixed with TaqMan® Reverse Transcription Reaction (Roche Applied Biosystems, Foster City, CA, USA) for cDNA synthesis as described in protocol given by the company. The final cDNA product was amounted to 25 µl and stored at -20°C up to subsequent quantitative real-time polymerase chain reaction.

Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (qPCR) was performed on ABI Prism 7700 (Applied Biosystems). The primer sequences were shown in Table 1. The predicated product size of the primers for TGF- α and EGFR are 350 base pairs and 170 base pairs respectively. The qPCR mix was made on the basis of the prescription from the supplier: 6 µl sterile water, 1 µl sense and 1 µl antisense primers, 10 µl Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Invitrogen) and 2 µl

target cDNA in a total volume of 20 µl. Ribosomal 18s was used as an internal control in each cDNA sample and qPCR protocol was as follows: 7 µl sterile water, 1 µl 18S rRNA endogenous control VIC / MGB probe (Applied Biosystems), 10 µl TaqMan® PCR Master Mix (Applied Biosystems) and 2 µl target cDNA in a total volume of 20 µl. qPCR run conditions were: 50°C for 2 min, 95 °C for 10min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min. Duplicate reactions were performed in each cDNA samples and relative mRNA expression level was analyzed according to the literature [22]. $2^{-[\Delta\Delta Ct]}$ (fold change) was chosen to represent the level of expression of mRNA. It was estimated by converting natural logarithm to its real value, which represented the normalized expression of the target gene in the test sample relative to the control sample. A fold change of more than 2 was considered as high level of expression (over-expression) and a change in between 1 and 2 was considered as expression within normal range. Fold change of less than 1 was considered as reduced expression.

Immunohistochemistry

Of the study populations, 59 PTC and 10 benign thyroid lesions were recruited for immunochemical studies. Formalin-fixed, paraffin-embedded thyroid tissues were cut with 4 µm thick. The sections were mounted on polylysine-coated slides

and stained with haematoxylin-eosin to confirm the histopathology. Sections from the same block were dewaxed in xylene and gradually hydrated. Antigen retrieval was achieved by microwaving in 0.01 M citrate buffer for 10 min at 95 °C. After microwave treatment, the slides were treated with hydrogen peroxide at room temperature for 30 min to block the endogenous peroxidase and followed by blocking with 2% bovine serum albumin, 10 % normal goat serum (Invitrogen) in phosphate buffer shock (PBS) at room temperature for an hour. The slides were then incubated for 18 hr at 4 °C in the primary anti-TGF- α monoclonal mouse antibody (1:100 dilutions, Cambiochem, San Diego, CA, USA) or anti-EGFR polyclonal rabbit antibody (1:50 dilutions, Santa Cruz Biotechnology, CA, USA) diluted with blocking solution. A thyroid cancer showing strong positive staining for the EGFR was used in every experiment as the positive control. For negative isotype controls, the sections were incubated in mouse immunoglobulin G (1:1500 dilutions, Zymed, San Francisco, CA, USA) or rabbit immunoglobulin G (1:1000 dilutions, Zymed) diluted with blocking solution.

After defrosting at 37°C for 30 min, the slides were washed with PBS and incubated with biotinylated secondary antibody (Dako Evision + System, Gloustrup, Denmark) for 45 min at 37°C. The slides were washed thoroughly with PBS and antibody reactions were examined by colour development using

3,3'-diamino-benzidine tetrachloride substrate solution (DAKO EnVision+ System).

Afterwards, the slides were briefly counterstained with haematoxylin, mounted in crystal mount, and cover-slipped in 50:50 xylen-Permount.

The immunochemical staining for TGF- α and EGFR were examined and scored by the author (AKYL) blinded to the qPCR results of the specimens. In both reactions, the staining was mainly in the cytoplasm. Thus, brown cytoplasmic staining was labelled as positive. The results of immunochemical staining were assessed semi-quantitatively divided into three categories: 0 to 30% positive (1+); 31 to 60% positive (2+) and 61 to 100% (3+).

Statistical analysis

All the clinical, pathological, follow-up, mRNA expression and protein expression data were computerized. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 17.0, SPSS Inc., Chicago, IL, USA). Fisher's exact test, chi-square test or likelihood ratio was used for categorical variables. Student t-test with Yates correction, Pearson correlation test or ANOVA was used for continuous variables. Survival analysis was tested using Kaplan-Meier and Cox regression. Significance level of the tests was taken at $p < 0.05$.

RESULTS

mRNA expression of TGF- α and EGFR in different thyroid tissues

The mRNA expression of TGF- α and EGFR was compared in 71 PTC, 68 adjacent non-cancer thyroid (in contra-lateral lobe) and 20 benign thyroid tissues. Significant difference in the mRNA expression level was found for TGF- α and EGFR in PTC against adjacent non-cancer thyroid (in contra-lateral lobe) and benign thyroid tissues ($p < 0.0001$ and $p < 0.05$ respectively) (Figure 1).

High level of expression of TGF- α mRNA was noted in 77% (55 of 71) of PTCs. The other PTCs showed either TGF- α mRNA in normal range (10%; $n=7$) or reduced level (13%; $n=9$). Also, high level of expression of EGFR mRNA was noted in 54% (38 of 71) of PTCs. The other PTCs showed either EGFR mRNA in normal range (6%; $n=4$) or reduced level (40%; $n=29$).

The correlations between the expression levels of TGF- α and EGFR mRNA in PTC and the adjacent morphologically non-cancer tissue were studied in 68 cases in which the adjacent non-cancer tissues were available (Table 2). In 25% (13 out of 52) of PTCs with high level of expression of TGF- α mRNA, paired tissue sampled from the adjacent non-cancer thyroid also showed high level of TGF- α mRNA expression. Also, in 63% (22 out of 35) of PTCs with high level of EGFR mRNA expression, tissue sampled from the adjacent non-cancer thyroid also showed high

level of EGFR mRNA expression.

Immunochemical detection of TGF- α and EGFR in thyroid tissues (Figure 2)

All of 59 PTC were stained positively by TGF- α and EGFR antibodies whereas only 30% of benign thyroid tissues were positive. The adjacent non-cancer tissues were either negative or showed only 1+ staining for both antibodies. For TGF- α protein expression in PTC, 10 % (n=6) has 1+ staining, 7% (n=4) had 2+ staining and 83% (n=49) had 3+ staining. For EGFR protein expression in PTC, 10 % (n=5) has 1+ staining, 7% (n=6) had 2+ staining and 83% (n=48) had 3+ staining.

Correlations between the expression of mRNA and proteins of TGF- α and EGFR

PTCs with 1+ expression of TGF- α protein had a mean fold change of TGF- α of 3.8 while those with either 2+ or 3+ TGF- α had a mean fold change of 15.9 (p=0.0001). Also, 89% (42 of 46) of patients with high level of expression of TGF- α mRNA showed either 2+ or 3+ TGF- α protein expression.

The fold change in EGFR mRNA was correlated with EGFR expression. In PTCs with 1+ expression of EGFR protein, the mean fold change was 2.2 while those with 2+ or 3+ expression of EGFR protein, the mean fold change was 7.25 (p=0.029). In addition, 93% (28 of 30) of patients with high level of expression of EGFR mRNA also showed either 2+ or 3+ EGFR protein expression.

The relationship between TGF- α and EGFR mRNA expression in PTC

The correlation between mRNA expression of TGF- α and EGFR in PTC was examined by Pearson correlation test. Positive correlation between these genes was observed ($p<0.0001$) (Figure. 3). Over-expression of both TGF- α and EGFR mRNA was noted in 52% (37 of 71) and normal or under-expression of both TGF- α and EGFR mRNA was observed in 21% (15 of 71) of the PTCs. Thus, 73% of patients with PTC had similar trend in expression of these mRNA.

The positive correlation between TGF- α and EGFR expression was also noted at the protein level ($p=0.0001$). High level of expression (3+) of both TGF- α and EGFR proteins were found in 75 % (44 of 59) whereas low level of expression (1+) of both proteins was noted in 8% (5 of 59) of patients with PTC. Overall, 83% of patients with PTC had a similar trend in expression of these proteins.

Association of TGF- α and EGFR mRNA in PTC with clinicopathological parameters

(Table 3)

The mean TGF- α fold change of PTCs showing lymphovascular permeation was 26 whereas mean fold change of PTCs without lymphovascular permeation was 12 ($p=0.02$). Other than this, the TGF- α mRNA expression did not correlate with other clinical and pathological parameters of the PTCs.

In conventional papillary thyroid carcinoma, approximately equal number of cases had high level of mRNA expression (n=28) and low level of mRNA expression (n=29) of EGFR. In papillary microcarcinoma, 7 had high level of mRNA expression and 2 had low level of mRNA expression of EGFR. All the 3 follicular variant of PTCs showed high level of mRNA expression while the 2 tall cell variant of PTCs revealed low level of mRNA expression of EGFR.

The clinical and pathological relevance of TGF- α and EGFR proteins expression

(Table 4)

In the patients with multi-focal PTC in the thyroid, 71% (20 of 28) had 3+ TGF- α protein expression whereas in the patients with solitary PTC in thyroid, 94% (29 of 31) had 3+ TGF- α protein expression (p=0.02). Other than this, the expression of TGF- α protein did not correlate with other clinical and pathological features.

The level of protein expression of EGFR was related to the both the TNM and the MACIS staging system. In TNM stage 1 cancer, 84% (27 of 32) had 3+ protein expression of EGFR. On the other hand, in TNM stage 4 cancer, 63% (5 of 8) had 3+ protein expression (p=0.02). Also, in thyroid cancers in the low MACIS staging group (less than 6), 86% (31 of 36) had 3+ protein expression of EGFR whereas in

thyroid cancers with high MACIS group (more than 8), 25% (1 of 4) had 3+ protein expression ($p=0.03$). Other than these, the levels of protein expression of EGFR did not correlate with other clinicopathological parameter of PTC.

The prognosis of patients with PTC in relationship with TGF- α and EGFR expression

The mean fold change of EGFR mRNA for patients with recurrent disease was 2 while that of other patients was 8. Thus, the difference in level of EGFR mRNA expression was significant between the 2 groups ($p=0.002$). Other than this, the survival or disease free survival rates of the patients with PTC were not related to the levels of protein or mRNA expression of either TGF- α or EGFR.

DISCUSSION

Up-regulation of EGFR or TGF- α has been found in papillary thyroid carcinoma [23-30]. In addition, co-expression of these genes in thyroid carcinomas was also noted [23, 24]. All these studies demonstrated the increased expression of EGFR or TGF- α in thyroid carcinoma through immunohistochemistry and slot-blot approach. However, these qualitative methods are hard to achieve statistical analysis. Furthermore, studies on TGF- α expression in thyroid carcinoma were based on small number of cases [23, 24, 28, 29]. To the best of our knowledge, the current study documented for the first time the presence and correlations of the mRNA expression of TGF- α and EGFR in a large cohort of patients with PTC by quantitative real-time PCR. Also, the levels of expression of the genes as determined by real-time PCR and immunohistochemistry, correlated. However, mRNA expression as detected by quantitative real-time PCR may give a more precise value of the expression of these genes in PTC.

In this study, we noted that a high portion of morphological non-cancer tissue adjacent to the thyroid cancer showed high expression of mRNA for both TGF- α and EGFR. It is worth noting that we have reported higher expression of VEGF protein in non-cancer thyroid tissue [31]. The findings can be explained by the presence of genetic changes in the tissue before the morphological appearance of cancer or by the

presence of intra-glandular spread of micro-metastases not detected by morphological examination. Thus, non-cancer thyroid tissue from cancer patient harbour genetic changes and cannot be used as negative control in the study of genetic changes in cancer.

Up-regulation of TGF- α mRNA was noted in PTC when compared with either benign thyroid lesions or paired non-cancer thyroid tissue adjacent to the PTC. Also, the higher levels of TGF- α mRNA in PTC correlated with higher levels of protein expression as detected by immunohistochemistry. Seventy-seven per cent of PTC had high level of TGF- α mRNA expression and 90% had 2+/3+ level of protein expression. Thus, TGF- α was confirmed to be increased in both mRNA and protein level in PTC.

We noted that higher level of TGF- α expression was associated with more frequent lympho-vascular permeation. On the other hand, higher level of protein expression of TGF- α expression was related to lower prevalence of macroscopically multi-focal cancer in the thyroid. Thus, the presence of macroscopically multi-focality of the thyroid cancer may be related to mechanisms other than lympho-vascular permeation. Overall, though TGF- α expression is not a prognostic parameter, it may play a role in micro-invasiveness in PTC.

EGFR mRNA and protein expression was more often noted in PTCs than benign

thyroid lesions. High level of expression of EGFR mRNA and protein was noted in 54% and 90% of PTCs. It is worth noting that higher level of EGFR protein expression was more important in early stage thyroid cancer. It is likely that EGFR expression was related to early phase of carcinoma progression. Also, EGRF expression has been shown to be independent factor for predicting cancer recurrences in PTC [25, 27]. In this study, we noted that the level of EGFR mRNA expression predicted thyroid cancers with recurrence. However, the level of expression was not related to the survival rate of patients with PTC. The results should be confirmed in a larger series with longer follow-up period.

EGFR protein expression can occur both in the cytoplasm and the membrane of the cells [32]. The switch from greater cytoplasmic EGFR to greater membranous EGFR expression occurs in progression in some cancers [33]. In this study, the staining EGFR in thyroid carcinoma was mainly cytoplasmic. This may in concur with the suggestion that EGFR protein expression is an early event in thyroid cancer.

Different variants of PTC had different clinical and pathological features [12]. In the current study, we noted that the level of expression of EGFR mRNA was different in conventional PTC, papillary micro-carcinoma, follicular variant of PTC and tall cell variant. It seems that higher expression of EGFR mRNA was noted in PTC variant with less biological aggressive variants. It is worth noting that genetic

differences have been observed in different variants of PTC [34-36]. However, more cases of histological variants of PTC may need to be studied to confirm the difference in EGFR mRNA expression in different histological variants of PTC.

To conclude, this is the first documented report demonstrating the increased levels of TGF- α and EGFR mRNA in thyroid carcinoma through a quantitative approach. The mRNA expression of these genes was also correlated with the protein expression. Up-regulated TGF- α and EGFR may be related to the pathological parameters of thyroid carcinoma. The finding of high level of expression of these genes in many PTCs may provide useful information when considering gene targeting therapies for this cancer.

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Table 1 **Primers used in this study**

Target mRNA	Gene specific primers (sense/antisense)	Expected size of PCR products (bp)
TGF- α	5'-TGTTCGCTCTGGGTATTGTG-3' 5'-GACCTGGCAGCAGTGTATCA-3'	350
EGFR	5'-TAACAAGCTCACGCAGTTGG-3' 5'-GTTGAGGGCAATGAGGACAT-3'	170

Table 2

Correlations of TGF- α and EGFR mRNAs expression in cancer and the tissue adjacent to the cancer

----- TGF- α mRNA expression level			----- EGFR mRNA expression level		
Cancer	Adjacent to cancer	No	Cancer	Adjacent to cancer	No
High	High	13	High	High	22
High	Normal	9	High	Normal	6
High	Low	30	High	Low	7
Normal	High	1	Normal	High	3
Normal	Normal	1	Normal	Normal	0
Normal	Low	5	Normal	Low	1
Low	High	0	Low	High	3
Low	Normal	1	Low	Normal	2
Low	Low	8	Low	Low	24
----- Total		68	----- Total		68

Table 3. Relationship between clinicopathological features of 71 papillary thyroid carcinomas and mRNA expression of TGF- α or EGFR (fold change)

Variable	Number	TGF- α expression		EGFR expression	
		Mean (SD)	p-value	Mean (SD)	p-value

Age					
≤ 45	31	12.03 (16.73)	0.43	4.44 (6.49)	0.14
> 45	40	15.90 (22.65)		8.88 (15.39)	
Gender					
Male	17	10.97 (11.48)	0.45	3.57 (3.39)	0.20
Female	54	15.23 (22.29)		8.00 (14.01)	
Tumour size					
$\leq 4\text{cm}$	62	15.05 (21.27)	0.36	7.37 (13.27)	0.45
$> 4\text{cm}$	9	8.43 (9.54)		4.00 (3.58)	
Multifocality					
Presence	30	15.38 (20.50)	0.68	7.17 (15.14)	0.89
Absence	41	13.35 (20.25)		6.77 (10.24)	
Extra-thyroidal invasion					
Presence	33	17.64 (21.87)	0.19	7.01 (10.95)	0.96
Absence	38	11.23 (18.49)		6.88 (13.76)	
Lymphovascular permeation					
Presence	13	25.80 (25.36)	0.02	8.00 (8.71)	0.74
Absence	58	11.61 (18.17)		6.71 (13.20)	
Lymph node metastasis					
Presence	39	13.76 (17.76)	0.84	5.14 (6.63)	0.18
Absence	32	14.75 (23.20)		9.13 (17.00)	
Distant metastasis					
Presence	1	13.22 (NA)	0.96	0.37 (NA)	0.60
Absence	70	14.22 (20.39)		7.04 (12.51)	
TNM staging					
I & II	38	12.94 (20.20)	0.57	7.11 (13.89)	0.90
III & IV	33	15.68 (20.50)		6.75 (10.77)	
MACIS score					
< 6	42	12.50 (19.44)	0.40	6.80 (13.23)	0.91
≥ 6	29	16.68 (21.45)		7.15 (11.44)	

SD= standard deviation; NA=not applicable

Table 4. Relationship between clinicopathological features of papillary thyroid carcinoma and TGF- α or EGFR protein expression

Variable	Number	TGF- α expression			EGFR expression		
		0-2+	3+	p-value	0-2+	3+	p-value
Age							
≤ 45	28	27	1	0.40	5	23	0.94
> 45	31	31	0		6	25	
Gender							
Male	15	15	0	0.58	2	13	0.82
Female	44	43	1		9	35	
Tumour size							
$\leq 4\text{cm}$	51	7	44	0.05	42	9	0.94
$> 4\text{cm}$	8	3	5		7	1	
Multifocality							
Presence	28	8	20	0.02	23	5	0.75
Absence	31	2	29		26	5	
Extra-thyroidal invasion							
Presence	27	5	22	0.41	24	3	0.53
Absence	32	5	27		25	7	
Lymphovascular permeation							
Presence	10	10	0	0.68	9	1	0.86
Absence	49	48	1		40	9	
Lymph node metastasis							
Presence	31	5	26	0.99	28	3	0.28
Absence	28	5	23		21	7	
Distant metastasis							
Presence	1	0	1	0.90	1	0	0.25
Absence	58	10	48		48	10	
TNM staging							
I	32	31	1	0.68	5	27	0.02
II	1	1	0		1	0	
III	18	18	0		2	16	
IV	8	8	0		3	5	
MACIS score							
< 6	36	4	32	0.07	5	31	0.03
≥ 6	23	6	17		6	17	

Figure Legends

Figure 1

mRNA expression of TGF- α (A) and EGFR (B) by Real-time RT-PCR in three groups of thyroid tissues: tumour (papillary thyroid carcinoma), adjacent (paired thyroid tissue from adjacent or contra-lateral lobe of papillary thyroid carcinoma) and benign (benign thyroid tissue). Y-axes are arbitrary units and independent for each component.

Figure 2

2A. Representative immunochemical staining for TGF α in two groups of papillary thyroid carcinoma: high level of expression, low level of expression and negative expression in benign thyroid tissues.

2B. Representative immunochemical staining for EGFR in two groups of papillary thyroid carcinoma: high level of expression and low level of expression as well as negative expression in benign thyroid tissues.

2C. High magnification of papillary thyroid carcinoma with high level of expression showing strong cytoplasmic expression of EGFR. The intranuclear pseudoinclusions of the cancer cells were also positive for EGFR,

Figure 3 Correlation between mRNA expression of TGF- α and EGFR in papillary thyroid carcinoma.