



The role of estrogen receptors isoforms in breast cancer

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ABSTRACT

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Background: Estrogen and progesterone receptor (ER/PR) status is an accepted predictive marker in breast cancer. It is well known that breast tumors, which are ER(+) are more likely to respond to endocrine therapy. However, certain percentage of ER(+)/PR(+) tumors do not respond to endocrine therapy. Identification of the second estrogen receptor, named estrogen receptor beta (ERβ), as well as the existence of numerous isoforms/splice variants of both ERα and ERβ, suggests that complex regulation of estrogen action exists. In this study, we analyze does the expression of two ERβ isoforms correlates with ERα/PR status.

Methods: Sixty samples of primary operable breast carcinomas were analyzed for ERα and PR protein levels and for mRNA expression of two ERβ isoforms (ERβ1 and ERβΔ5). ERα and PR proteins were measured by classical biochemical techniques, and ERβ mRNAs were measured by real-time RT-PCR.

Results: Tumors are divided in three groups according to relative level of mRNA for ERβ1 and ERβΔ5. We found that there is no correlation of ERβ1 mRNA expression with ERα and PR protein levels. We confirmed the existence of inverse correlation of ERβΔ5 with PR and of ERβΔ5 with ERα in the group of postmenopausal patients. In the subsets of tumors defined by ERα/PR status, we found that percentage of tumors, which concomitantly expressed high levels of both transcripts, are parallel with those that do not response to tamoxifen treatment.

Conclusion: Inverse correlation of ERα with ERβΔ5 and PR with ERβΔ5 isoform suggests that ERβΔ5 may have inhibitory effect on ERα activity in postmenopausal patients. In addition, we point out that determination of expression profiles of ERα and ERβ isoforms in the defined groups of patient are necessary for elucidating its involvement in endocrine resistance.

KEY WORDS: Breast Neoplasms; Receptors, Estrogen; Receptors, Progesterone; Antineoplastic Agents, Hormonal; Reverse Transcriptase Polymerase Chain Reaction

INTRODUCTION

Estrogen receptor (ER) status of breast tumor (expressed as + or – status) is a well-accepted predictor of response to endocrine therapy. In addition, a down-stream marker of functional ER signaling (1), progesterone receptor (PR), is measured in breast cancer (BC) biopsies and surgical samples. Now, it is known that two receptors for estrogen exist: ERα and ERβ, encoded by two different genes: ERα is encoded by gene located on chromosomal loci 6q25.1 (2), and the ERβ is encoded by gene located on loci 14q22-24 (3). The existence of numerous isoforms/splice variants of both ERα and ERβ, suggests that complex regulation of estrogen action exists. The exact biological significance of isoforms and its splice variants of both receptors (ERα and ERβ), is still unclear, but it seems that their existence may have regulatory role in the response to estrogen. Both genes, ERα and ERβ, have complex “system” of multiple promoters and differential splicing in 5'-UTR region (4). Exon deletions or duplications are second mechanism that potentially generates changes in reading frame, and, accordingly, exchanged proteins (5,6). In addition, five ERβ isoforms (designated as ERβ1 - ERβ5) originate by alternative usage of five 8th codons (7,8). It has been shown that the expression of ERα increases during the process of cancerogenesis, but the expression of ERβ seems to decreases. Estrogen receptor β is under intensive investigation and its role in BC appears to be of predictive value, too. It is reasonable to propose that ER status should now include both receptors, ERα and ERβ.

In this study, we measure expression levels of mRNA of two ERβ variants ERβ1 and ERβΔ5. We also define the cut-off values for ERβ1 and ERβΔ5 mRNA expression in systematic sample of invasive BC. In addition, we compared ERβ status with ERα and PR status. For this purpose, we quantified the expression levels of mRNA of two isoforms of ERβ gene in 60 samples of primary operable BC samples and in adjacent normal tissue by the real-time RT-PCR. This study was performed by using the sensitive and sequence specific assays based on TaqMan methodology. Relative levels of ERβ1 and ERβΔ5 variant were measured by assays designed to detect these transcript at unique regions.

MATERIAL AND METHODS

Patients

We analyzed 60 samples obtained after surgery from patients with primary breast tumors, hospitalized at the Institute of Oncology and Radiology of Serbia in Belgrade. The study had received Institutional Review Board approval according to the National Health Regulation. Adjacent normal tissues are obtained after total mastectomy. Tissue was stored at liquid nitrogen until RNA and protein isolation. The patients all met the following criteria: primary operable unilateral invasive BC, without previous treatment.

Steroid receptor assay

Steroid receptors, ER α and PR were measured by a five-point dextrane-coated charcoal assay in a cytosol fraction of frozen tumor tissue as previously described (9).

RNA Isolation and cDNA synthesis

The approximately 50–100 mg of tissue frozen in liquid nitrogen was pulverized in cold mortar vessel and extraction of total RNA was performed with acid-phenol guanidine method (10). Quality of RNA preparation was verified on agarose gels stained with ethidium bromide. RNA was dissolved again and concentration was determined spectrophotometrically. Total RNA (1 μ g) was reverse transcribed in 20 μ l reaction volume with Omniscript RT Kit (Qiagen, Hilden, Germany) using the 10 μ M random hexamer and 1 μ M oligo(dT)(15) primer according to manufacturer conditions (reverse transcription was performed 60 min on 37°C).

Real-time PCR analysis

All PCR reactions were performed using a 7000 Sequence Detection System (Applied Biosystems). PCR was carried out in 25 μ l reaction volume containing the 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 1x TaqMan Pre-Designed Gene Expression Assay specific for target transcript sequence and cDNA diluted with water (1:10). Relative quantity of target transcripts ER β 1 or ER β Δ 5 in each sample was expressed as an N-fold differences relative to calibrator, or 1x sample, according to equation: $N = 2^{-(\Delta C_{t\text{sample}} - \Delta C_{t\text{calibrator}})}$. ΔC_t values of samples and calibrator were determined by subtracting of average Ct value of target transcripts (ER β 1 and ER β Δ 5) from the average Ct value of β -actin gene (endogenous control) (11).

Statistical analysis

Since levels of expression show non-Gaussian distribution, nonparametric tests (Spearman, Mann-Whitney and Chi square) were used for the analysis of correlation with clinical and histopathological parameters.

RESULTS

Relative quantities of ER β 1 and ER β Δ 5 mRNA were expressed as n-fold difference in relation to calibrator and normalized to the β actin as a reference gene. Distributions of RNA expression levels for both transcripts were the same as distribution of ER α and PR proteins and according to Kolmogorov-Smirnov test, values vary significantly from the pattern of a normal distribution.

Expression of ER β 1 and ER β Δ 5 mRNA in normal and malignant breast tissue

Concerned with fact that there are no cut-off values for ER β expression of mRNA level, and that ΔC_t method of relative quantification is applied, we defined three levels of expression. In this study we classified the BC samples according to expression of ER β 1 and ER β Δ 5 mRNA as "low expressed" (Tu1), "medium expressed" (Tu2), and "high expressed" (Tu3). Samples with "high expression" (Tu3) of ER β 1 were defined as those in which more than three fold differences (in respect to calibrator) were detected; "medium expression" (Tu2) were those between one to three fold differences, and "low expression" (Tu1) were those with less than one fold difference. In the same way, the expression level is defined for the ER β Δ 5 expression, but for slightly different expression range: Tu3 more than six fold difference; Tu2 between two and six fold difference, and Tu1 less than two fold difference. This classification and numbers of samples in each expression group were showed in Table 1. Expression levels of ER β 1 and ER β Δ 5 mRNA in these three groups of samples were compared with expression level in healthy mammary tissue (N), Table 1. Samples in "high expression" group (Tu3) does not differ significantly from the expression level in healthy mammary tissue (Tu3 vs. N, Mann-Whitney for β 1, $p=0.315$, Figure 1; Tu3 vs. ER β Δ 5, Mann-Whitney for β Δ 5, $p=0.078$, Figure 2). Samples in "medium expression" group (Tu2) and in "low expression" group (Tu1) significantly vary from the expression level in healthy

mammary tissue. For ER β 1 Tu2 group vs. N, $p=0.023$, and Tu1 group vs. N, $p=0.001$ (Mann-Whitney), Figure 1. For ER β Δ 5 Tu2 group vs. N $p=0.006$ and Tu1 vs. N, $p=0.002$ (Mann-Whitney). Expression between "low expression" and "medium expression" groups does not differ significantly neither for ER β 1, nor for ER β Δ 5.

It is apparently that Tu3 tumors (those do not differ from expression level in normal tissue) could be considered as "positive" for ER β expression, and Tu2 and Tu1 could be considered as "negative" for ER β expression.

Table 1. Expression of ER β 1 and ER β Δ 5 mRNA in breast cancer and in healthy mammary tissue

Number of samples		Mean*	Median*	Range*
ERβ1				
Breast cancer tissue				
(+)	High expressed (T3) (34)	12,340	6,928	3,091-70,427
	medium expressed (Tu2) (14)	2,060	2,035	1,124-2,966
(-)	low expressed (Tu1) (12)	0,523	0,581	0,000-1,000
	Healthy mammary tissue (N)	50,208	23,154	0,989-205,372
ER$\beta$$\Delta$5				
Breast cancer tissue				
(+)	High expressed (Tu3) (26)	21,074	9,962	6,272-102,508
	medium expressed (Tu2) (25)	3,584	3,203	2,132-5,831
(-)	low expressed (Tu) (9)	1,108	1,007	0,000-1,754
	Healthy mammary tissue (N)	108,811	48,694	2,662-358,389

*All values are expressed in relative expression level (n-fold difference in respect to calibrator) and obtained from 100 ng of total RNA.

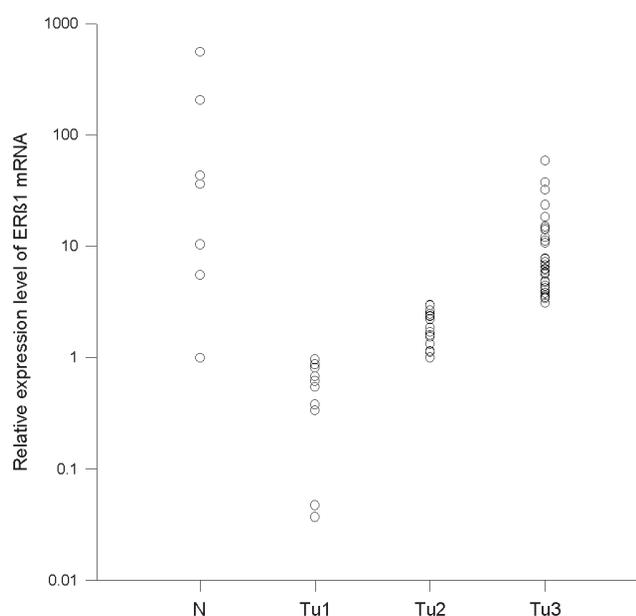


Figure 1. Three groups of breast cancers according to expression of ER β 1 isoform mRNA (Tu1, Tu2 and Tu3) compared with healthy mammary tissue (N)

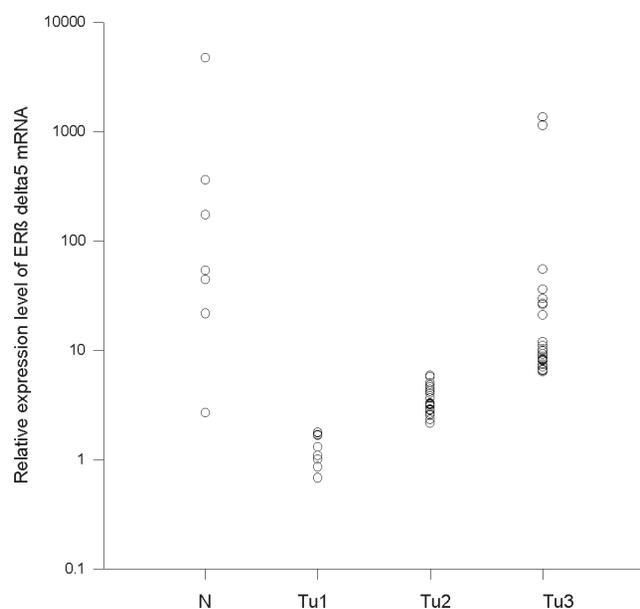


Figure 2. Three groups of breast cancers according to expression of ER β Δ 5 isoform mRNA (Tu1, Tu2 and Tu3) compared with healthy mammary tissue (N)

Correlation of ER β 1 and ER β Δ 5 mRNA expression with the status of ER α and PR

We examined does the expression status of ER β 1 and ER β Δ 5 mRNAs correlates with ER α /PR status. According to ER α status 36 samples were positive (≥ 10 fmol/mg of total cytosol proteins) and 24 were ER α -negative. According to PR status, 20 samples were PR-positive (≥ 20 fmol/mg of total cytosol proteins) and 40 were PR-negative.

Analysis of association between ER α and PR protein levels shows no correlation with expression of the wild type of estrogen receptor beta (ER β 1).

We found correlation between ER β Δ 5 mRNA and PR protein in the subgroup of postmenopausal patients (ER β Δ 5 vs. PR, Chi square, $p=0.005$). Moreover, in this group of patients significant inverse correlation exist between ER β Δ 5 mRNA and ER α (Spearman, $\rho=-0.335$, $p=0.04$). There is no correlation between ER β Δ 5 mRNA and ER α or PR in the group of premenopausal patients (results not shown).

In order to examine does the ER β expression correlates with established percentage of endocrine unresponsive patients, we analyze expression of both ER β transcripts in groups defined by the ER ER α /PR status. We found that the percentage of tumors with concomitantly high expression level of both transcripts, in the groups of ER-positive/PR-positive, ER-positive/PR-negative and ER-negative/PR-negative patients, are 22, 38 and 40%, respectively. Assignment of the tumors with high expression of ER β 1 and ER β Δ 5 (Tu3) in the above mentioned groups of patients are parallel to the percentage of endocrine unresponsive ones.

DISCUSSION

Majority of available data about ER β expression in clinical samples come from the immunohistochemical studies. Quantification of ER β expression on RNA level is justified, since the ER β mRNA increases in parallel with the increase in protein level as showed by Cheng et al (12), indicating that regulation of ER β expression is on transcriptional level.

In this study, we performed the specific and sensitive TaqMan pre-designed assay for qPCR to quantify mRNA of ER β 1 (wt isoform) and ER β Δ 5, which is a splice variant without fifth exon of ER β 2 isoform. ER β Δ 5 mRNA encoded truncated protein without ligand binding domain.

In examined samples of BC we found that there are two groups, according to expression of ER β 1 and ER β Δ 5: *i*) those that are not different compared to normal mammary tissue (referred as ER β -positive or "high expressed" (Tu3)), Table 1, Figure 1 and 2.; *ii*) and those in

which expression levels significantly varies from normal mammary tissue (referred as ER β -negative or "medium expressed" (Tu2) and "low expressed" (Tu1)), Table 1, Figure 1 and 2. We found no correlation between expression of ER β 1 mRNA, measured by qRT-PCR and ER α measured by ligand binding assay. Other authors also report (in immunohistochemical studies) that there is no correlation between ER α and ER β expression on protein level (13,14). The absence of correlation of ER β 1 expression with clinical and histopathological parameters (our unpublished results), indicate that its expression level could be an independent predictive marker in BC, but its predictive value remains to be established.

Interestingly, little is known about expression of ER β Δ 5 in the clinical samples. Deletions of fifth exon results in frame shift in reading frame of protein, generating five alternative amino acids, and stop codon after them, which results in truncated protein without ligand binding domain. In cell transfection studies (16), it was shown that ER β Δ 5 truncated protein possesses dose dependent, inhibitory activity against and ER α in 293T cell line. A similar activity, also in eukaryotic cell culture system, has its counterpart, ER α Δ 5 variant against the ER α on ERE reporter genes.

Our finding of inverse correlation of ER β Δ 5 with PR and with ER α in postmenopausal patients are in concordance with *in vitro* study of Inoue and coworkers (16) and may reflect the inhibitory activity of this truncated protein *in vivo* against transcriptional activity of ER α receptor. Inverse correlation of PR and ER β 2 was also reported by Saji and coworkers (17). Their data, together with our result that ER β Δ 5 are in inverse correlation with PR *in vivo* suggest that high expression of some ER β isoforms may underlie the emergence of ER α -positive/PR-negative cells. Inverse association between expression of ER β Δ 5 variant and levels of PR protein may be the consequence of inhibition of estrogen-induced activity of ER α by hetero-dimerization with truncated protein encoded by β Δ 5 splice variant as proposed by Inoue (16). Our finding that this inverse correlation comes from postmenopausal subset of patients, points out that this inhibitory activity might be detectable *in vivo* only when levels of circulating estrogen is low.

In addition, we found that in the subsets of patients defined by the ER/PR status, percentage of concomitantly "highly expressed" ER β 1 and ER β Δ 5, are parallel to the established percentages of endocrine unresponsive patients. Poola and coworkers (15) recently published similar results where they show that two isoforms of ER β receptor (β 1 and β 5) are highly expressed in ER α -negative tumors. Biochemical explanation for this occurrence is that in the absence of ER α the ER β can be mediator of estrogen signaling as suggested by Poola (15). Moreover, there are data that tamoxifen may have agonistic effect on ER β in HeLa cells and BC cell lines. At the same time, high level of ER β Δ 5 variant (which dimerizes with ER α (16)) may suppress the ER α activity. Although it is widely documented that the decreased expression of ER β correlates with tumor emergence and progression, the role of this receptor in tamoxifen resistance remains unclear.

Our approach in defining of ER β mRNA positive and negative is based on comparison between normal and malignant tissue is justified, since we obtained results comparable with data obtained in immunohistochemical studies. This approach may be helpful in future studies dealing with clinical significance of ER β .

In conclusion, we point out the necessity for analyzing the complete isoform profiles of ER β , ER α and PR in clinical samples, since it is possible that one pattern of isoforms expression may be a cause of tamoxifen resistance and the other might be a marker of sensitivity.

Note

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