

## Can the Expression Pattern of Estrogen Receptor Alpha and Beta Proteins in Papillary Thyroid Carcinoma be of Prognostic Value?

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**Abstract:** Increasing incidence of papillary thyroid carcinoma (PTC) in women suggests a potential role for estrogen and its receptor (ER) subtypes. In this study, we investigated the functional activity and expression pattern of estrogen receptor subtypes in PTC. We measured serum and thyroid tissue estradiol (E2) level, functional activity of ER by assessing its ligand binding activity (LBA), expression level of ER  $\alpha$  and ER  $\beta$  proteins in 68 patients with PTC. ER LBA significantly increased in all PTC patients tested. ER  $\alpha$  expression increased in 58.82% male and 50.98% female PTC. ER  $\beta$  expression decreased in 72.54% female but increased in 88.23% male PTC samples indicating a sex specific differential expression. In female PTC increased ER  $\alpha$  positively correlated with LBA. ER  $\beta$  negatively correlated with LBA and ER  $\alpha$  subtype. In male PTC, increased ER  $\alpha$  positively correlated with ER  $\beta$ . However, no significant correlation was observed between ER LBA and either of ER subtypes.

**Conclusions:** Change in the level of expression of ER subtypes in PTC tissues significantly affects papillary thyroid tumor growth with ER  $\alpha$  showing pro-mitogenic property, while ER  $\beta$  appears to be antimitogenic. Hence, analyzing the expression pattern of estrogen receptors may have good prognostic value in the diagnosis of PTC.

**Key words:** 17 $\beta$ -Estradiol, ER  $\alpha$ , ER  $\beta$ , thyroid carcinoma.

### INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and its prevalence is continuously increasing over the past three decades worldwide<sup>1</sup>. The age-adjusted incidence rate of thyroid cancer has been increasing worldwide mainly due to the rise of PTC in females<sup>2</sup>. Peak increase in the incidence of PTC in women during the active reproductive period indicates activation of sex steroid associated signaling pathways in the progression of the disease. The biological effect of 17 $\beta$ -estradiol (E2) results from its specific binding to estrogen receptor (ER) subtypes,  $\alpha$  and  $\beta$ , which belong to the nuclear receptor superfamily of ligand regulated transcription factors<sup>3</sup>. Studies from our laboratory and other investigators indicated the mitogenic effect of E2 on immortalized human thyroid cancer cell lines and in normal thyroid follicular cells<sup>4-10</sup>. The precise role of ER subtypes responsible for PTC tumor growth is not well defined. In this study, we tested the association of ER  $\alpha$  and ER  $\beta$  in modulating PTC tumor growth by correlating its expression with the ligand binding activity (LBA) in human PTC.

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### MATERIAL AND METHODS

#### Human Papillary Thyroid Carcinoma tissues

This study included human PTC tissue samples collected from 68 patients (male-17; female-51) who underwent surgical resection at the Department of Endocrine Surgery, Madras Medical College, Chennai, India (Table 1). The tumor type was confirmed based on the histopathological report from the Department of Pathology, Madras Medical College. Normal tissues removed from the opposite lobe of the thyroid gland of 13 patients subjected to total thyroidectomy (3 males, mean age 35.0 $\pm$ 12.0; and 10 females, mean age 28.2 $\pm$ 4.28) and confirmed based on the histopathological report were used as controls. This study was carried out in accordance with the Helsinki Declaration (2000) of the World Medical Association, and was approved by our institutional ethical committee for research in humans (UNOM/HEC/2003/9). Written informed consent was obtained from all patients. Thyroid tissues and blood samples were collected as described previously<sup>4</sup>.

#### Enzyme Linked Immunosorbent Assay (ELISA)

Serum and thyroid tissue E2 was assayed by ELISA according to the manufacturer's instruction (DRG Instruments GmbH, Marburg, Germany).

#### Radio receptor assay

The functional activity of ER in terms of its LBA was quantified in thyroid tissue using [<sup>3</sup>H] E2 as elaborated earlier (Banu et al., 2002, Stanley et al., 2010).

**Table 1:** Patient baseline characteristics, serum and papillary thyroid carcinoma (PTC) tissue  $17\beta$ -estradiol (E2) level.

Variable	Male	Female
Age (years)	38.8±3.6	36.2±2.5
Height (cm)	164±2.38	157.1±1.74
Weight (kg)	57.8±2.59	54.3±2.42
Serum Estradiol (pg/ml)	23.0±1.72	82.52±5.3
Tissue Estradiol (pg/g)	24.1±1.52	93.98±5.3

### Western blot analysis

Total protein from thyroid tissue was separated on 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was then used for detection of ER  $\alpha$  (MC-20) or ER  $\beta$  (H10) by western blotting. The band intensity of respective protein was normalized with  $\beta$ -actin and the results were expressed as optical density units relative to  $\beta$ -actin.

### Statistical analysis

All data were analyzed using SPSS@17.0 students version (SPSS Inc., Chicago, USA). Mann-Whitney U test for non-parametric samples and MedCalc@9.6.0.0 statistical software (MedCalc, Mariakerke, Belgium) were used to compare the means of normal and PTC tissue samples. Pearson's correlation coefficient analysis

was performed to assess the strength of association between ER LBA and ER subtypes. Results were considered statistically significant at  $P < 0.05$ .

## RESULTS

### Patient baseline characteristics, serum and tissue E2

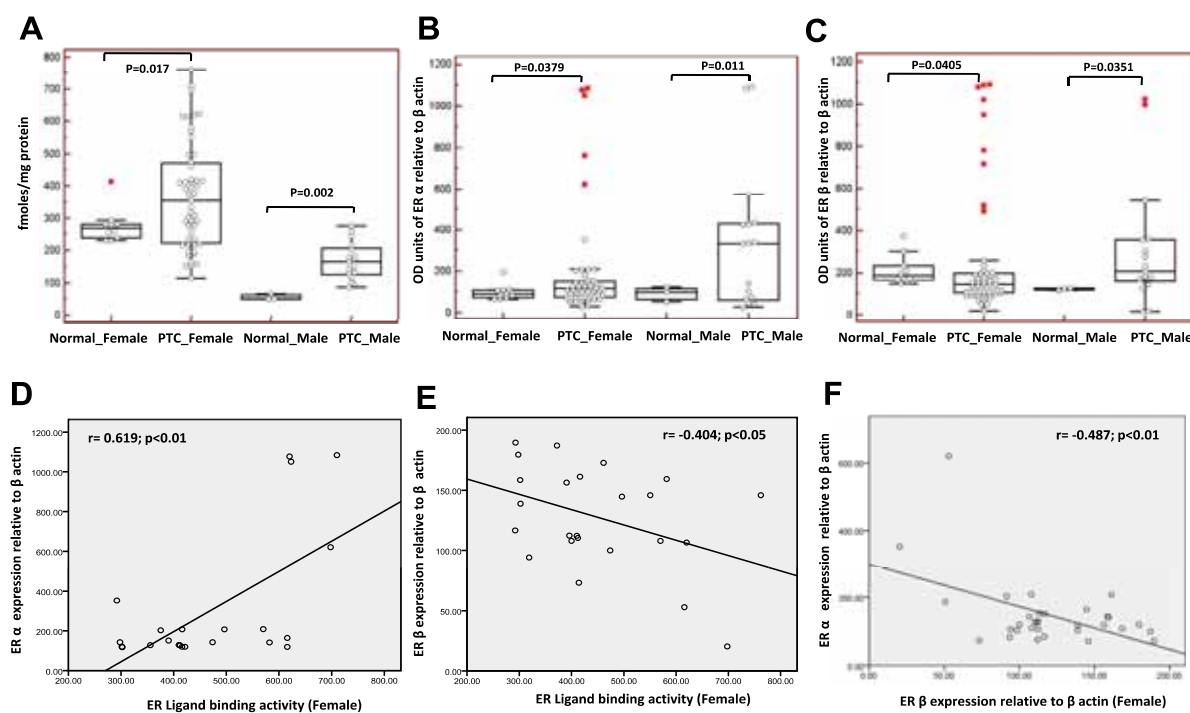
Anthropometric measurement details about the patients are given in Table 1. Serum and thyroid tissue E2 levels (bioavailability) remained within the normal level in male and female PTC.

### ER ligand binding activity and expression of ER subtypes in human PTC tissues

There was a significant increase in the LBA of ER in both female (66.66%) and male (100%) PTC when compared to normal thyroid tissue ( $P < 0.05$ ) (Fig. 1A, Table 2). ER  $\alpha$  expression significantly increased in a majority of PTC irrespective of the sex (Female-50.98%; Male-58.82%) when compared to normal thyroid tissue ( $P < 0.05$ ) (Fig. 1B, Table 2). Unlike ER  $\alpha$ , ER  $\beta$  showed a sex specific variation in its expression level, it significantly decreased in female (72.54%) but increased in male (88.23%) PTC, when compared to normal thyroid tissue (Fig. 1C, Table 2).

### Association of ER subtypes with ER LBA in human PTC tissues

Pearson's correlation analysis showed a significant positive



**FIG1:** Functional activity of ER, expression of ER subtypes (ER  $\alpha$  and ER  $\beta$ ) in human PTC tissues. ER LBA (A); ER  $\alpha$  (B); ER  $\beta$  (C). The box and whisker plot comprises three components. The horizontal line in the box indicate the central tendency of location (median value), a box to indicate variability (standard error) that represents values from the lower to the upper quartiles (25th to 75th percentile), the line extends from the minimum (5th percentile) to the maximum (95th percentile) values, excluding 'outside' values (<) i.e., values smaller than the lower quartile minus 1.5-fold the inter quartile range, or larger than the upper quartile plus 1.5-fold the inter quartile range, plotted as a square marker. Please refer Table 1 for number of cases and characteristics of tumor samples. Correlation between ER  $\alpha$  protein expression and ER ligand binding activity in female PTC (D). Correlation between ER  $\beta$  protein expression and ER ligand binding activity in female PTC (E). Correlation between ER  $\alpha$  and ER  $\beta$  subtypes in female PTC (F).

correlation between increased ER LBA and ER  $\alpha$  protein expression ( $r=0.619$ ;  $P<0.01$ ), whereas there was a negative correlation between increased ER LBA and ER  $\beta$  protein expression ( $r=-0.404$ ;  $P<0.05$ ), indicating that increased LBA may result from ER  $\alpha$  subtype in PTC females (Fig. 1D, E). We then analyzed the relation between ER  $\alpha$  and ER  $\beta$  in individual samples. ER  $\beta$  protein showed a negative correlation with ER  $\alpha$  in PTC from female patients ( $r=-0.487$ ;  $P<0.01$ ) (Fig. 1F). In PTC males, no significant correlation was observed between ER LBA and either of ER subtypes (Fig. 2A, B). Interestingly, in most of the PTC samples from male with overexpression of ER  $\alpha$ , there was a positive correlation with ER  $\beta$  ( $r=0.813$ ;  $P<0.01$ ) (Fig. 2C).

**DISCUSSION**

Our results indicate a boost in ER LBA with a parallel increase in ER  $\alpha$  expression in human PTC tissues. We reported a positive and

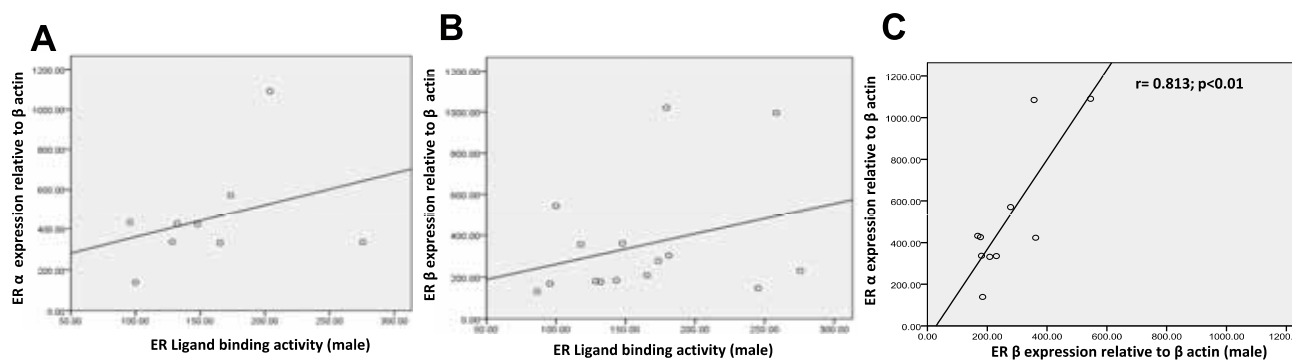
direct effect of  $17\beta$ -E2 on N-bis (2-hydroxypropyl) nitrosamine (DHPN)-induced thyroid tumorigenesis in rats, which was associated with an increased ER level in thyroid tumor tissue<sup>12</sup>. Our earlier studies also showed a homologous up regulation of ER in rat thyrocytes and thyroid carcinoma cell lines after E2 stimulation to activate its mitogenic effect supporting the clinical observation<sup>6,7</sup> that overexpression of ER  $\alpha$  may be responsible for E2-induced growth and progression of PTC in females. Subsequently, a consistent higher ER  $\alpha$  labeling index in PTC tissue of premenopausal women than normal thyroid was reported by Kawabata<sup>13</sup> validating the view that ER  $\alpha$  plays a major role in thyroid tumorigenesis and can be used as a differential diagnostic marker to assess the disease prognosis.

Functionally, ER  $\alpha$  and ER  $\beta$  exerts opposite effects in many cell types, with ER  $\alpha$  showing a pro-mitogenic effect in the breast, uterus and prostate while, ER  $\beta$  showing antimitogenic effect in the prostate, mammary gland, colon and lung<sup>14</sup>. The opposing effect of the two ERs was also reported in

**Table 2:** The overall trend and the percentage of difference obtained in various assays.

Parameters	PTC	Trend	% samples
Number of Samples analyzed	Male - 17		
	Female - 51		
Serum Estradiol concentration	Male	~	100%
	Female	~	100%
Tissue Estradiol Concentration	Male	~	100%
	Female	~	100%
ER Ligand Binding Activity	Male	↑	100%
	Female	↑	66.66%
		~	3.92%
		↓	29.42%
ER $\alpha$ protein expression	Male	↑	58.82%
		~	11.76%
		↓	29.41%
	Female	↑	50.98%
		~	33.33%
		↓	15.68%
ER $\beta$ protein expression	Male	↑	88.23%
		~	Nil
		↓	11.76%
	Female	↑	17.64%
		~	9.8%
		↓	72.54%

PTC, Papillary thyroid carcinoma; ER – estrogen receptor  
 ↑ - Increased level; ↓ - Decreased level; ~ - Unaltered level.



**Fig. 2:** Correlation between ER  $\alpha$  protein expression and ER ligand binding activity in male PTC (A). Correlation between ER  $\beta$  protein expression and ER ligand binding activity in male PTC (B). Correlation between ER  $\alpha$  and ER  $\beta$  subtypes in male PTC (C).

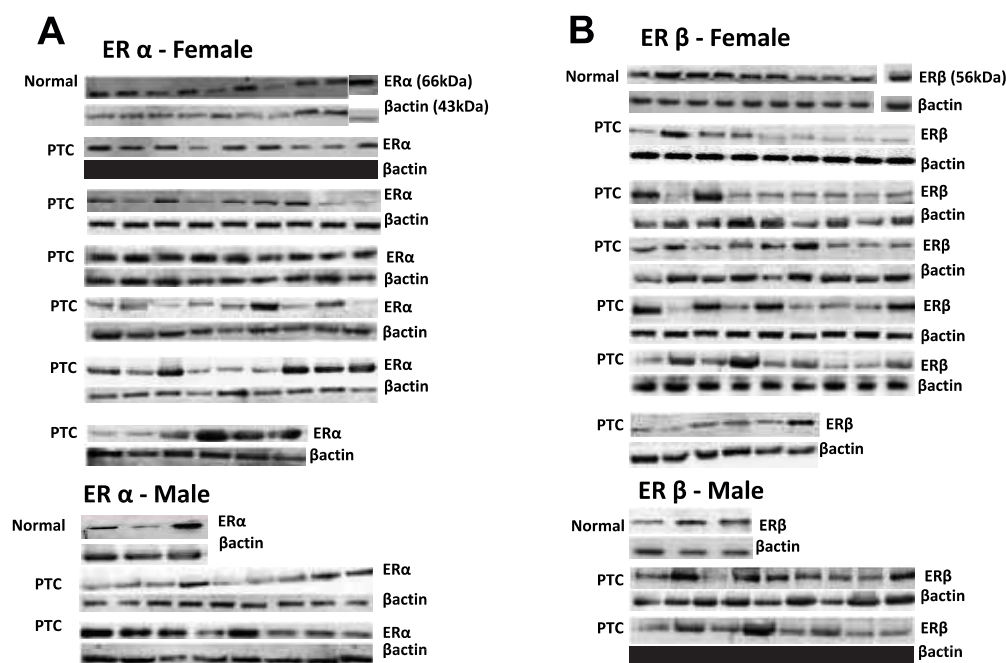


Fig.3: Immunoblots of ER  $\alpha$  (A) and ER  $\beta$  (B) in normal and PTC tissues.

various thyroid cancer cell lines<sup>10</sup>. An increase in the ratio of ER  $\alpha$ : ER  $\beta$  expression may therefore, favor thyroid tumor progression in female PTC. Consequently, loss of ER  $\beta$  in female PTC individuals appears to be a primary factor in facilitating the mitogenic effect of ER  $\alpha$  in thyroid tumor progression. The protective effect of ER  $\beta$  seems to be compromised in subjects expressing a normal ER  $\alpha$  where ER  $\beta$  expression is low. Such upset in the ER  $\alpha$ : ER  $\beta$  ratio was more conserved among females than males. In our previous study, we observe decreased expression of AR in most of the PTC females and overexpression of AR in PTC males emphasizing that AR expression pattern in thyroid cancer tissues of men and women may predispose to the sex specific incidence of thyroid tumors<sup>11</sup>. Thus, in men who have a relatively low incidence of PTC than women, AR appears to play a major role in promoting the progression of the cancer, despite a parallel increase in ER subtypes. However, investigations in more number of samples may throw more light on the issue. In the light of our earlier report on AR protein<sup>11</sup>, results from the present study on ER subtypes suggest that a fine balance between AR and ER subtypes may determine the nature and progression of thyroid cancer in humans. The differential expression pattern of these receptors between male and female PTCs may contribute to the sex specific variation in the incidence of PTC with female predominance. The present study also suggests that increased expression of ER  $\alpha$  with a general decline in the expression of ER  $\beta$  as a possible mechanism underlying the progression of papillary thyroid tumor in women as in the case of classical estrogen dependent tumors like breast cancer. Alterations in the ratio of ER  $\alpha$ : ER  $\beta$  might determine the growth and progression of PTC with ER  $\alpha$  acting as pro-mitogenic factor. We suggest that ER  $\alpha$  represents a novel prognostic marker for PTC. Taken together, our earlier findings of varied expression of AR protein in malignant and non-malignant thyroid tissues, depending upon the expression of micro RNA 124 a<sup>11</sup> and the present study, it is suggested that sex steroid receptors status may have good prognostic value in the diagnosis of thyroid cancer.

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