

Influence of thyroid hormone receptors on breast cancer cell proliferation

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Background: The involvement of thyroid hormones in the development and differentiation of normal breast tissue has been established. However, the association between breast cancer and these hormones is controversial.

Therefore, the objective of the present study was to determine the protein expression pattern of thyroid hormone receptors in different human breast pathologies and to evaluate their possible relationship with cellular proliferation.

Patients and methods: The presence of thyroid hormone receptors was evaluated by immunohistochemistry and western blot analysis in 84 breast samples that included 12 cases of benign proliferative diseases, 20 carcinomas *in situ* and 52 infiltrative carcinomas.

Results: TR- α was detected in the nuclei of epithelial cells from normal breast ducts and acini, while in any pathological type this receptor was located in the cytoplasm. However, TR- β presented a nuclear location in benign proliferative diseases and carcinomas *in situ* and a cytoplasmic location in normal breast and infiltrative carcinomas. The highest proliferation index was observed in carcinomas *in situ*, although in infiltrative carcinomas an inverse correlation between this index and the TR- α expression was encountered.

Conclusions: The results of this study reveal substantial changes in the expression profile of thyroid hormone receptors suggesting a possible deregulation that could trigger breast cancer development.

Key words: breast, cancer, proliferation, thyroid hormone receptors

introduction

The thyroid produces and releases into the circulation two potent hormones, triiodothyronine (T3) and thyroxine (T4), which have an influence on the basal metabolism or the oxygen consumption in nearly all body tissues. Thyroid hormones also play a part in linear growth, brain function (including intelligence and memory), neural development, dentition and bone development [1].

Many studies *in vitro* and *in vivo* have related thyroid hormones and human cancer since Beatson [2] described the use of thyroid extracts for metastatic breast cancer treatment. Abundant data indicate that thyroid status affects tumour formation, growth and metastasis in experimental animals and humans [3, 4]. However, the relationship between thyroid status and the pathogenesis of human breast cancer is not yet understood [4].

Hormone dependency of human mammary neoplasia and controversial data on the relationship between the thyroid status of the patient and neoplastic illness [4–6] have suggested that

thyroid hormone receptors (TR- α and TR- β) could become a marker and a therapeutic target like the estrogen and progesterone receptors (ER and PR). TRs mediate multiple effects of T3 on the phenotype, proliferation and gene expression of cultured normal mammary epithelial cells [7–9]. However, only a few reports have described the presence of TR in breast tumours [10, 11] and breast cancer cell lines [12, 13]. López-Barahona et al. [8] have demonstrated that the overexpression of TRs affects the normal phenotype of mammary epithelial cells and Martínez et al. [14] reported that the addition of thyroid hormones at non-physiological concentrations can alter their proliferation. This effect has been observed in neuroblastoma cells in which the existence of a transcriptional cross-talk between the TRs and the *ras* oncogene has been demonstrated, influencing processes such as cell proliferation, transformation or tumorigenesis [15].

We therefore focused our study at the protein level to elucidate the location or distribution of TRs in different breast pathologies and to evaluate their possible relationship with the cellular proliferation by immunohistochemistry and western blot analysis in order to determine the possible influence of TRs in breast cancer development and progression.

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patients and methods

Breast samples used in this study were collected from 84 patients diagnosed by the Pathology Service of the Hospital Príncipe de Asturias of Alcalá de Henares. Glandular breast lesions were classified as follows: 12 cases of benign proliferative diseases including ductal and lobular hyperplasia, apocrine metaplasia, fibroadenoma and fibrocystic changes; 20 carcinomas *in situ* (CIS) classified as low, moderate and high grade (five, six and nine cases, respectively); and 52 infiltrative carcinomas (IC) (30 ductal and 22 lobular). Removal of tissues and the study of archive samples were carried out with the consent of the patients' relatives and the permission of the ethics committee of the hospital.

Each specimen was divided into two approximately equal portions: one portion was processed for immunohistochemistry (10% formalin fixed and paraffin embedded) and the other portion was frozen in liquid nitrogen and maintained at -80°C for western blot analysis.

immunoblotting

For western blot analysis, tissues were homogenized in the extraction buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM 1,4-dithio-L-threitol, and 0.2 M ethylenediamine tetracetic acid, pH 8) in addition to a cocktail of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ of leupeptine and 1 $\mu\text{g}/\text{ml}$ of aprotinin) and phosphatase inhibitors (200 $\mu\text{g}/\text{ml}$ of sodium fluoride and 50 $\mu\text{g}/\text{ml}$ of sodium orthovanadate) in the presence of 0.5% Triton X-100 and 0.1% SDS. Homogenates were centrifuged for 10 min at 9000 g at 4°C . Supernatants were mixed with an equivalent volume of sodium dodecyl sulfate (SDS) buffer (10% SDS in Tris/HCl, pH 8, containing 50% glycerol, 0.1 mM 2-beta-mercaptoethanol and 0.1% bromophenol blue). The mixture was then denatured for 4 min at 96°C , and aliquots of 50 μg protein were subjected to SDS-PAGE (9% acrylamide gel). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes (0.2 μm) in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS and 20% methanol) for 4 h at 250 mA. Briefly, the blots were blocked in 5% Blotto (Santa Cruz Biotechnology, CA, USA) for 1 h and after blocking, the sheets were incubated with primary antibodies diluted 1:1000 in blocking solution 1:9 overnight at room temperature.

After extensive washing with Tris-buffered saline/Tween 20, the membranes were incubated with swine anti-rabbit for TR- α and rabbit anti-mouse for TR- β biotinylated immunoglobulins (Dako, Barcelona, Spain) diluted 1:2000 in blocking solution 1:9 and afterwards an extensive wash with streptavidin-peroxidase (Zymed Laboratories Inc, CA, USA) at 1:10 000 dilution in the same blocking solution 1:9. Finally, the membranes were developed with an enhanced chemiluminescence (ECL) kit, following the procedure described by the manufacturer (Amersham, Buckinghamshire, UK).

immunohistochemistry

Following deparaffinisation, sections 5- μm thick were hydrated and incubated for 20 min in 0.3% H_2O_2 to inhibit endogenous peroxidase activity. To retrieve the antigen the sections were incubated with 0.1 M citrate buffer (pH 6) for 10 min at 96°C . After rinsing in TBS buffer, the slides were incubated with 3% normal donkey serum (NDS) in TBS for 1 h to prevent non-specific binding of the first antibody. Afterwards, the sections were incubated with the mouse monoclonal TR- β (Santa Cruz), proliferating cell nuclear antigen (PCNA) (Zymed) and rabbit polyclonal TR- α (Santa Cruz) primary antibodies. All of them were diluted at 1:200 in TBS containing 0.3% NDS. Sections for TRs were incubated with primary antibodies overnight at room temperature, and at 4°C for PCNA.

The sections were then washed in TBS and incubated for 1 h with either swine anti-rabbit (for TR- α) or rabbit anti-mouse (for TR- β and PCNA) biotinylated immunoglobulins (Dako), diluted 1:500 in TBS plus

0.3% NDS. After washing in TBS, the sections were incubated with streptavidin-peroxidase (Zymed) for 1 h and developed with 3,3'-diaminobenzidine (DAB) using the glucose oxidase-DAB-nickel intensification method [16]. After this, sections were dehydrated and mounted in DePex (Probus, Badalona, Spain).

To assess the specificity of immunoreaction, negative and positive controls were used. For negative controls, adjacent sections of different pathology (benign proliferative diseases, ductal and lobular carcinomas) were incubated omitting the primary antibody. As positive controls, sections of rat thymus and human thyroid gland were processed with the same antibodies anti-TRs.

quantitative evaluation of cell proliferation

The percentages of PCNA-immunostained nuclei (PCNA labelling index) [17] were calculated in each section using the formula: number of labelled nuclei/total number (labelled + unlabelled) of nuclei \times 100. Measurements were carried out using an Olympus microscope equipped with a \times 20 lens and using the stereologic software Motic Images Advanced 3.2 (Motic China Group Co., Ltd). This program allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. An average of 20 fields per section was scanned. The systematic field selection with a random start assures that cell proliferation estimates were representative of all the breast tissue.

statistical methods

Statistical associations between categorical variables (i.e. TR- α and TR- β expression and breast pathology types) were assessed by means of Pearson's chi-square test with a 5% significance level. Median of quantitative variables (i.e. cell proliferation indexes) among the three breast pathologies (i.e. benign breast disease, carcinoma *in situ*, infiltrative carcinoma) were compared by means of non-parametric Kruskal-Wallis test followed by post-hoc comparisons between pairs using the Mann-Whitney *U*-test. To adjust the significance level for multiple comparisons, Bonferroni correction was applied using a corrected significance level of 0.017. All statistical analyses were performed using SPSS 12.0.

To analyse the possible correlation between the thyroid hormone receptors expression and the different clinicopathological characteristics of the patients with infiltrative carcinomas, the Spearman correlation was realised.

results

western blot analysis

Western blot analysis was performed to evaluate the presence or absence of TRs and the possible modifications on their expression between the different pathologies analysed. We have observed that all samples studied showed a single band for each receptor at approximately 55 KDa (Figure 1). Moreover, TR- α showed a higher expression in benign breast diseases (BBDs) and carcinomas *in situ* (CIS) than in infiltrative carcinomas (IC). No changes in the intensity of TR- β immunoreaction were detected.

immunohistochemical study of thyroid hormone receptors

No background immunoreaction to TR antibodies was observed in any of the negative controls (Figure 2A). TR receptors were located in the nuclei of follicular cells in human thyroid (Figure 2B).

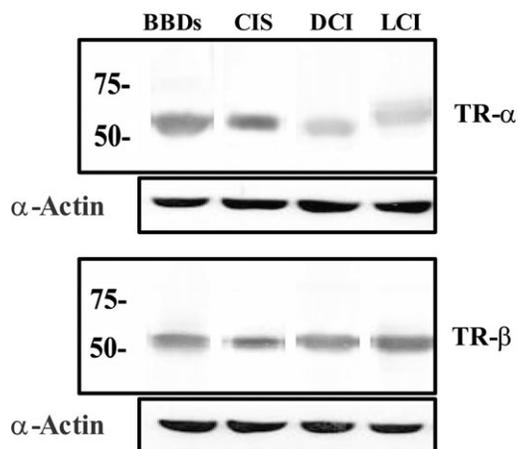


Figure 1. Western blot analysis of TR- α and TR- β . BBDs, benign breast diseases; CIS, carcinomas *in situ*; IDC, infiltrative ductal carcinomas; ILC, infiltrative lobular carcinomas.

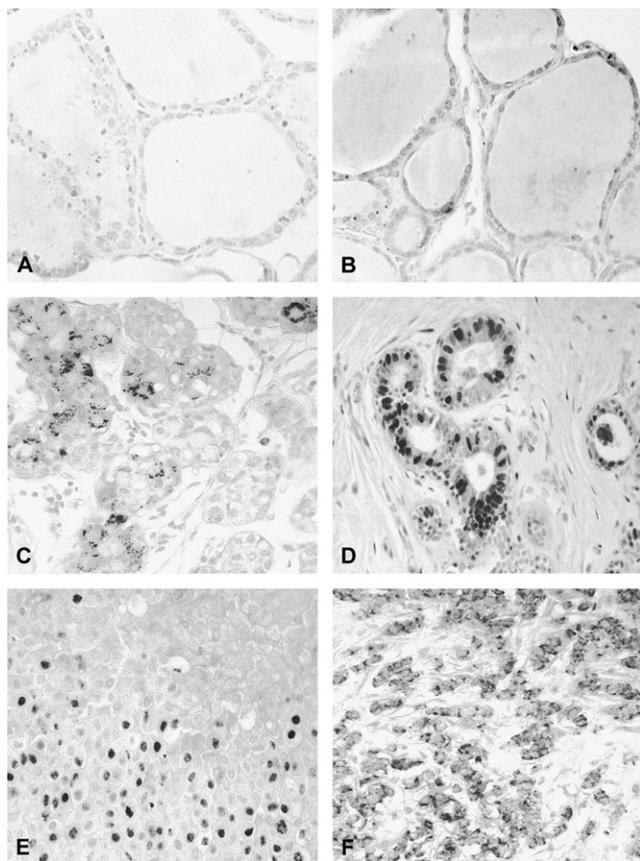


Figure 2. Immunohistochemistry of thyroid hormone receptors. (A) Negative control section from human thyroid gland showing no immunoreaction when it was incubated without the primary antibody ($\times 300$). (B) Positive control section from human thyroid gland. A positive reaction to TR- α antibody was observed in the nuclei of follicular cells. The colloid was slightly immunostained ($\times 300$). (C) Carcinoma ductal *in situ* showing a cytoplasmic immunoreaction to TR- α ($\times 300$). (D) Normal human mammary gland presenting a strong nuclear immunostaining to TR- α ($\times 250$). (E) TR- β with nuclear location in the carcinomas *in situ* ($\times 400$). (F) Infiltrative lobular carcinoma of small cells showing a cytoplasmic immunoexpression of TR- β ($\times 250$).

TR- α was the most abundant isoform of thyroid receptors in all breast lesions analysed. The highest percentage of cases immunostained for this isoform was recorded in BBDs, although this immunoexpression was always observed in the cytoplasm (Figure 2C). However, in the epithelial cells from normal breast ducts and acini present in these samples, a nuclear immunoreaction was observed (Figure 2D).

A small percentage of patients showed a positive immunoreaction for TR- β . In BBDs and CIS a nuclear immunoreaction was encountered (Figure 2E). In all pathology types, a cytoplasmic immunolabelling was also observed (Figure 2F). All carcinomas *in situ* analysed presented a pattern of immunostaining for these receptors independent of its histological grade.

The results of immunohistochemical analysis are shown in the Table 1. No statistical association was found between thyroid hormone receptors expression and the different breast pathology types analysed (chi-squared tests $P = 0.177$ for TR- α and $P = 0.168$ for TR- β).

proliferation index

Carcinomatous lesions showed an increased number of PCNA-immunoreactive nuclei compared with benign breast diseases. In these lesions, the highest proliferation index was recorded in *in situ* carcinomas. The median value of proliferation indices for each pathology group was 2.39 for benign breast diseases, 31.07 for carcinomas *in situ* and 10.29 for infiltrative carcinomas. Overall, there were statistical differences among the breast pathologies ($P < 0.001$, Kruskal–Wallis test). Post-hoc pair-wise comparisons (Mann–Whitney test with Bonferroni correction) showed significant differences between BBDs and CIS ($P < 0.001$) and between CIS and IC ($P < 0.001$).

We looked for a correlation between the proliferation index and the expression of thyroid hormone receptors in the different breast pathologies. We compared PCNA expression between samples with different TR- α and TR- β expression patterns in the three breast pathology subgroups and we found that only in the samples obtained from infiltrative carcinomas was PCNA expression statistically higher in the samples without TR- α expression than those that expressed TR- α (median PCNA expression 25.49 versus 8.65, $P = 0.004$, Mann–Whitney test).

The Spearman correlation between different clinical data—such as the age of patients, the tumour stage, the lymph node status, estrogen and progesterone receptors status, disease-free survival, proliferation index calculated previously—and the expression of both thyroid receptors

Table 1. Percentage of positive patients for thyroid hormone receptors in different pathologies of the breast

	BBDs (n = 12)		CIS (n = 20)		IC (n = 52)	
	N	C	N	C	N	C
TR- α	0 (0%)	11 (91.7%)	0 (0%)	15 (75%)	0 (0%)	34 (65.4%)
TR- β	2 (16.7%)	4 (33.3%)	4 (20%)	5 (25%)	1 (2%)	13 (25%)

BBDs, benign breast diseases; CIS, carcinomas *in situ*; IC, infiltrative carcinomas; n, number of cases; N, nuclear immunoreaction; C, cytoplasmic immunoreaction.

isoforms showed that only TR- α presented a direct significant statistical correlation with disease-free survival ($P = 0.0138$) and an inverse correlation with PCNA index ($P = 0.0234$).

discussion

The expression of thyroid hormone receptors seems to be an important point in the characterization of breast carcinomas. The results of this study reveal substantial changes in the expression profile of these receptors since TR- α was detected in the nuclei of epithelial cells from normal breast ducts and acini while in any pathological type this receptor was located in the cytoplasm. However, TR- β presented a nuclear location in benign proliferative diseases and carcinomas *in situ* and a cytoplasmic location in normal breast and infiltrative carcinomas.

Studies about thyroid hormone receptors expression in human breast cancer are scarce. Only a few reports describe the presence of TR in breast tumours [10, 11] and breast cancer cell lines and they are mainly focused on the gene expression between normal and carcinomatous samples [15, 18].

Recent studies have demonstrated that the presence of thyroid abnormalities may influence breast cancer progression [19, 4]. Saraiva et al. [20] observed that postmenopausal breast cancer patients have a significantly increased thyroid hormone/estradiol ratio, suggesting a possible tumour growth-promoting effect caused by this misbalance.

In different carcinomas types including breast cancer, González-Sancho et al. [21] have demonstrated the existence of specific alterations in TR- α and TR- β gene expression, suggesting that the deregulation of target genes transcription of the thyroid hormones could be implicated in the genesis of these neoplasias. We have observed that the expression of these receptors suffers important modifications in the different pathologies. Thus, only normal breast ducts and acini showed nuclear immunoreaction for TR- α and this location was changed in any pathological situation, as in benign breast diseases in carcinomatous lesions. For TR- β this nuclear immunoreaction was located in benign breast diseases and carcinomas *in situ*, and infiltrative carcinomas were always located in the cytoplasm.

The nuclear localization of TR- α in the nuclei of epithelial cells from normal mammary gland, seems to be related to an adequate balance between proliferation and differentiation regulated by the formation of heterodimers RXR-TR that act as suppressors of *ras* oncogen. However, the cytoplasmic location of TR- α in all human breast lesions studied could suggest the alteration of this balance, crucial in the cancer genesis. These location changes might begin by deletions in the receptor molecule promoting a cytoplasmic accumulation, taking into account that this receptor is localized in the nucleus in the absence of ligand [22].

García-Silva and Aranda [15] related the expression changes of thyroid hormone receptors to proliferation and tumoral transformation mechanisms, suggesting that TRs bring about the potent suppressors role of the *ras* oncogen actions. The proteins activated by Ras induce the cyclin D1 expression, and T3 seems to be crucial in the mechanism that blocks the proliferation dependent of Ras. However, the repression of cyclin D1 levels by T3 is reverted when cyclin is overexpressed

[15]. Cyclin D1 is frequently overexpressed in breast carcinomas and its overexpression seems to be correlated with a poor prognosis [23]. This fact is associated with the inverse correlation that we have encountered between the proliferation index and the TR- α expression in breast tumours. Nevertheless, many mutations of thyroid hormone receptors and methylations in its promoters have been described in different carcinoma types [18, 24]. Thus, we have suggested that the expression loss of these receptors previously proposed by García-Silva and Aranda [15] or its cellular location changes and, therefore, changes in its functionality, could increase the tumoral development or promote an advantage in the cellular transformation.

The nuclear expression of TR- β in the carcinomas *in situ* of the human mammary gland reflects its potential biological role in the tumoral progression. The loss of nuclear immunostaining observed in the infiltrative carcinomas, could be the result of either an alteration in the normal splicing mechanism as reported for *mdm2* and *ER α* [25, 26] or a gene biallelic inactivation observed by Li et al. [24] in 25% of primary tumours that they analysed. This suggests that possible epigenetic changes in the promoter region of TR- β could induce the gene inactivation in the first steps of aggressive carcinoma development.

In this study we have observed that the percentage of positive patients for both isoforms of thyroid hormone receptors was diminished from benign breast diseases to infiltrative carcinomas. However, Puzianowska-Kuznicka et al. [27] observed in the majority of examined renal clear cell carcinomas (RCCCs), that the amount of TR α 1 protein is increased while the amount of TR β 1 protein is decreased. It should be noted, however, that there are RCCCs with a decreased amount of TR α 1 protein or RCCCs characterized by TR β 1 protein overexpression. These observations suggest that the TRs present a cell-specific expression.

All of these data reveal substantial changes in the expression profile of thyroid hormone receptors suggesting a possible deregulation of its target genes that could trigger breast cancer development. Although it would be necessary to amplify this study with a higher number of patients to correlate their expression with possible clinical implications, we hypothesise that these receptors may serve as new therapeutic targets in the treatment of breast cancer.

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