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# The significance of the expression of ERRα as a potential biomarker in breast cancer

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ABSTRACT

It was shown the functional crosstalk between ERRα and ERα in breast cancer, however, the biological significance of estrogen-related receptor α (ERRα) remains largely unclear. Therefore, we examined the expression of ERRα in 39 primary human breast cancer tissues and 19 matched normal tissues using RT-PCR and immunohistochemistry in the context of the aromatase, ERα and proliferation markers (c-myc, Ki-67) expression. Compared to the normal breast tissue, breast cancer tissues showed a slightly higher expression level of ERRα mRNA (mean 46.2 ± S.D. 42.0, 57.7 ± S.D. 58.7, respectively). However, ERRα mRNA levels in breast cancer tissues showed greater diversity than in normal tissues. Immunohistochemical analysis of breast cancers revealed perinuclear and cytoplasmic localization of ERRα. Our study shows that there is no correlation between ERRα and ERα expression. We demonstrated a positive correlation between ERRα and c-myc at the transcriptional level and statistically significant positive correlation between aromatase and the ERRα at protein level.

It seems that ERRα could play an important role in the alternative pathway to classical estrogen receptors-dependent pathway in cell signaling. Development and use of ERRs modulators might lead in the future to design new well-tolerated and individualized therapeutic agents.

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## 1. Introduction

Estrogens and their receptors play a pivotal role in the development and the physiological function of mammary gland as well as in the etiology of breast cancer. Until recently, the main research and clinical interest were focused on the classical estrogen transduction pathway through two nuclear estrogen receptors alpha and beta (ERα and ERβ). In order to exert a specific biological effect, ERs require a ligand activation, and then the complex-receptor ligand can bind to the promoter site of target genes containing ERE (estrogen-response element). This classical pathway is necessary to induce the expression of ERE-dependant genes (such as *pS2*, *bcl-2* or *Cathepsin D*) and probably may be critical for many effects of estrogens in mammary gland physiology: development, during pregnancy lactation and involution [1].

In breast cancer cells estrogens promote cell proliferation through the stimulation of G1-to-S-phase transition modifying the expression of hormone-responsive genes and alter cytoarchitectural and phenotypic properties of these cells. The classical pathway seems to be misregulated and altered and less important in the stimulation of cell proliferation in favor of the "non-classical" pathways through kinase cascades and "transcription factor crosstalk" with AP-1, Sp-1 or NFκB [2–4]. Many of these transcription factors which stimulate proliferation or which can increase cell survival and metastasis are overexpressed in breast tumor.

The cellular estrogenic signaling pathway has been more intricate through identification of orphan receptors, known as estrogen-related receptors (ERRs). ERRs belong to an orphan receptor family, which is composed of three members: α, β and γ [5,6]. Structurally and functionally ERRs are close relatives to classical estrogen receptors. Indeed it has been shown that in some cellular and promoter contexts ERRs crosstalk with estrogen pathway [7–9]. Furthermore, ERRs can also interfere with other steroid signaling pathways through stimulation the expression of androgen-responsive genes in prostate cancer [10]. In contrast to the ligand-dependent estrogen receptors, ERRs are in a permanent active configuration [11,12]. ERRs are ready to recruit coregulatory proteins such as PGC-1 alpha, PGC-1 beta, GRIP1/SRC2 which enhance the transcriptional activity of ERR or interact with the corepressor RIP140 which represses their transcriptional activity

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[11,13,14]. The existence of natural ligand for the ERR is doubtful. The ligand binding pocket of ERR is very small, however experimental data show that the plasticity of this pocket could allow larger compounds to act as natural or pharmacological ligands [15–17].

ERRs bind to a specific DNA nucleotide sequence of ERE (estrogen-response element) presented in some genes but also bind to another DNA response element referred to ERRE (estrogen-related receptor response element), that is recognized also by ERα but not by ERβ [8,18].

It is well known that ERRα modulates estrogen receptor (ER)-mediated activity and also may modulate estrogen-regulated gene expression by several mechanisms: share target genes, coregulatory proteins, ligands and competition for binding in ERE, ERRE, or through tethering via protein–protein interactions to the promoter site in ER-target genes. It is shown that the transcriptional activity of several genes is regulated by ERRα as well as by ERα like pS2, breast cancer marker lactoferrin and osteopontin [6,9,11,13,14]. Limited studies show that ERRs may be involved in the development and progression of breast and ovarian cancer [19]. Thus ERRα could be potential target for therapy of these cancers.

Recently it was shown that ERRα actively participates in local mammary steroidogenesis by the stimulation of the transcription of aromatase gene and the induction of steroid sulfotransferase gene [20,21]. Intratumoral production of estrogens occurs as a result of the aromatisation of C19 steroids into estrogens, and this is catalyzed by the cytochrome P450-aromatase [22,23]. The overexpression of aromatase in the site of tumor may enhance a local production of estrogen which would in turn stimulate breast cancer progression.

One of the important growth regulatory genes which is induced through "transcription crosstalk estrogen-dependent pathway" is *c-myc*. The studies in recent years showed that transcriptional factor c-Myc participates in most aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis [24]. In clinical studies concerning breast cancer biology *c-myc* gene amplification has been associated with progression from in situ to the invasive stage of breast tumor [25], markers of aggressive phenotypes [26,27], and poor prognosis [26,28,29]. A cohort study of 217 primary breast cancer samples demonstrated that *c-myc* mRNA and c-Myc protein expression are detectable in the majority of breast cancers, both ER(+) and ER(−) [30]. There is close correlation between mRNA expression and protein expression of c-Myc. Up till now it was thought that c-Myc might be one of the factors responsible for the initiation of the proliferation process in estradiol-independent manner. However, recent data challenge the view that c-Myc overexpression is predominantly a feature of ER(−) cancers [26]. McNeil et al. proposed that c-Myc expression is necessary for the estrogen-induced cell cycle progression [30]. And it is thought that estrogens can induce the growth of breast cancer cells through ER indirect DNA-binding to other transcription factors and expression of *c-myc*, not through the "classical" pathway of estrogen signaling.

The aim of our study was to assess the utility of ERRα as a breast cancer biomarker in the context of the aromatase, ERα, proliferation markers expression and clinicopathological features.

## 2. Patients and methods

39 specimens of primary breast cancer tissues were obtained from females who underwent radical surgical treatment. Specimens matched (adjacent) mammary gland tissues were available from examination in 19 of these 39 cases. Specimens for RNA isolation were snap-frozen and stored at −80 °C until use.

Tumor samples were cut into 5 μm thick sections and stained with hematoxylin–eosin. Histopathological examination was based on the WHO and pTN classification of breast tumors [31]. Our study comprised only invasive ductal carcinomas, representing G2 (19 patients, cases) and G3 (20 patients) grade. Histopathological grading (G) was performed according to the Bloom and Richardson system [32]. There were 53.8% (21/39) tumors in pT1 stage and 46.2% (18/39) in pT2 stage. Patients had not received any preoperative chemo- or hormonotherapy. The age of patients ranged from 33 to 83 years, with a mean age of 51.9 years. The local ethical committee approved the protocol of this study.

### 2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen breast and matched tissues according to Chomczynski and Sacchi method [33]. RNA integrity was verified by electrophoresis in 1.5% agarose gel and staining with ethidium bromide, and by amplification of housekeeping gene, GAPDH. RNA was quantified spectrophotometrically at 260 nm. 1 μg of total RNA was used to prepare cDNA. cDNA synthesis was performed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP mix (Promega), 2.5 μM oligo $dT_{15}$, 20 U RNasin Ribonulease Inhibitor (Promega), 100 U MMLV reverse transcriptase (Promega) in a final volume of 40 μl using MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA). For reverse transcription, the mixtures were incubated at 42 °C for 60 min and then heated at 95 °C for 5 min and finally rapidly cooled at 4 °C.

### 2.2. Evaluation of ERRα mRNA expression

To determine the mRNA level of ERRα we used Assays-on Demand Gene Expression Assay Mix (Applied Biosystems). All real-time PCR reactions were performed using ABI Prism 7000 Sequence Detection System (PerkinElmer Applied Biosystems, USA). For each PCR run, a master mix was prepared with 10 μl 2× Taq Man Universal PCR Master Mix (Applied Biosystems), 1 μl 20× Assays-on Demand Gene Expression Assay Mix (Applied Biosystems), 5 μl cDNA and sterile water to final volume of 20 μl. The relative quantification was given by the ratio between the mean value of the target gene and the mean value of the reference gene (GAPDH) for each sample. The relative amount of PCR product generated from each primer set was determined on the basis of the Ct value.

### 2.3. Evaluation of aromatase mRNA expression

The aromatase and GAPDH standards were prepared as it was previously described by Bouraïma et al. [34]. In order to prepare the aromatase standard the primers (sense CA5: 5′GCT TTG AGA AGG ATA GGC CTT CAT TAA C3′; antisense CAST: 5′GCA AGT GGC TGA GGC ATA AAT CGT TT G CCA CAG ACA GAT CAT ATG TAG AC3′) were designed in the way that they could allow to amplify the standard and target aromatase sequence using the same pair of primers in one tube. PCR was carried out in final volume of 50 μl using 25 pmol of each of the primers, 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer and 5 μl cDNA as a template. PCR was carried out under the following conditions: 5 min at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 60 °C, 1 min extension at 72 °C for 30 cycles, with an additional 10 min extension for the last cycle. Amplified products were subjected to electrophoresis on a 2% agarose gel, extracted and purified from agarose slices using DNA Gel Extraction Kit, quantified by the use of One Dscan/Zero Dscan software and then diluted in sterile water. To determine amounts of aromatase mRNA quantitative competitive PCR was performed in final volume of 50 μl using 25 pmol of each of the primers (sense CA5: 5′GCT TTG AGA AGG ATA GGC CTT CAT TAA C3′; antisense CA3: 5′GCA AGT GGC TGA GGC ATA AAT CG3′), 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer, 5 μl cDNA from studied tissues and 5 μl diluted standard in the

same tube. For each analysed tissue several dilutions of aromatase standard were used (0.05–50 fg). All the results obtained in [fg] were calculated into fmol of aromatase per μg total RNA.

The specific primers for GAPDH standard building (sense Bene 1P1B: 5′TCA TCC ATG ACA ACT TTG GTA TCG TGC GGC ATC AGA GCA GAT TGT ACT GAG3′; antisense Bene 2M1B: 5′GTG CTC AGT GTA GCC CAG GAT GCG GGG AAA CGC CTG GTA TCT TTA TAG TCC3′) were designed so that the 5′ ends of sense and antisense primers were complementary to GAPDH mRNA sequence and 3′ ends of these primers were complementary to pBR322 sequence. At 5′ and 3′ ends PCR product contained complementary sequences to specific GAPDH primers used in quantitative competitive PCR. PCR for GAPDH standard building using pBR322 vector as a template and purification of GAPDH standard were carried out under the same conditions as described for aromatase standard building.

To determine amounts of GAPDH mRNA quantitative competitive PCR was performed in final volume of 50 μl using 25 pmol of each of the primers (sense Bene 1: 5′TCA TCC ATG ACA ACT TTG GTA TCG TG3′, antisense Bene 2: 5′GTG CTC AGT GTA GCC CGG ATG C3′), 40 μM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 μl 10-fold PCR buffer, 5 μl cDNA from studied tissues and 5 μl diluted standard in the same tube. For each analysed tissue two dilutions of GAPDH standard were used (0.5 and 50 pg). All the results obtained in [pg] were calculated into pmol of GAPDH per μg total RNA. PCR was carried out under the same conditions as described for aromatase standard building. Amplified products were separated on a 4% agarose Methafor gel and quantified by the use of One Dscan/Zero Dscan software. All results of aromatase mRNA expression levels were corrected by GAPDH mRNA expression levels.

### 2.4. Evaluation of c-myc mRNA expression

The relative level of c-myc mRNA was examined using a semi-quantitative RT-PCR method. PCR was carried out in a total reaction vol of 20 μl, and so-called hot-start technique was employed. The reaction mixture contained 2 μl 10-fold PCR buffer, 2 μl cDNA, 40 μM of each dNTP, 1 unit of HotStarTaqDNA polymerase (Qiagen GmbH, Hilden, Germany) and 200 nM of each primer.

The expression of the housekeeping gene, β-actin, was considered as a semi-quantitative control. Specific primers used were as follows—sense: 5′CAC CAG CAG CGA CTC TG3′, antisense: 5′GCA GGA TAG TCC TTC CG AG3′ for c-myc generating a specific 222 bp fragment; sense: 5′CCA GAT CAT GTT TGA GAC CT3′, antisense: 5′GCA CAG CTT CTC CTT AAT GT3′ for β-actin amplifying 292 bp fragment. PCR was carried out under the following conditions: 15 min at 94 °C in order to activate HotStarTaq DNA polymerase, 1 min denaturation at 94 °C, 1 min annealing at 65 °C, 1 min extension at 72 °C for 40 cycles, with an additional 5 min extension for the last cycle. Controls with water replacing template were included in all experiments. Amplification products were separated on a 2% agarose gel. Ethidium bromide-stained gels were visualised

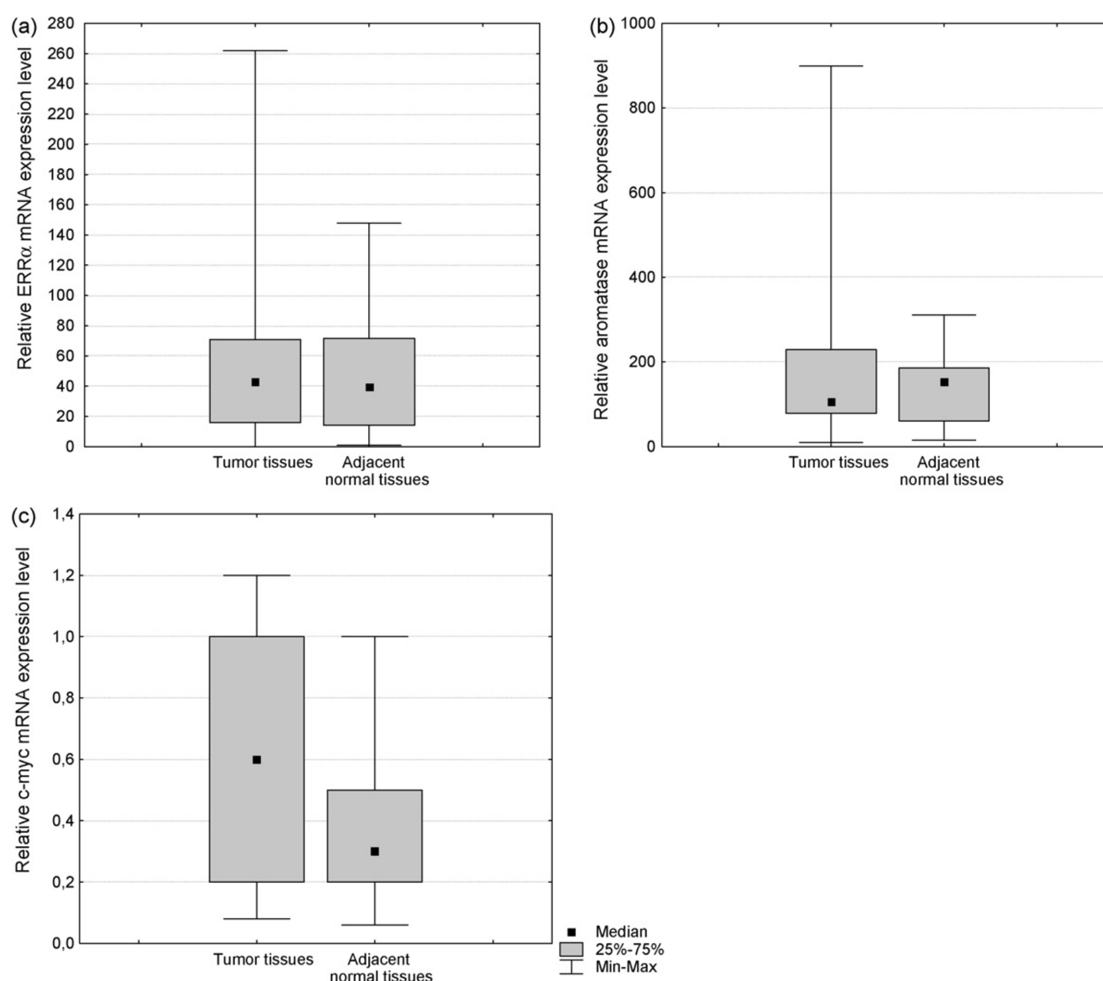


**Fig. 1.** Analysis of ERRα, aromatase and c-myc mRNA expression levels in breast cancer and adjacent normal tissues. (a) Slightly higher expression level of ERRα in breast cancer tissues compared to normal breast; (b) higher mean values of aromatase transcripts in cancer tissues compared to aromatase mRNA level in normal tissues, however median values represent opposite effects due to the asymmetric layout of aromatase transcripts values in cancer tissues compared to control tissues; (c) overexpression of c-myc mRNA in breast cancer tissues in comparison with normal surrounded tissues.

under UV illumination, photographed and for each sample the intensity of the signal was measured using One Dscan/Zero Dscan software (Scanalytics Inc., USA). Ratios of the corresponding peak areas, c-myc/β-actin, were calculated for each sample and used for quantitative calculations and comparisons.

### 2.5. Immunohistochemistry

For immunohistochemical studies we selected two representative tissue sections and it was performed as described by us previously in details [35]. Following markers were investigated: ERRα, aromatase (ARO), ERα and Ki-67. ERRα was detected with a rabbit polyclonal antibody (Ab) (E0406, Sigma–Aldrich, Germany) at dilution 1:200. Aromatase expression was assessed using a rabbit polyclonal Ab R-10-2 against cytochrome P450-aromatase at dilution 1:800 (a generous gift from Dr. Yoshio Osawa, Hauptman-Woodward Medical Research Institute, Buffalo, NY, USA). This antibody raised in rabbit, was generated against human placental P450arom that had been previously purified by immunoaffinity using a monoclonal antibody to P450arom. ERα was assessed using mouse monoclonal Ab F-10 (Santa Cruz, USA), dilution 1:200; and Ki-67, mouse monoclonal Ab MIB-1 (Dako, Denmark), dilution 1:100.

The sections were deparaffinized in xylenes and rehydrated through graded alcohols. After antigen unmasking and endogenous peroxidase removal, nonspecific binding was blocked by incubating the slides for 1 h with 1.5% normal serum in PBS. Next, the sections were incubated with the primary antibodies using staining chamber (The Binding Site, United Kingdom). Primary antibodies were diluted in PBS. The studies for ERRα and ARO were performed with EnVision system (Dako, Denmark), for ERα with avidin–biotin-peroxidase complex (ABC Staining System, Santa Cruz, USA), and for Ki-67 with streptavidin–biotin-peroxidase complex (LSAB kit, Dako, Denmark) to reveal Ab–antigen reactions. Staining was routinely developed using 3,3′-diaminobenzidine as a chromogen (Dako, Denmark). Sections were counterstained with hematoxylin. Breast tissues previously classified as positive for the studied markers were used as positive controls and for protocol standardization. In negative controls, primary Abs were omitted. Two pathologists (SS and KM) evaluated immunostainings with the use of light microscopy (20× and 40× objectives). The evaluation of studied proteins was analyzed in 10 different tumor fields and the mean percentage of cancer cells with positive staining was evaluated. The sections were classified as positive if at least 10% of cells expressed the studied antigen.

### 2.6. Statistical analysis

The mean values ± standard deviation (S.D.) were calculated. The results were analyzed using the Mann–Whitney test and Spearman correlation test, accepting $p < 0.05$ as significant.

## 3. Results

### 3.1. Expression of ERRα, aromatase and c-myc mRNA

Real-time RT-PCR technique revealed the presence of transcript of ERRα in all analyzed the breast cancer tissues (Fig. 1a). ERRα mRNAs were also detected in all matched normal mammary gland tissues (Fig. 1a). Compared to the normal breast tissue (mean 46.2 ± S.D. 42.0), breast cancer tissues showed a slightly higher expression level of ERRα (mean 57.7 ± S.D. 58.7; Table 1).

To evaluate the levels of aromatase mRNA expression, the curve 100–10 fg to 1–0.1 fg (prepared as described in Section 2) was used. The all results obtained in [fg] were calculated into fmol of aromatase per μg of total RNA, and then corrected by the level of

**Table 1**
Comparison of ERRα, aromatase and c-myc mRNAs expression levels in studied breast cancer and control tissues.

| Analyzed genes | Tumor tissues | | | Adjacent normal tissues | | |
|---|---|---|---|---|---|---|
| | Mean | ±S.D. | Median | Mean | ±S.D. | Median |
| *ERRα* | 57.7 | 58.7 | 42.94 | 46.2 | 42.0 | 41.7 |
| *aromatase* | 194.85 | 219.9 | 105.5 | 143.45 | 86.7 | 155.5 |
| *c-myc* | 0.58 | 0.37 | 0.6 | 0.38 | 0.25 | 0.3 |

GAPDH. All of analyzed breast tumors and as well as matched normal tissues showed expression of aromatase transcripts (Fig. 1b). Mean values of aromatase transcripts in cancer tissues (mean 194.85 fg/μg of RNA ± S.D. 219.9) was higher to aromatase mRNA level in normal tissues (mean 143.45 fg/μg of RNA ± S.D. 86.7; Table 1).

We also showed expression of *c-myc* mRNA in all studied tissues (Fig. 1c). In the analyzed breast cancer tissues expression transcript level was higher (mean 0.58 ± S.D. 0.37) than in normal surrounded tissues (mean 0.38 ± S.D. 0.25; Table 1).

### 3.2. Immunohistochemical analysis of studied proteins

Immunohistochemical analysis of breast cancers revealed perinuclear and cytoplasmic localization of ERRα (Fig. 2a and b), while the expression of aromatase was predominantly cytoplasmic (Fig. 2c and d). 62% of analyzed cases were positive for ERRα. Immunohistochemical analysis of breast tumor sections revealed positive aromatase expression in all of examined tissues. Weak (1+) aromatase expression was detected in the cytoplasm of the 12.9% tumor cases, whereas moderate (2+) and strong (3+) expression was found in the 48.4% and 38.7% studied tumors, respectively. Nuclear immunostaining for ERα (Fig. 2e) and Ki-67 (Fig. 2f) was observed in 67% and 93.5% of analyzed cancers, respectively. After omission of primary antibodies in negative controls, specific staining was abolished.

### 3.3. Correlation of ERRα, aromatase and c-myc expression with selected clinical and pathological features

Statistically significant negative correlation was found between ERRα mRNA expression and age of the patients ($p < 0.0049$, $r = -0.49$). *C-myc* mRNA expression was also negatively correlated with patients' age ($p < 0.007$, $r = -0.479$). There was no association between ERRα, aromatase, *c-myc* expression and lymph node status (pN), tumor size (pT) as well as tumor differentiation (G) (Table 1). Analysis of relationships did not reveal significant correlations between ERRα and ERα, ERRα and Ki-67, aromatase and ERα as well as aromatase and Ki-67 (Table 1).

## 4. Discussion

Molecular profiling analysis shows that biology of breast cancer differs from each other and differs from normal tissue. The first identified distinct segregator divided breast cancers into two main biologically different categories: classified by the presence (+) or absence (−) of active estrogen receptor. Although it is undoubtedly true that the development of human breast cancer is a complex process where estradiol and estrogen receptors play a crucial role in the growth of breast tumor cells there is some data that indicate growing significance of orphan receptors in carcinogenesis in mammary gland. The cellular signal pathway connected to ERRs is less known than classical ER-dependant pathway. Almost all organs express ERRs at the same level and it is known that ERRα participates in the regulation of fatty acid oxidation, mitochondrial biogenesis and oxidative phosphorylation [36,37]. Thus,

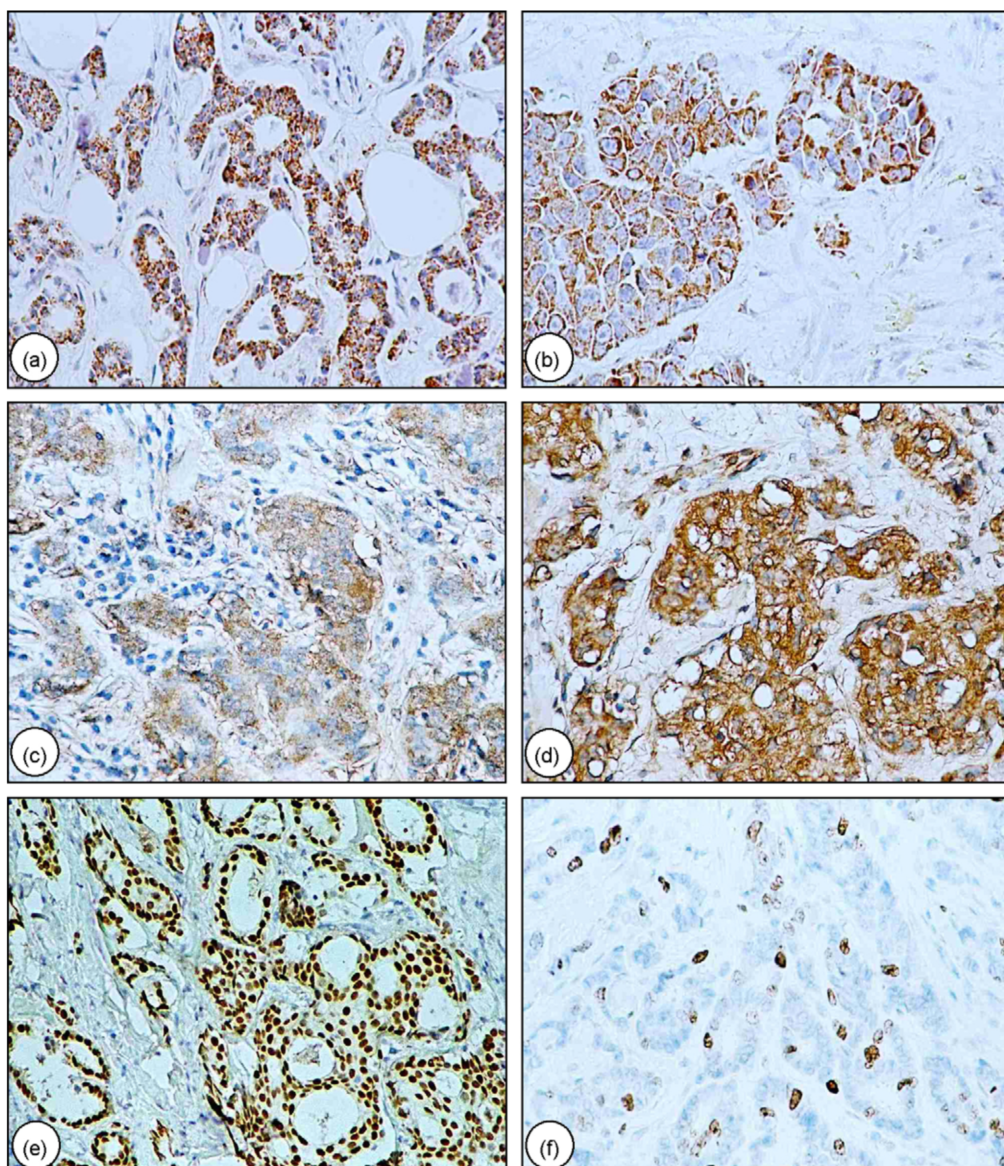


**Fig. 2.** Strong perinuclear ad cytoplasmic microgranular immunostaining for ERRα in moderately (a) and poorly (b) differentiated breast cancer; representative aromatase expression assessed as weak (1+; c) and strong (3+; d) cytoplasmic staining; positive nuclear immunostaining for ERα in majority of breast cancer cells (e); Ki-67 nuclear expression in breast cancer cells (f). Original magnifications: (a, c–f) 200×; (b) 400×.

the expression level of ERRα may be an important factor in the regulation of the energy metabolism in the tumor. Furthermore, the recent data have shown that ERRs can stimulate HIF-induced transcription in the tumor [38]. Giguère established a role for ERRα as a modulator of ERα signaling [39]. It was shown that functional crosstalk between ERRα and ERα can also lead to cell-specific estrogenic response. It is still an open question how important this ERR–ER crosstalk in breast tumor biology is.

Suzuki et al. suggested that ERRα mainly modulates ERα-mediated ERE-dependent transcription and changes the expression of estrogen-responsive genes in breast cancer cells [40]. ERα and ERRα can act on DNA as homodimers as well as heterodimers, and this competition is dependent on the molar ratio of each receptor in the cell and also on the cell type and the cell context. Both, ERα and ERRα can directly compete for binding ERE in promoter region. ERRα has a potential to modulate genes transcription (such as lactoferrin, pS2 or aromatase) through binding to ERE in the absence of estrogens that is in accordance with previous *in vitro* study suggesting that activity of ERRα could also depend on serum compounds [9,20,41–43]. Thus, another regulating factors can modulate the ERRα action [44].

Two recent studies independently revealed that ERRα is involved in breast cancer progression [40,45]. Ariazi et al. demonstrated inverse relationship between ERRα and ERα in 38 cases of examined breast cancer tissues and suggested that ERRα might function as a possible unfavorable marker in breast cancer [45]. In current study we did not observe any relationship between ERRα expression and tumor grade. However, we showed that mRNA ERRα expression was negatively correlated with patients' age. It is not surprising in the light of facts that in younger women estrogen-independent tumors are more frequently observed. We did not confirm these results. Our study shows that there is no correlation between ERRα and ERα expression. In our earlier investigation we demonstrated that all of examined breast tumors express mRNA ERα but in "estrogen-independent" tumors, ERα protein was absent in Western Blot and immunohistochemical studies [35]. Our present results show that ERRα mRNA was expressed in all tumors, similarly to ERα mRNA in our previous study, and ERRα protein

assessed by immunohistochemistry was detected in the perinuclear area of carcinoma cells in 68% of studied tissues. Furthermore, in estrogen-independent tumors that did not synthesize functional ERα protein, ERRα protein was always expressed but no significant correlation was found between the ERα-negative tumor status and the ERRα expression level. It is probable that in such ER(−) tumors ERRα takes over the function of ERα in the activation of estrogen-responsive genes without the activation by a ligand. The recent interesting data on function of ERRα in endometrial cancer revealed that ERRα probably regulates the growth of malignant endometrial cells in the absence or presence of estrogen in a different manner. The cells stably overexpressing ERRα grew more slowly than the control cells in the presence of estrogen [46]. Interestingly, as opposed negative correlation in primary and metastatic breast cancer between expression of ERα and Ki-67, expression of ERRα did not associate with Ki-67 status. In our study we did not show any correlation between ERRα expression and the proliferation marker Ki-67 in breast cancer tissue. Previously we noted a negative correlation between the expression of ERα and Ki-67 in primary and metastatic breast cancer [35].

Our results showed that genes connected to estrogen signaling in breast cancer cells (such as aromatase and *c-myc*) may be regulated not only through the well characterized estrogen receptor pathway but also through the pathway connected with orphan receptors such as ERRs. DeNardo et al. suggested that the induction of *c-myc* expression by estrogens occurs through the "non-classical" pathway. Estrogen induction of *c-myc* expression does not require ERα DNA binding to its promoter [1]. In our study we showed a positive correlation between ERRα mRNA and *c-myc* mRNA. It was shown that the overexpression of c-Myc (induced as well as constitutive) in estrogen-dependent MCF-7 cells is also involved in partial resistance to the antiestrogen therapy using ICI 182,780 [30]. Furthermore, similarly to ERRα, *c-myc* expression level is negatively correlated with patients' age.

It is known that locally the production of estrogen in the estrogen-dependent breast tumor is one of the important factor that stimulates the tumor growth and additionally the high level of metabolites of estradiol inside the tumor can have a genotoxic effect and therefore can intensify the malignancy of the tumor. Local production of estrogen within the tumor is regulated also through aromatase. It was established that interleukins and other cytokines released from carcinoma and inflammatory cells within the tumor had been indicated to potently induce aromatase expression in adipose fibroblast cells [47]. Yet, the relationship between aromatase and nuclear receptors in parenchymal and cancer cells of mammary gland has remained to elucidate. Our study revealed that all of the examined tumors showed mRNA and protein expression of aromatase. We showed a statistically significant positive correlation between aromatase and the ERRα assessed by the immunohistochemical study. However, we did not observe any correlation at the transcription level. It is probably due to heterogenity of studied tumor samples containing both carcinoma and stromal cells. Miki et al. using laser capture microdissection confirmed a positive correlation between ERR and aromatase also at the gene level in human breast carcinoma or parenchymal cells, but not with stromal cells or whole breast tissue [48]. Simpson et al. postulated that in post-menopausal women, induction of steroidogenic enzymes may be an important effect of ERRα–ERα crosstalk [47]. Therefore, ERRα is the potential key regulator of intratumoral estrogen production in breast cancer cells. Beyond the local role of ERRα in the mammary gland in the post-menopausal women, Seely et al. documented that ERRα induces transcription of the steroid sulfotransferase SULT2A1 within the adrenal glands. SULT2A1 maintains the high level of peripheral DHEAS which is an important factor in estrogen synthesis in certain tissues [21]. Another important mechanism of SULT2A1 action is the inactivation of tamoxifen and raloxifene [49]. Taken together, ERRα by the activation of SULT2A1 participates in the enhancement of estrogen production and may decrease efficacy of these SERMs, and this fact can partly explain the resistance of breast cancer cells expressing ERRα to SERMs therapy. The elevated expression of ERRs in the tumor tissue and their possibility of interaction with active ERα protein probably can alter the response of cancer cells to SERMs treatment and may be one of the reasons which leads to the resistance of breast cancer cells to SERMs therapy. Furthermore, it is probable that, likely to ovarian cancer cells [50], breast cancer cells that express ERRα can develop resistance to tamoxifen therapy because ERRα-mediated activities could compensate for the weak expression or the complete loss of ER signaling. On the other side, it has been shown that XCT790, synthetic compound that disrupts interaction between ERRα and its coactivator PGC-1α can potentiate the effect of antiestrogen treatment in ERα (+) cancers [51]. Lanvin et al. proposed that in such tumors where two receptors form ERRα–ERα heterodimers which probably protect each other from anatgonist-degradation, pretreatment with XCT790 enhances the efficacy of ICI182,780 therapy [51].

On the other side, especially in the ERα (−) cells ERRα can play an important role in the alternative pathway in estrogen signaling to initiate the mechanisms regulating cell progression. Probably, in younger women, expression of these factors is more altered, and therefore it may be connected with more malignant phenotype of tumor. Owing to the fact that ERRs are proven druggable targets, it is hoped that development and use of ERRs modulators will lead to new well-tolerated therapeutic approaches to treat breast cancer. Given the potential crosstalk in estrogen-signaling pathway between ERs, ERRs, aromatase, *c-myc*, other transcriptional factors, and subtle differences between molecular profiling, treatment plans in the future should be individualized to each patient.

## Conflict of interest

None declared.

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