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Thyroid hormone receptor (TR)alpha and TRbeta expression in breast cancer

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Summary. There is evidence that breast cancer patients suffer from thyroid disorders. However, the relation between thyroid receptor (TR) expression and breast cancer remains unknown so far. Therefore, the aim of this study was an immunohistochemical analysis of TR expression in breast cancer patients.

Materials and methods: The expression of the combined antibody TRalpha1 and 2 and TRalpha1 or 2 alone as well as the expression of combined TRbeta1 and 2 and TRbeta1 or 2 alone was investigated with specific monoclonal or polyclonal antibodies in 82 patients. All patients presented with a first diagnosis of sporadic breast cancer. The ABC method was used for staining and staining intensities were analyzed using the IRS score.

Results: Both TRalpha and TRbeta were expressed in the nuclei of breast cancer cells. In 24% (28/78) of the slides TRalpha1 and 2 IRS was positive. Immunopositivity for TRalpha1 was found in 55/78 slides, for TRalpha 2 in 54/79 slides (71 and 68%, respectively). The expression of TRbeta1 and 2 showed a positive detection in 33/77 (43%) of the slides, for TRbeta1 it was 43/79 (54%), for TRbeta2 60/76 (79%).

Significant correlations of the expression of TRs - especially TRalpha2 - were found with further prognostic histopathological parameters such as tumor size, axillary lymph node involvement, grading and hormone receptor status. Multivariate analysis showed a trend for TRalpha2 as an independent predictor of disease-free and overall survival.

Discussion: Our results revealed specific alterations in the expression of TRs - especially of TRalpha2 - in breast cancer patients, suggesting it as a marker with possible prognostic validity.

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Introduction

Breast cancer is the most frequent malignant tumor in women worldwide. So far, pathological findings like axillary lymph node involvement, hormone receptor (estrogen (ER) and progesterone receptor (PR)) status and differentiation grade have been established as important prognostic factors.

There is evidence of a connection between thyroid disorders and breast cancer (Rasmusson et al., 1987; Turken et al., 2003). A distinct link does exist between thyroid hormones and breast cancer. This is reflected in higher incidences of breast cancer in patients with thyroid dysfunction compared to healthy controls (Rasmusson et al., 1987; Giani et al., 1996; Shering et al., 1996; Smyth et al., 1998; Schernhammer et al., 2001; Turken et al., 2003; Kuijpens et al., 2005). Recent studies showed increased serum levels of thyroid-stimulating hormone (TSH) with subclinical or manifest hypothyroidism in 10% - 19.7% of breast cancer patients (Limanova et al., 1998; Jiskra et al., 2003). Furthermore, associations with hyperthyroidism, hypothyroidism and thyroiditis have been reported, but no convincing evidence exists for a causal role for overt thyroid disease in breast cancer (Smyth, 1997). In our former prospective study of thyroid disorders in breast cancer patients (Ditsch et al., 2010) patients with therapeutic

adjusted hypothyroidism had TSH levels within the normal range, but hypothyroidism was more frequent in breast cancer patients than in patients with a Carcinoma in situ of the breast, women with benign breast tumour and healthy controls. Thyroid dysfunction (especially hypothyroidism) seems to appear during long-term follow-up of breast cancer patients (Bruning et al., 1985). Furthermore fT4 and TSH are predictive indicators of therapeutic response and prognosis of patients with recurrent breast cancer (Yokoe et al., 1996).

While performing their function using thyroid hormones, the role of thyroid receptors (TRs) is poorly understood and the etiologic and prognostic relevance is unknown.

TRs are ligand-modulated transcription factors regulating the expression of target genes upon binding to specific sequences, known as hormone response elements (Muñoz and Bernal, 1997).

The nuclear receptors of thyroid hormones regulate the expression of specific cellular genes by interacting with distinct DNA sequences. They are ligand-activated transcription factors, which regulate the transcription of target genes. TRs are encoded by two genes – TR alpha and beta – located on human chromosomes 17 and 3 (Silva et al., 2002). They have three major isoforms: TRalpha1, TRalpha2 and TRbeta1 (Ling et al., 2010) with high homology in amino acid composition. The most diversified region between TRalpha and TRbeta is located in the N-terminal area, related to their trans-activation activity (Lazar, 1993; Truss and Beato, 1993).

The hormone dependency of the mammary gland and the functional similarity of TRs and ER/PR (both act in the nucleus as transcription factors) have led to the hypothesis that TRs may be a prognostic marker in breast cancer patients (Li et al., 2002a). López-Barahona et al. (1995) have demonstrated that the overexpression of TRs affects the normal phenotype of mammary epithelial cells and Martinez et al. (2000) reported that the addition of thyroid hormones at non-physiological concentrations can alter mammary epithelial cell proliferation. Furthermore, thyroid hormones or their antagonists that act via TRs have been suggested as a possible treatment for metastatic breast cancer (Beatson, 1896) or could be used as prophylactic agents for breast and genital cancer (Loeser, 1954).

Most investigations of TR expression in breast cancer were performed in vitro (Zhou-Li et al., 1992). Few studies demonstrated over-expression in epithelial cells of the breast (Lopez-Barahona et al., 1995) or breast tumors (Smallridge and Latham, 1980).

The rationale for this study with the detection of TR in breast cancer tissue was the known increased incidence of breast cancer in association with altered thyroid hormone levels (acting through TR) and the functional relationship of TRs and ER/PR. In breast cancer, ER/PR detection is associated with prognostic relevance (Knight et al., 1977; Maynard et al., 1978; Pichon et al., 1980), and it has long been known that overexpression is treatable with anti-hormonal therapy (De Sombre et al., 1978; Osborne and McGuire, 1979). The prognostic relevance of ER/PR is considered to be independent of estrogen and progesterone hormone blood level, therefore we focused only on immunohistochemical detection of TRs and correlated the staining results with the clinical parameters of breast cancer patients.

Materials and methods

Patients

82 patients with a first diagnosis of sporadic breast cancer were chosen for a frequency-matched analysis of a patient group treated from 1990-2000 in the Department of Obstetrics and Gynecology of the LMU in Munich, Germany. To obtain similar patient subgroups, patients were analyzed by histopathological type, axillary lymph node involvement, grading and hormone receptor status (HR). For histopathological type only the most frequent group with invasive ductal carcinoma of the breast was chosen. Subgroups were based on lymph node involvement, three differentiation grades (G1, 2, 3), and HR status. To compare newer and older diagnostic findings each subgroup contained a maximum of ten patients. Table 1 shows the possible numbers of patients complying with the requirements. Patients with known inherited breast cancer were excluded. The following clinical and histological parameters were obtained for every patient: age, year of first diagnosis of breast cancer, tumor size, histopathological type, lymph node status, presence of metastases, histological grading, as well as ER/PR. Results for Her-2/neu detection were not available because they were not routinely determined in Germany before 2001. TNM classification was according to the WHO System (Boecker, 2002). Histological grading was determined according to the Bloom and Richardson

Table 1. Patient cohort with classification of the subgroups.

LNI	Grading	HR	n
negative	1	negative	1
negative	1	positive	8
negative	2	negative	10
negative	2	positive	10
negative	3	negative	3
negative	3	positive	10
positive	1	negative	0
positive	1	positive	0
positive	2	negative	10
positive	2	positive	10
positive	3	negative	10
positive	3	positive	10

LNI: lymph node involvement, HR: hormone receptor (ER/PR), n: number of patients

modification of the Elston and Ellis grading (Elston and Ellis, 2002). Hormone receptor status was determined by immunohistochemistry on paraffin-embedded material. Slides were regarded as HR-positive in the event of positive staining in ≥10% of the tumor cell nuclei. Tumor-related and clinical data were available from all of the patients.

Follow-up took place after 10-20 years (median 12 years) after the first diagnosis of breast cancer by contacting the patients by mail or phone or by getting the data from the Bavarian tumor register.

Immunohistochemistry

Immunohistochemistry was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex with the use of the mouse IgG Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Mouse monoclonal antibodies used for these experiments are listed in Table 2.

Paraffin-embedded tissue sections were dewaxed using xylol for 15 min, rehydrated in a descending series of alcohol (100%, 96% and 70%), and subjected to epitope retrieval for 10 min in a pressure cooker using sodium citrate buffer (pH 6.0) containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water. After cooling, sections were washed twice in PBS. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with diluted normal serum (10 ml PBS containing 150 μl horse serum; Vector Laboratories) for 20 min at room temperature. Sections were then incubated at room temperature for 60 min with the primary antibodies. After washing with PBS, sections were incubated in diluted biotinylated serum (10 ml PBS containing 50 μl horse serum; Vector Laboratories) for 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (diluted in 10 ml PBS; Vector Laboratories) for 30 min and repeated washing steps with PBS, visualization was performed with substrate and the chromagen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8-10 min. Sections were counterstained with Mayer's acidic hematoxylin and dehydrated in an ascending series of alcohol (70-100%). After xylol treatment, sections were covered. Negative controls were performed by replacing the primary antibody with isotype matching control antibodies of the same species (DAKO, Hamburg, Germany). Positive stained cells showed a brownish color (Fig. 1a,b), slides without detection of stained cells showed a blue color (Fig. 1c). As antibodies the combined TRalpha1 and 2 and the antibodies TRalpha1 or 2 were immunostained. Furthermore, the combined TRbeta1 and 2 and the antibodies TRbeta1 or 2 were immunostained. An appropriate positive control was used for immunohistochemical staining (placental tissue, Fig. 1d). For negative controls (colored blue) isotype matching control antibodies of the same species (DAKO, Hamburg, Germany) were applied (Fig. 1e).

The immunoreactive score (IRS) was assigned according to Remmele and Stegner (1987). The intensity and distribution patterns of specific immunohistochemical staining were evaluated using a semi-quantitative assay and used to assess the expression pattern of various marker molecules such as steroid receptors (Jeschke et al., 2005; Mylonas et al., 2005). The IRS score was calculated by multiplication of the optical staining intensity (graded as 0=none, 1=weak, 2=moderate and 3=strong) and the percentage of positively stained cells (0=no staining, 1=≤10%, 2=11-50%, 3=51-80%, and 4= >81% of cells stained). Sections were examined using a Leitz (Wetzlar, Germany) microscope equipped with a CCD color camera (JVC, Victor Company of Japan, Japan).

For survival analysis, two subgroups were analyzed: IRS scores of 0-1 were classified as TR negative, IRS scores of 2-12 as TR positive.

Statistical analysis

Statistical analysis was performed with SPSS for Windows (SPSS, Chicago, Illinois, USA, version 17). Variables were described using adequate measures of location and variability. Group comparisons regarding quantitative or ordinal analysis variables were based on Mann-Whitney U tests or Kruskal-Wallis tests as appropriate. Correlations between ordinal variables were

Table 2. Antibodies and working concentrations.

Antibody	Clone (Isotype)	Working dilution	Source
TRalpha1/2	Polyclonal Rabbit IgG	1:200	Abcam Cambridge, MA USA
TRalpha1	Polyclonal Rabbit IgG	1:1000	AbDSerotec
TRalpha2	Monoclonal Rabbit IgG1/1330	1:200	AbDSerotec Oxford, United Kingdom
TRbeta1/2	Polyclonal Rabbit IgG	1:200	Zytomed Berlin, Germany
TRbeta1	Polyclonal Rabbit IgG	1:200	Millipore, Schwalbach, Germany
TRbeta2	Polyclonal Rabbit IgG	1:100	Millipore, Schwalbach, Germany

Antibodies used for immunohistochemical analysis.

quantified using Spearman's correlation coefficients. Analysis of survival times were shown for each TR. Survival curves were estimated according to Kaplan-Meier and compared with the logrank test. Multivariate analyses to control for the established predictor "tumor size" relied on a Cox proportional hazards model based on logrank tests. Cox regression analysis was used to compare the differences in DFS and OS. Therefore, data of each TR were divided into 2 groups (IRS negative versus IRS positive). In all analyses, p values <0.05 were regarded as statistically significant. Due to the exploratory nature of our analyses, we did not adjust for

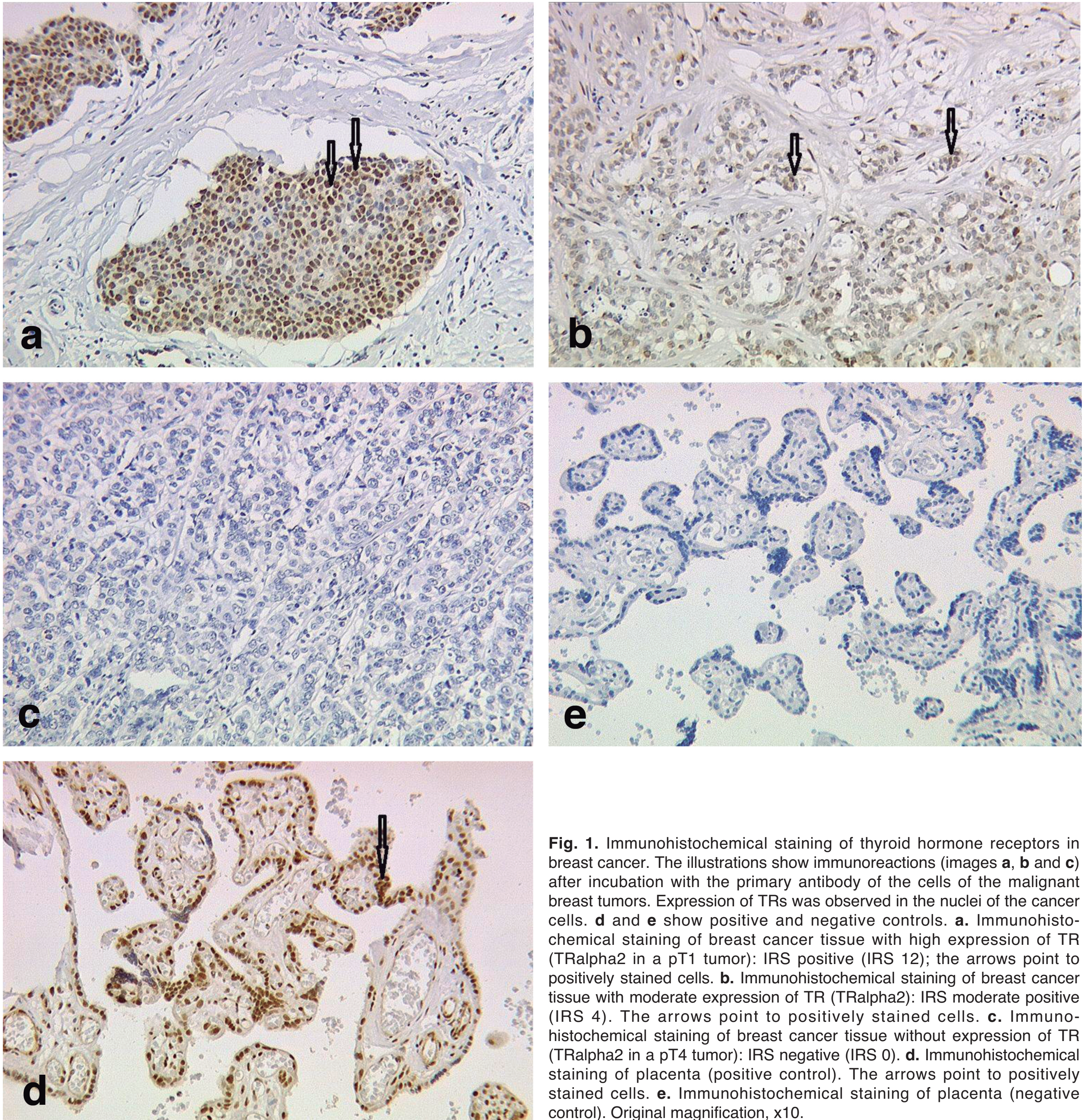


Fig. 1. Immunohistochemical staining of thyroid hormone receptors in breast cancer. The illustrations show immunoreactions (images **a**, **b** and **c**) after incubation with the primary antibody of the cells of the malignant breast tumors. Expression of TRs was observed in the nuclei of the cancer cells. **d** and **e** show positive and negative controls. **a.** Immunohistochemical staining of breast cancer tissue with high expression of TR (TRalpha2 in a pT1 tumor): IRS positive (IRS 12); the arrows point to positively stained cells. **b.** Immunohistochemical staining of breast cancer tissue with moderate expression of TR (TRalpha2): IRS moderate positive (IRS 4). The arrows point to positively stained cells. **c.** Immunohistochemical staining of breast cancer tissue without expression of TR (TRalpha2 in a pT4 tumor): IRS negative (IRS 0). **d.** Immunohistochemical staining of placenta (positive control). The arrows point to positively stained cells. **e.** Immunohistochemical staining of placenta (negative control). Original magnification, x10.

multiple testing.

Results

Clinical and histopathological data

The age of the patients was 68±13.6 years (mean±standard deviation). As expected, most of the patients had a pT1c (n=44, 54%) or pT2 (n=17, 21%) tumor stage. Less frequent were pT1b tumors (n=15, 18%). Tumor stages pT1a (n=1, 1%), pT4b (n=2, 2%) and pT4d (n=3, 4%) were rare. 46% had one or more positive lymph nodes - pN1a (n=2), pN1bi (n=4), pN1bii (n=1), pN1biii (n=13), pN1biv (n=6), pN2a (n=12). About 90% of the patients had a differentiation grade of 2 (n=40) or 3 (n=33). With regard to ER/PR status, the levels were positive in 48 out of 82 patients (59%).

Expression of thyroid receptors

Immunohistochemical staining of 82 slides was done. As a function of the different groups three to six slides could not be used for analysis because of the detachment of tissue from the slides (Table 3). IRS of 0-1 were classified as "negative-stained", IRS of 2-12 as "positive-stained".

In breast cancer tissue immunohistochemically expression of all TRs was found at different levels. The distribution of negative and positive staining varied between the different TRs (Table 4). Median IRS was 1 for the combined antibodies (TRalpha1 and 2 and TRbeta1 and 2). For the single TRalpha 1 or 2 and the single TRbeta1 or 2 median IRS ranged between 3 and 4.

The clinical parameters were correlated with expressions of TRalpha1 and 2, TRalpha1 or 2, TRbeta1 and 2, TRbeta1 or 2 (Table 5). Only data for significant correlations are shown.

Eleven out of 82 (13.4%) patients were lost to follow-up. Twenty-three patients (28%) had disease recurrence. Five out of 17 patients developed hepatic, seven bone, three pulmonary and two multiple metastases. Twelve patients had locoregional recurrence. Twenty-eight out of 82 (34%) patients died during follow-up.

TRalpha1and 2

For TRalpha1 and 2 an IRS of 0 was mainly present. The maximum IRS was 6, median IRS was 1. Analysis of clinically important prognostic factors such as tumor size, axillary lymph node involvement, grading and ER/PR status showed no correlations.

Twenty patients had a relapse - 15 within the IRS negative group, 5 within the IRS positive group. 24 patients died - 17 (71%) within the IRS negative group (after a time of 19 years 61% of the IRS positive group versus 72% of the IRS negative group were alive). Mean DFS was 13.7 years for the group with negative IRS versus 11.9 years for the group with positive IRS. Mean OS showed comparable results: 13.9 years for the group with negative IRS versus 10.9 years for the group with positive IRS.

As shown in Kaplan-Meier-curves for both DFS (Fig. 2a) and OS (Fig. 2b) no significant differences were found between patients with positive or negative

Table 3. Total immunostaining results.

antibodies	n (total of 82)	%
TRalpha1 and 2	78	95
TRalpha1	78	95
TRalpha2	79	96
TRbeta1 and 2	77	94
TRbeta1	79	96
TRbeta2	76	93

n= number of slides.

Table 4. Immunostaining results of the different antibodies.

antibodies	n IRS (positive)	% IRS (positive)	n IRS (negative)	% IRS (negative)
TRalpha1 and 2	19	24	59	76
TRalpha1	55	71	23	29
TRalpha2	54	68	25	32
TRbeta1 and 2	33	43	44	57
TRbeta1	43	54	36	46
TRbeta2	60	79	16	21

n= number of slides IRS= Immunoreactive score, positive IRS: 2-12, negative IRS: 0-1.

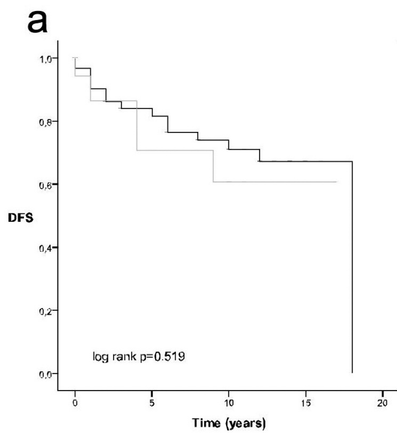
Table 5. Correlations of TR antibodies and histopathological data.

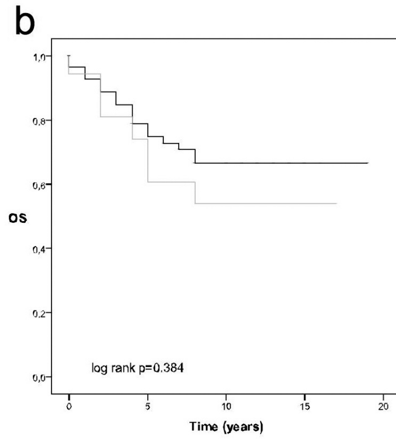
antibodies	Tumor size (pT)	LNI	Differentiation grade	ER/PR
TRalpha1 and 2	ns	ns	ns	ns
TRalpha1	cc:-0.357 p=0.001	ns	ns	ns
TRalpha2	cc:-0.329 p=0.003	cc:-0.487 p=0.002	cc:-0.542 p=0.009	cc:0.248 p=0.028
TRbeta1 and 2	ns	ns	ns	cc:-0.349 p=0.002
TRbeta1	cc:-0.293 p=0.009	ns	ns	cc:0.252 p=0.025
TRbeta2	cc:- 0.314 p=0.006	ns	ns	ns

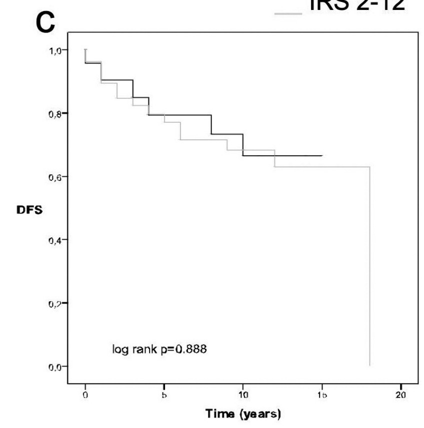
LNI: lymph node involvement, ER: Estrogen receptor, PR: Progesterone receptor, cc: coefficient of correlation (negative correlations are in italics), ns: not statistically significant.

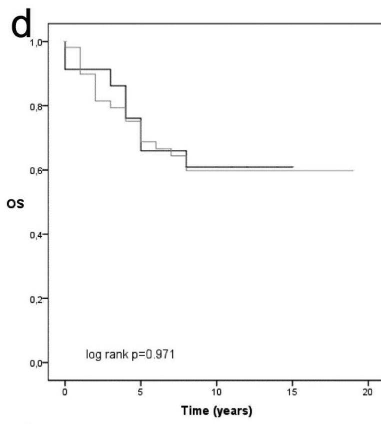
__ IRS 0-1

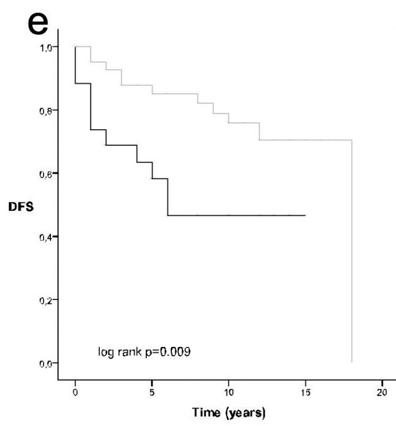
__ IRS 2-12

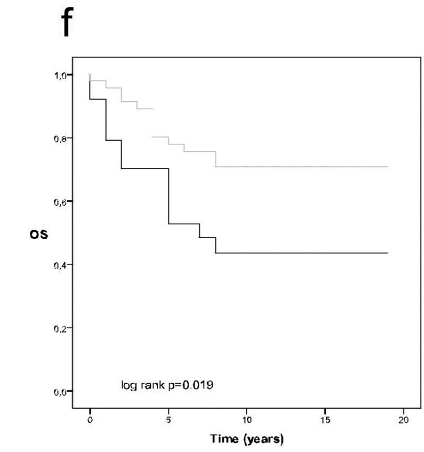


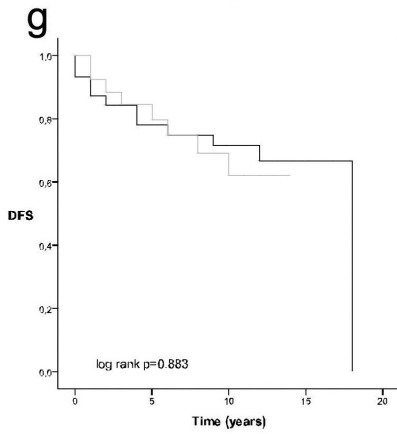


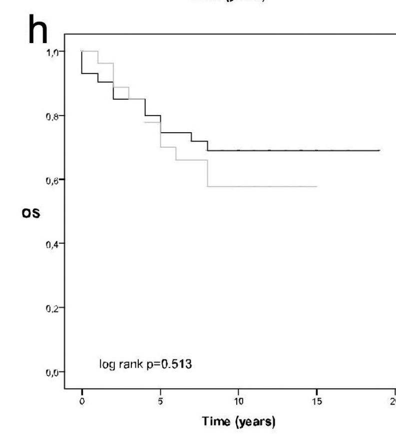


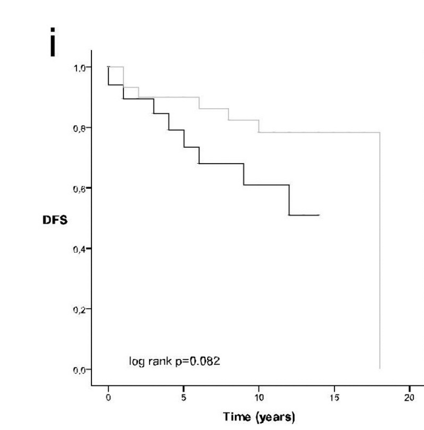


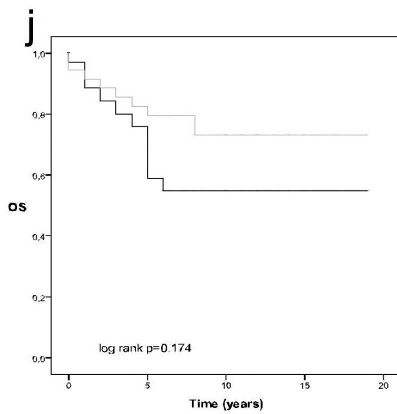


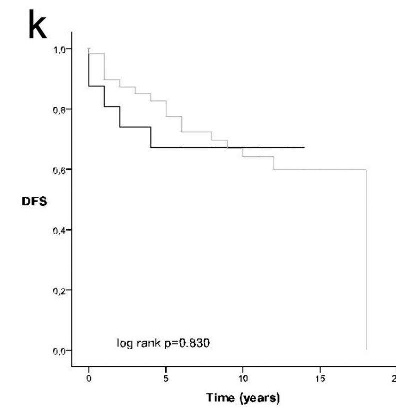


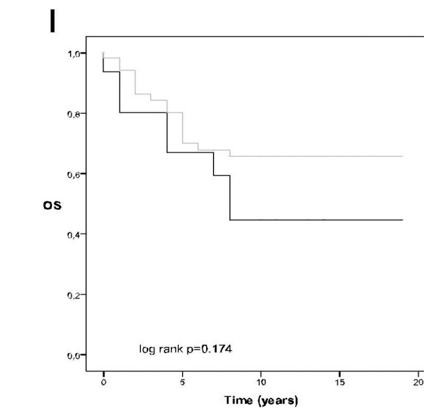












IRS of TRalpha 1 and 2 (DFS: log rank: p=0.519, OS: log rank: p=0.384).

TRalpha1and 2 had no significant relevance for prognosis shown in univariate and multivariate analysis (Table 6 and 7).

TRalpha1

TRalpha1 expression showed unequal results with regard to the combined antibody TRalpha1 and 2. In contrast to TRalpha1 and 2 the IRS results of TRalpha1 moved to higher IRS levels with high variability. A maximum score of 12 was reached. In addition, a median IRS of 4 was reached in contrast to a median score of 1 for the combined antibody. Correlation tests with other histopathological parameters showed a negative correlation between TRalpha1 expression and tumor size (Table 5).

In contrast to TRalpha1 and 2 expression, most patients who relapsed or died were classified with positive IRS. Although more patients relapsed or died within the group of positive IRS, the percentage of patients without event was equally distributed in the group of IRS negative and IRS positive patients (after a time of 15 years 74% versus 73% for DFS and 65 versus 66% for OS). Mean DFS was 11.6 years for the group with negative IRS versus 13.1 years for the group with positive IRS. For OS similar results were shown (10.6 years for the negative IRS group and 12.8 years for the positive IRS group). There were no prognostically

Table 6. Progression-free survival - univariate (uv) and multivariate (mv) analyses.

Histopathological parameters	p (uv)	HR (uv)	95%CI (uv)	p (mv)	HR (mv)	95%CI (mv)
Tumor size (pT)	0.001	1.48	1.18-1.85	0.017	1.44	1.07-1.95
Lymph node involvement (LNI)	0.009	3.52	1.37-9.01	0.250	1.87	0.64-5.43
Grading	0.516	1.24	0.65-2.38	0.894	0.95	0.41-2.16
Estrogen-/Progesterone receptors	0.777	0.88	0.38-2.07	0.503	1.30	0.53-3.69
TRalpha1 and 2	0.525	1.40	0.50-3.90	0.233	1.21	0.88-1.67
TRalpha1	0.889	1.07	0.41-2.79	0.601	1.05	0.89-1.23
TRalpha2	0.015	0.34	0.15-0.81	0.074	0.84	0.69-1.02
TRbeta1 and 2	0.885	1.07	0.43-2.68	0.668	0.95	0.76-1.19
TRbeta1	0.090	0.41	0.15-1.16	0.091	0.83	0.66-1.03
TRbeta2	0.833	0.90	0.33-2.45	0.99	1.00	0.86-1.17

uv: univariate analysis, mv: multivariate analysis, p: significance, HR: Hazard Ratio, CI: Confidence Interval.

Table 7. Overall survival - Univariate (uv) and multivariate (mv) analyses.

Histopathological parameters	p	HR	95%CI	p	HR	95%CI
Tumour size (pT)	0.005	1.38	1.10-1.73	0.320	1.17	0.85-1.61
Lymph node involvement (LNI)	0.033	2.33	1.07-5.04	0.398	1.48	0.60-3.67
Grading	0.861	0.95	0.54-1.67	0.364	0.73	0.37-1.44
Estrogen-/Progesterone receptors	0.976	0.99	0.46-2.11	0.776	1.13	0.50-2.56
TRalpha1 and 2	0.397	1.46	0.61-3.53	0.470	1.11	0.83-1.49
TRalpha1	0.972	1.02	0.44-2.32	0.796	1.02	0.89-1.16
TRalpha2	0.027	0.42	0.19-0.91	0.062	0.86	0.73-1.01
TRbeta1 and 2	0.523	1.31	0.58-2.96	0.880	1.02	0.84-1.23
TRbeta1	0.189	0.55	0.23-1.34	0.716	0.97	0.83-1.13
TRbeta2	0.191	0.57	0.25-1.32	0.097	0.88	0.76-1.02

uv: univariate analysis, mv: multivariate analysis, p: significance, HR: Hazard Ratio, CI: Confidence Interval.

Fig. 2. Kaplan-Meier estimates of disease-free survival (DFS) and overall survival (OS) by TR categories (IRS). The black line shows results for IRS 0-1, the grey line shows results for IRS 2-12. The graph depicts the estimated survival probability as function of the time after first diagnosis (in years). The steps mark the observed event times. Data were obtained from patients with or without immunohistochemical detection of TRalpha1 and 2 (**a, b**), TRalpha1 (**c, d**), TRalpha2 (**e, f**), TRbeta1 and 2 (**g, h**), TRbeta1 (**i, j**), TRbeta2 (**k, l**). Data are illustrated as Kaplan-Meier curves (black colored line for IRS 0-1, grey colored line for IRS 2-12). For each TR data are presented for cumulative DFS and OS as a function of time. TRalpha1 and 2 and cumulative DFS (**a**), TRalpha1 and 2 and cumulative OS (**b**); TRalpha1 and cumulative DFS (**c**), TRalpha1 and cumulative OS (**d**) TRalpha2 and cumulative DFS (**e**), TRalpha2 and cumulative OS (**f**), TRbeta1 and 2 and cumulative DFS (**g**), TRbeta1 and 2 and cumulative OS (**h**), TRbeta1 and cumulative DFS (**i**), TRbeta1 and cumulative OS (**j**), TRbeta2 and cumulative DFS (**k**), TRbeta2 and cumulative OS (**l**).

relevant differences between groups with negative or positive IRS (DFS: log rank: p=0.888, Fig. 2c; OS: log rank: p= 0.971, Fig. 2d).

For TRalpha1 no clinical benefit (univariate and multivariate analysis) was reached for DFS or OS (Tables 6, 7).

TRalpha2

Based on the results of the single TRalpha1 the IRS of TRalpha2 also showed high variability and a maximum of 12. Median IRS (IRS of 4) showed equal results as for TRalpha1.

Significant negative correlations between TRalpha2 expression and clinical-histopathological parameters did exist for tumor size, axillary lymph node involvement and grading (Table 5). This could be clearly demonstrated for tumor size and TRalpha2 expression - TRalpha2 levels were higher in pT1- tumors (median IRS 6) compared to pT2-4 tumors (p=0.024, Mann-Whitney U test). TRalpha2 expression had a positive association with ER/PR expression of the tumors (Table 5).

Twenty-two patients relapsed (11 from each group), 26 patients died (13 from each group). 56% of the TRalpha2 IRS-negative group lived without recurrent local or distant disease within 15 years after first diagnosis of breast cancer. Regarding the group with positive TRalpha2 expression, there were 79% without recurrent disease. For OS these differences are comparable with 58% of the IRS negative and 79% of the IRS positive group.

Based on the data of TRalpha2 the group with positive IRS had a longer DFS (log rank: p=0.009, Fig. 2e) and OS (log rank: p=0.019, Fig. 2f) than patients without expression of TRalpha2. Mean DFS showed significant better results with 14.5 years in contrast to 8.4 years for the negative IRS group. Mean OS differed significantly between 10.1 and 14.6 years of the group with positive IRS. TRalpha2 had prognostic relevance in univariate analysis and showed a trend for prognostic relevance in multivariate analysis for both DFS and OS (Tables 6, 7).

TRbeta1 and 2

TRbeta1 and 2 expression was comparable to those for the combined TRalpha1 and 2. IRS 0 was mainly present and the median score was 1. Analysis of TRbeta1 and 2 expression and the histopathological parameters showed only a negative correlation with ER/PR expression (Table 5).

Results for DFS and OS are comparable to those of the combined TRalpha1 and 2. Twelve patients out of the group with negative IRS and 8 patients out of the group with positive IRS relapsed, mean survival was 13.4 years for the negative IRS group versus 10.7 years for the positive IRS group. 73% versus 76% lived without recurrence after 15 years. Twenty-three patients died – 12 of them within the IRS negative group. Mean survival was 14.2 versus 10.6 years in favor of the IRS negative group. 73% within the IRS negative group versus 67% within the IRS positive group were alive. Overall the results of DFS (log rank: p=0.883, Fig. 2g) and OS (log rank: p= 0.513, Fig. 2h) did not show significant differences between the IRS negative and IRS positive group.

Patients with TRbeta 1 and 2 expression had no survival benefit in univariate and multivariate analysis (Tables 6, 7).

TRbeta1

The median value of IRS of TRbeta1 expression was 2, the maximum score was 12.

Analysis of individual TRbeta1 expression showed a negative correlation with tumor size. Furthermore, there was a positive correlation between TRbeta1 and ER/PR status (Table 5).

Nine patients out of the group with negative IRS and 7 patients out of the group with positive IRS relapsed, mean survival was 10 years for the negative IRS group versus 15.2 years for the positive IRS group. In favor of the IRS positive group 63% versus 81% lived without recurrence after 19 years. Eleven patients died within the IRS negative group, 9 died within the IRS positive group, mean survival was 12 versus 14.9 years within the IRS positive group. As shown for DFS, follow-up after 19 years was in favor of the IRS positive group (67% versus 75%).

TRbeta 1 expression was associated with a slight benefit in patients with positive IRS in contrast to patients without expression, but only for DFS (log rank: p=0.082, Fig. 2i), not for OS (log rank p=0.174, Fig. 2j). No clinical benefit (univariate and multivariate analysis) was reached for DFS or OS (Tables 6, 7).

TRbeta2

IRS of TRbeta2 could be detected from a minimum value of 0 until a maximum value of 12, medium IRS was 3. Clinical correlation data showed only significant results for tumor size (Table 5).

Twenty patients had a relapse - 15 of them within the IRS negative group. 25 patients died - 8 within the IRS negative group. Mean DFS was 9.9 years for the group with negative IRS versus 12.9 years for the group with positive IRS, and after 15 years DFS was equally balanced in both groups (69% versus 72% within the IRS positive group). Mean OS showed comparable results, 10.9 years for the group with negative IRS versus 13.7 years for the group with positive IRS. In contrast to DFS OS was in favor of the IRS positive group (50% versus 72%). For both – DFS (Fig. 2k) and OS (Fig. 2l) – no significant differences were found between patients with positive or negative IRS of TRbeta2 (DFS log rank: p=0.830; OS log rank: p=0.174). TRbeta2 showed no significance in univariate

and multivariate analyses (Tables 6, 7) and therefore presented without prognostic relevance.

Discussion

In our study we demonstrated a statistical correlation of both TRalpha and TRbeta expression with clinical and histopathological parameters. Negative correlation of TRalpha1 with tumor size was shown. TRalpha2 expression had negative associations with tumor size, axillary nodal status and grading. Furthermore, TRalpha2 expression had a positive association with ER/PR expression of the tumors.

TRbeta1/2 expression showed a negative correlation with ER/PR expression whereas TRbeta1 expression was positively correlated with ER/PR expression. As shown for TRalpha1 or 2, TRbeta1 or 2 had a negative correlation with the tumor size.

Patients in the group with positive expression of TRalpha2 had a trend to a longer disease-free survival, while tumor size remained the strongest prognostic factor in multivariate analysis.

In multivariate analysis TRalpha2 showed a trend toward being an independent prognostic factor for overall survival. In this patient cohort all other TRs did not influence the prognosis in breast cancer.

In contrast to earlier studies of thyroid receptor expression in breast cancer focused on detection in breast cancer cell lines (Silva et al., 2002) our findings were based on immunoreactive exploration of malignant breast tumors.

Studies by Smyth (Smyth, 1997) and Shering (Shering et al., 1996) showed an association between thyroid disorders and progressive disease in breast cancer patients. Details of associations with hyperthyroidism, hypothyroidism, thyroiditis and thyroid enlargement (up to 45.5% of the patients with breast cancer in contrast to 10.5% of healthy controls) and non-toxic goiter (more than twice as common in breast carcinoma patients) were reported previously (Shering et al., 1996).

A study by Lemaire and Baugnet-Mahieu (1986) showed TR expression in 100% of samples of primary breast cancer in contrast to significantly lower expression in non-tumor tissue. In our study, investigation of the different kinds of TRalpha and beta in this cohort of breast cancer patients showed them to be present in about 24-79%.

Most studies focusing on TRalpha and TRbeta expression in breast cancer patients did not differentiate between the TR isoforms such as alpha1 and alpha2 or beta1 and beta2, but focused only on one TR isoform (mainly beta1) (Li et al., 2002b).

In contrast to a former study with detection of TRs mainly in the cytoplasm of breast cancer tissue (Conde et al., 2006), in our study the expression of TRalpha and TRbeta was only present in the nuclei of the malignant breast tumor cells with an overall low median IRS. Conde et al. (2006) showed TRbeta1 immunostaining in the cytoplasm of invasive breast carcinomas. The location of TRalpha varied in different pathologies with cytoplasmic accumulation in breast cancer tissue.

In agreement with a study of 93 cases of breast cancer by Lemaire and Baugnet-Mahieu (1986), a negative relation was found between the nuclear TR receptor level and the involvement of axillary lymph nodes. Furthermore the results were similar regarding the possible relationship between TR and HR. These data from 1986 are the only ones that support our results regarding the relationship between TRs and known prognostic histopathologic factors, but the authors did not differentiate between the different individual TRs. In contrast to these older TR-undifferentiated results, most of the newer studies investigated only the possible correlations between single TR isoform expression and clinical parameters (Silva et al., 2002).

In another study, TRbeta1 expression was shown as a possible suppressor of tumor invasiveness and metastasis (Martinez-Iglesias et al., 2009) in the breast cancer cell line MDA-MB-468, which is known to be highly invasive. The trend toward a higher expression of TRbeta1, as well as of TRalpha1 or 2 and TRbeta2 in smaller tumors in our study population could support these findings.

In contrast to our findings, Silva et al. did not show a significant correlation between TRbeta1 expression and any clinical or histopathological parameter (Silva et al., 2002). The reasons for such differences in receptor expression of the different TRs are not completely understood. Maybe they are due to differences in methods. Our findings showed a negative correlation of TRbeta1/2 with HR, but TRbeta1 and TRalpha2 correlated positively. Therefore, our data are in line with other studies investigating thyroid hormone receptor genes in breast cancer and partly show a lack of nuclear immunostaining (Li et al., 2002b) as a result of an alteration in the normal splicing mechanism as detected, for example, for ERalpha (Fasco et al., 2000) or a gene biallelic inactivation (Li et al., 2002b). The combined antibodies TRalpha1 and 2 and TRbeta1 and 2 detect a mixed epitope of both TRalpha1 or 2 and TRbeta 1 or 2. If this mixed epitope is only present in a part of the tumor, the staining results could be different from these with the single TRalpha1 or 2 or TRbeta1 or 2 (with a higher rate of positive measurements). Maybe this could also explain the differences between the correlations of the single TRs with the combined TRs.

A study of Cerbon et al. (1981) could not find a correlation of triiodothyronine receptor and ER/PR concentration, but this study did not differentiate between the different TRs. Furthermore these data stand in line with our results for the combined TRalpha1 and 2 and TRbeta1 and 2.

Summarizing the collected data, we assume a relationship of all single TRs between each other because there were significant negative correlations with tumor size and significant positive correlations with ER/PR expression. In contrast, this could not be detected

for combined TRs. This leads to the assumption that single TR detection could provide more specific results than detection of the combined TRs.

Especially, TRalpha2 showed significant correlations with all of the other histopathological data. In univariate analysis TRalpha2 showed an effect on prognosis, which is confirmed in multivariate analysis only as a trend toward being a prognostic factor for disease-free and overall survival. This might be due to the limited sample size. Significant differences in TRalpha2 expression between the 2 categories in multivariate analysis lead to the assumption that patients with a high expression of TRalpha2 have a better prognosis than patients with low levels of TRalpha2 expression.

The different background (e.g. protein or gene detection) of former studies underlines the difficulties in comparing the data and making definitive statements concerning TR as a possible prognostic factor in breast cancer.

To conclude, the expression of TRalpha2 may have a role in the pathophysiology of malignant breast tumors and may have prognostic relevance. To permit valid conclusions and to confirm this assumption for TRalpha2, further studies with amplification of the patient cohort should be part of ongoing research.

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