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Differential expression patterns of estrogen receptor (ER)-β splice variants between papillary thyroid cancer and nodular thyroid goiter

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The aim of this study was to investigate the expression patterns of estrogen receptor (ER) β1 (wild-type ERβ) and ERβ2 (ERβcx) in papillary thyroid cancer (PTC) and nodular thyroid goiter (NTG), and to explore the reasons for the higher incidence of PTC in women of reproductive age.

Material/Methods: ERβ1 and ERβ2 expression was examined immunohistochemically on paraffin-embedded thyroid tissues from 106 patients with PTC and 30 patients with NTG.

Results: There was significant difference in the subcellular localization of ERβ1 ($P<0.001$), but not in the positive percentage, between PTC and NTG specimens. No significant difference was found in the positive percentage or the subcellular distribution of ERβ2 expression between PTC and NTG specimens. Both nuclear and nucleocytoplasmic ERβ1 expressions were significantly lower in PTC lesions than in NTG tissue ($P<0.001$ and $P<0.05$, respectively), while ERβ2 expression was significantly higher in the former than the latter ($P<0.05$). ERβ1 expression in reproductive-aged (18~45 years) female patients with PTC was lower than that in age-matched male patients ($P<0.05$), while ERβ2 expression had the opposite expression profile ($P<0.05$). There was no significant difference in ERβ1 and ERβ2 expression between reproductive-aged and advanced reproductive-aged (>45 years) female patients with PTC.

Conclusions: This preliminary study indicates that the expression patterns of ERβ1 and ERβ2 differ between malignant PTC lesions and benign NTG tissue, and their expression might be involved in the female predominance of PTC during the reproductive years. The clinical and biological significance of these results await further investigation.

key words: **estrogen receptor β • splice variants • thyroid gland • tumors • goiter**

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Background

Thyroid cancer represents the most frequent endocrine malignancy, and its incidence has significantly increased in the past several decades [1]. Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for 80% of all thyroid cancers. The incidence of thyroid cancer is 3 to 5 times more frequent in women than in men. This female predominance, which is greatest during reproductive age, is observed in all geographical areas and ethnic groups [2]. The use of oral contraceptives appears to result in a moderately increased risk of developing thyroid cancer [3–5]. An elevated risk was also reported in women who used estrogens for gynecological problems, but not for low-dose estrogen replacement therapy in postmenopausal women [3,6]. In fact, the incidence of thyroid cancer decreases after menopause [7]. The difference in incidence between the sexes suggests that the growth and progression of thyroid tumors is influenced by female sex hormones, particularly estrogen, which has been clearly implicated in the development and progression of breast, endometrial, and prostate cancers [8–10].

Estrogens are the predominant sex hormones in females. They exert their action in target tissues via binding to 1 of the 2 estrogen receptors (ER), ERα or ERβ. Like other steroid hormone receptors, ERs act as dimers to regulate transcriptional activation. Full transcriptional activation by ERs is mediated by synergism between 2 activation domains, Activation Function-1 (AF-1) at the N terminus and AF-2 in the ligand-binding domain. Both ERα and ERβ contain the potent AF-2 function, but unlike ERα, ERβ seems to have a weaker corresponding AF-1 function, and thus depends more on the ligand-dependent AF-2 for its transcriptional activation function [11]. Recently, several splice variants of ERβ with truncations or insertions in the C-terminal ligand-binding domain (ERβ1~ERβ5) have been described and widely studied in breast, endometrial, and prostate cancers [8–10]. Among the exon 8 splice variants of ERβ, the expression and function of ERβ2 (ERβcx) is well-documented. It appears that ERβ2 lacks the AF-2 core region and does not bind to ERβ ligands, but does bind to estrogen responsive elements (EREs) as a homodimer and as a heterodimer with either wild-type ERα or ERβ [12,13]. ERβ2 preferentially forms a heterodimer with ERα rather than with ERβ, inhibiting DNA binding by ERα. ERβ2 shows a dominant negative activity on transactivation mediated only by ERα [12,14,15]. The possible molecular mechanism is that ERβ2 induces proteasome-dependent degradation of ERα, presumably through the formation of ERβ2/ERα heterodimers [16].

ERs have been described in both neoplastic and non-neoplastic human thyroid tissues by immunohistochemical studies. In PTC, ERα expression is significantly higher in premenopausal women than in post-menopausal women and in men of various ages [17]. No consistent findings were reported about the correlation between ERβ expression and clinicopathological findings including age, menopausal status, sex, and/or histological type of thyroid lesions [17,18]. To the best of our knowledge, the expression of ERβ splice variants in PTC has not yet been evaluated by immunohistochemistry in the English literature.

In this paper, we used immunohistochemical method to determine the expression of ERβ1 (wild-type ERβ) and the C-terminal truncated splice variant ERβ2 (ERβcx) in benign and malignant thyroid tissues, and to further explore the reasons for the higher incidence of PTC in women of reproductive age.

Material and Methods

Clinical material

Thyroid specimens were obtained from 106 patients with PTC and 30 patients with NTG who were first admitted to our hospital with ⩽3 years' duration and who underwent a standard thyroidectomy between 2007 and 2010. Patient records were obtained from the Medical Records Department at our hospital and also from pathology reports. Their diagnoses were confirmed by histopathological examination. None of these patients had a history of familial thyroid cancer or neck external irradiation. The patients were divided into 2 groups on the basis of age: reproductive-aged patients (18~45 years) and advanced reproductive-aged patients (>45 years). Of the 106 PTC patients, there were 50 reproductive-aged female patients (mean age, 32.50±8.71 years; age range, 18~45 years), 39 advanced reproductive-aged female patients (mean age, 56.87±7.89 years; age range, 46~81 years), and 17 reproductive-aged male patients (mean age, 32.88±7.17 years; age range, 23~45 years). NTG patients comprised 30 reproductive-aged patients (mean age, 38.43±4.90 years; age range, 24~45 years).

Immunohistochemistry

Immunohistochemical staining was performed for both ERβ1 and ERβ2 to evaluate immunoreactivity in the abovementioned tissue samples. Paraffin-embedded tissue sections fixed in formalin were used. Slides were deparaffinized, rehydrated, and subjected to microwave heat antigen retrieval in 10 mM citrate buffer (pH 6.0) for 20~25 min. After blocking endogenous peroxidase activity, sections were incubated with primary antibodies against ERβ1 (1: 20; Clone PPG5/10, Serotec) and ERβ2 (1: 400; Clone 57/3, Serotec) overnight at 4°C. After washing with PBS, staining was performed by the Elivision™ plus two-step system (Maixin Bio, China). Immunoreactivity was visualized using the chromogen 3,3'-diamino-benzidine (DAB) (Maixin Bio, China). Slides were then counterstained with hematoxylin, washed, dehydrated with alcohol and xylene, and mounted onto coverslips. Appropriate positive and negative controls were run simultaneously with the patient specimen.

Evaluation of immunohistochemistry

Immunohistochemistry staining for ERβ1 and ERβ2 was interpreted using the Allred score [19,20]. Briefly, a proportion score (PS) represented the estimated proportion of tumor cells staining positive, as follows: 0 (none); 1 (1/100); 2 (1/100 to 1/10); 3 (1/10 to 1/3); 4 (1/3 to 2/3); and 5 (>2/3). An intensity score (IS) represented the average intensity of the positive cells: 0 (none); 1 (weak); 2 (intermediate); and 3(strong). The proportion and intensity scores were then added to obtain a total score (TS), which could range from 0 to 8, and "positive" is defined as scores ⩾3. Two investigators (Dong WW and Huang YH) independently

Table 1. Subcellular distribution of ERβ1 and ERβ2 in PTC and NTG.

	ERβ1				ERβ2			
	Nu	Cyto	Nu+Cyto	—	Nu	Cyto	Nu+Cyto	—
NTG	23 (76.7)	0 (0.0)	5 (16.7)	2 (6.7)	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
PTC	7 (14.0)	5 (10.0)	36 (72.0)	2 (4.0)	49 (98.0)	0 (0.0)	1 (2.0)	0 (0.0)
P	0.001*	——	0.014**	——	0.03#	——	——	——

Numbers in parentheses represent percentages. Nu – positive nuclear staining of ERβ splice variants; Cyto – positive cytoplasmic staining of ERβ splice variants; Nu+Cyto – positive nuclear and cytoplasmic staining of ERβ splice variants; '–' – negative staining of ERβ splice variants. * difference in ERβ1 nuclear staining between NTG and PTC; ** difference in ERβ1 nuclear and cytoplasmic staining between NTG and PTC; # difference in ERβ2 nuclear staining between NTG and PTC.

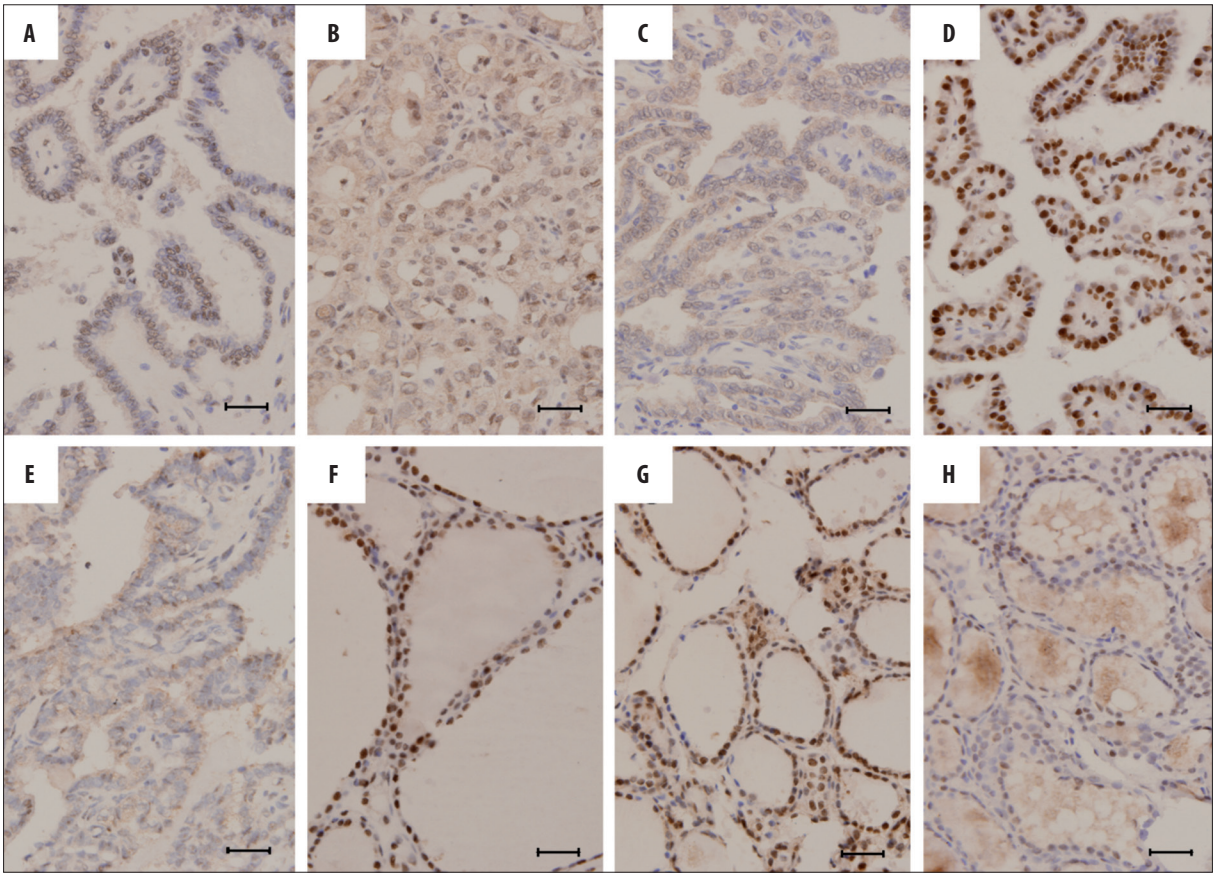

Figure 1. The subcellular distribution of ERβ1 and ERβ2 in PTC and NTG. Nuclear (**A**), nucleocytoplasmic (**B**) and cytoplasmic (**C**) staining of ERβ1 and nuclear (**D**) and nucleocytoplasmic (**E**) staining of ERβ2 in PTC. Nuclear (**F**) and nucleocytoplasmic (**G**) staining of ERβ1 and nuclear (**H**) staining of ERβ2 in NTG. Scale bar =5 μm.

evaluated the tissue sections. If the TS differed between the 2 investigators, a third investigator (Li J) evaluated the tissue sections, and a consistent result was rendered.

Statistical analysis

Descriptive statistics were used according to the distribution of variables. The Mann-Whitney U test was used for comparison of the immunohistochemistry scores. The χ^2 test or Fisher's exact test was used for the comparison of the frequency or proportions of single variables. Analyses were performed with SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). P values less than.05 were considered statistically significant.

Results

Expression of ERβ splice variants in reproductive-aged female patients with PTC and NTG

The subcellular distribution of ERβ1 and ERβ2 in PTC and NTG are shown in Table 1. Figure 1 illustrates the subcellular localization of ERβ1 and ERβ2 in PTC and NTG. There was significant difference in the subcellular localization of

ERβ1 (χ^2=34.069, *P*<0.001), but not in the positive percentage (χ^2=0.281, *P*=0.628; Fisher's Exact test), between PTC and NTG specimens. No significant difference was found in the positive percentage or subcellular distribution of ERβ2 between PTC and NTG specimens.

Nuclear ERβ1 expression in PTC was significantly lower than that in NTG (Z=–3.213, *P*=0.001), and nucleocytoplasmic ERβ1 expression in PTC was also lower than that in NTG (Z=–2.557, *P*=0.014). ERβ2 expression in PTC was higher than that in NTG (Z=–2.170, *P*=0.030).

Association between expression of ERβ splice variants and gender and age of patients with PTC

ERβ1 expression in reproductive-aged female patients with PTC was lower than that in corresponding male patients (Z=–2.387, *P*=0.017). On the contrary, ERβ2 expression in reproductive-aged female patients with PTC was higher than that in age-matched male patients (Z=–2.141, *P*=0.032). There was no significant difference in ERβ1 and ERβ2 expression between reproductive-aged and advanced reproductive-aged female patients with PTC.

Discussion

Unlike the traditional ERα, which is localized in the nuclei of cancer cells, ERβ splice variants have been detected in both the nucleus and cytoplasm of cancer cells [8,21]. However, the expression pattern of ERβ splice variants in thyroid cancer has not been adequately explored. Our data show nuclear and cytoplasmic expression of ERβ1 and ERβ2 were found in both PTC and NTG specimens, except cytoplasmic expression of ERβ2 in NTGs. Specifically, ERβ1 was predominantly localized in the nucleus of NTG cells, and in both the nucleus and cytoplasm of PTC cells, whereas ERβ2 was primarily localized in the nucleus of both NTG and PTC cells. It has been reported that ERβ isoforms (ERβ1, ERβ2, ERβ3, and ERβ5) are detected only in the nuclei of Barrett's metaplasia cells negative for dysplasia, and a significant number of carcinomas show cytoplasmic ERβ immunoreactivity, which is especially the case with ERβ2 [21]. It is speculated that perhaps there is a different level of expression of ERβ splice variants between normal or benign tissue and cancer tissue where certain isoforms with predominantly cytoplasmic immunoreactivity are associated with the malignant phenotype [22]. It may be ERβ1 in PTCs and ERβ2 in esophageal adenocarcinomas.

Consistent with findings in breast cancer, ERβ2 protein levels significantly increased in ductal carcinoma *in situ* (DCIS) and invasive breast cancer, compared to the adjacent normal gland [23]. We also found a decrease in both nuclear and nucleocytoplasmic ERβ1 expression and an increase in ERβ2 expression in PTC compared to NTG, indicating a role for ERβ2 in carcinogenesis that opposes the protective effect of wild-type ERβ1. However, the role of ERβ2 is quite the opposite in endometrioid carcinoma. Chakravarty et al reported that ERβ2 expression was decreased in endometrioid carcinoma compared to proliferative endometrium, and was decreased in higher grade tumors [24]; indicating that the roles of ERβ splice variants are specific to cancer types.

Currently, it is widely believed that ERβ has limited importance in thyroid cancer since no significant difference has been found in its expression between males and females, in benign versus malignant lesions, or in premenopausal versus postmenopausal females [18]. However, these data may reflect the fact that the presence of ERβ splice variants was not taken into consideration. In prostate cancer, no significant correlation was found between ERβ1 or ERβ2 expression and age [25]. In primary colorectal cancer, no association between sex and ERβ1 and ERβ2 protein expression was found [26]. However, a positive correlation between age and ERβ1 expression was identified in invasive breast cancer [27]. In our study we found that ERβ1 and ERβ2 expression was associated with sex but not with age, which may partly explain the female predominance of PTC during the reproductive years. Thus, the associations between ERβ splice variants with sex and age warrant further investigation.

In our study we determined the localization and expression pattern of ERβ splice variants and its significance in PTC. However, we did not study the relationship between ERβ splice variants and ERα, as all ERβ splice variants inhibit ERα transcriptional activity. We plan to study the association between ERβ splice variants and ERα in PTC in future studies.

Conclusions

This study investigated the expression pattern of ERβ splice variants in various types of thyroid lesions at the protein level using specific well-validated antibodies. Furthermore, it highlights the importance of ERβ splice variants in the development of PTC. The female predominance of PTC during the reproductive years may be associated with the expression of ERβ splice variants; thus, their clinical and biological significance merit further investigation.

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