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LEVELS OF ESTROGEN RECEPTOR B SPLICE VARIANT (ERBΔ5) MRNA CORRELATES WITH PROGESTERONE RECEPTOR IN BREAST CARCINOMAS

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Abstract - It is well known that breast tumors which are estrogen positive ER(+) are more likely to respond to hormone therapy. However, a certain percentage of ER(+)/PR(+) tumors do not respond to this 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 a complex regulation of estrogen action exists. In this study, we analyzed the expression ratio of ERβ1 isoform and ERβΔ5 splice variant mRNAs, and its correlation with ER/PR status by quantitative RT-PCR and clinical and histopathological parameters. We found that the relative proportion of ERβΔ5 in the total ERβ1 transcript "pool" inversely correlates with the PR level (ρ = -0,359, p< 0,003, Spearman). It may be that the ERβΔ5 variant modulates the ERα activity of downstream targets. In addition, we suggest that the determination of the expression profiles of ERα and ERβ isoforms and splice variants in the defined groups of patients are necessary for elucidating their involvement in endocrine resistance.

Key words: Estrogen receptor β, progesterone receptor, quantitative RT-PCR, breast cancer, normalization of splice variant to wt isoform

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INTRODUCTION

The estrogen receptor (ER) status of a breast tumor (expressed as + or – status) is a well-accepted predictor of the response to hormone therapy. In addition, the progesterone receptor (PR), the downstream marker of functional ER signaling, is measured in breast cancer (BC) biopsies and surgical samples. Two receptors for estrogen exist: ERα and ERβ which are encoded by two different genes (*ESR1* and *ESR2*) (Enmark 1997; Menasce 1993). The presence of numerous isoforms/splice variants of both ERα and ERβ suggests complex regulation of estrogen action. The exact biological significance of isoforms and the splice variants of both ERα and ERβ receptors is still unclear but it seems that their existence may regulate the response to estrogen. Both *ESR1* and *ESR2* genes have a complex organization of multiple promoters and differential splicing in the 5'-UTR region (Lewandowski 2002). Exon deletions or duplications are a second mechanism that potentially generates changes in the open reading frame and, accordingly, leads to different forms of proteins (Bollig 2000; Poola 2002a). In addition, five ERβ isoforms (designated as ERβ1 - ERβ5) originate by alternative usage of the eighth coding exon (Moore 1998; Ogawa 1998). It has been shown that the expression of ERα increases during the process of carcinogenesis, but the expression of ERβ seems to decrease (Bardin 2004). The estrogen receptor β is under intensive investigation and its role in BC appears to be of additional predictive value. It is reasonable to propose that the ER/PR status should now include both ERα and ERβ receptors together with some of their isoforms and functionally active splice variants, as well as the PR isoforms A and B.

In this study the expression levels of ERβ1 isoform mRNA, which is considered as the wt form of the receptor β, and ERβΔ5 mRNA (splice variant without 5th exon) were measured. In order to investigate if the ratio of ERβ1 and the splice variant ERβΔ5 is related to clinical and histopathological parameters, as well as to ERα/PR status, we analyzed their expression by the method of quantitative RT-PCR, using TaqMan assays. The relative levels of ERβ1 and ERβΔ5 variants were measured by assays designed to detect these transcripts at unique regions. The isoform ERβ1 was detected by assay to be located on the exon 7/8b junction (the population of transcripts with 8b exon is considered a wild-type isoform with intact ligand binding domain and AF-2 domain). Exon 5, ERβΔ5, was detected by an assay designed to cover the junction of coding exons 4 and 6.

PATIENTS AND METHODS

Sixty eight samples obtained after surgery from patients with primary breast tumors (adenocarcinomas) were analyzed. The patients all met the criterium of primary operable unilateral invasive BC without previous treatment. All tumor samples were microdissected and only regions with more than 70 % of malignant epithelial cells were used for RNA isolation. Adjacent normal tissues were obtained after total mastectomy from ten patients. Tissue was stored in liquid nitrogen until RNA and protein isolation. The study had received Institutional Review Board approval according to the National Health Regulation and informed consent was obtained from all patients.

Steroid receptors

ER and PR were measured by a five-point dextrane-coated charcoal assay in a cytosol fraction of frozen tumor tissue according to EORTC recommendations (Eortc 1980).

RNA Isolation and cDNA synthesis

About 50 – 100 mg of frozen tissue (frozen in liquid nitrogen) was pulverized in a cold mortar vessel and extraction of the total RNA was performed by the acid-phenol guanidine method (Chomczynski 1987). The quality of the RNA preparation was verified on agarose gels stained with ethidium bromide. Aliquots of the RNA samples used in real time analysis were treated with DNAse I (Fermentas, Vilnius, Lithuania) and DNAse I was heat-inactivated after treatment according to manufacturer instructions. Another phenol extraction was performed, followed by precipitation of the RNA. The RNA was dissolved again and its concentration was determined spectrophotometrically. One μg of the total RNA was reverse transcribed with 10 μM random hexamers and 1 μM oligo dT(15) primers in a 20 μl reaction volume using Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time PCR analysis

All PCR reactions were performed using a Prism 7000 Sequence Detection System (Applied Biosystems). PCR was carried out in a 25 μl 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). The TaqMan assays used for PCR were: Hs01100359_m1 for detection of the ERβ1 variant, and Hs01105521_m1 for ERβΔ5. The relative levels of ERβΔ5 in each sample were expressed as N-fold difference to (ERβ1) and splice variant (ERβΔ5) according to the equation: $N = 2^{-(CtER\beta1 - CtER\beta\Delta5)}$ (Applied Biosystems, User Buletin#2, 2001), and relative to the calibrator, (sample with the highest difference value, lowest ERβΔ5 to ERβ1 ratio), according to the equation: $N = 2^{-(\Delta Ct\ sample - \Delta Ct\ calibrator)}$.

Validation experiment

In order to check the amplification efficiency of the two assays (ERβ1 and ERβΔ5), serial dilutions of the sample were made and amplified by each of the assays (Applied Biosystems, User Buletin#2, 2001). The slopes of the curves obtained were calculated by the ABI Prism 7000 software. Results and Ct values were exported into MS Excel and ΔCt

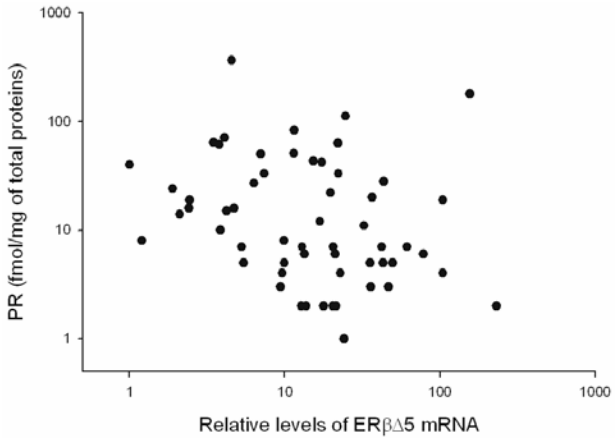

Figure 1. Scatter plot showing the association of ERβΔ5 mRNA relative levels with the PR protein level in 67 samples of BC (Spearman, **ρ**= - 0,359, **p**=0.003).

(average Ct β1 – average Ct β2Δ5) values were calculated for each dilution.

Statistical analysis

Nonparametric tests (Spearman, Mann-Whitney tests and Kruskal-Wallis Anova on Ranks) were used for the analysis of the correlation of the expression of ERβΔ5 with PR, and clinical and histopathological parameters.

RESULTS

The level of ERβΔ5 mRNA (splice variant) normalized to ERβ1 (full-length receptor) was calculated as N-fold difference in relation to the calibrator sample. The obtained value, which represents the assignment of Δ5 splice variant in the total ERβ1 transcript "pool", was analyzed as a continuous variable for correlation with PR, and clinical and histopathological parameters. Before quantification, the experiment of validation was performed and reaction efficiency was determined for both assays. The resulting slopes were: -3.6328, for assay detecting the ERβ1, and -3.6811, for assay detecting the ERβΔ5; the resulting slope difference between the ERβ1(wt) and ERβΔ5 assay was 0.048, which suggests an acceptable difference in reaction efficiency. The expression of ERβ1 and ERβΔ5 mRNAs was detected in all samples with lower Ct values for ERβ1 in all cases except one, where ERβΔ5 had the lower Ct value. This sample was excluded from the analysis.

When analyzed as a continuous variable, the proportion of ERβΔ5 mRNA is associated with the PR protein level - these two variables are in inverse correlation (Spearman, ρ= -0.359, p=0.003) in the whole examined group of patients (Figure 1).

There are no statistically significant differences in ERβΔ5 mRNA expression between groups of patients with different histopathological characteristics: IDC and ILC (invasive ductal and invasive lobular carcinoma), or within the groups defined by nodal status and tumor size. The correlation with tumor grade was not analyzed since most of the samples were of grade II. Although not significant, in this cohort we detected a trend (p=0.072, Mann-Whitney) towards higher ERβΔ5 mRNA expression in postmenopausal patients (median relative expression level 20.5, 42 cases) compared to premenopausal ones (median relative expression level 11.2, 18 cases).

Furthermore, we analyzed the expression level of ERβΔ5 in cancer samples in comparison with healthy mammary tissue. The median ERβΔ5 mRNA expression in the samples of healthy mammary tissue was 35.3. We chose the arbitrary cut-off value of 20 to divide cancer samples into two groups. In 46% of the samples (31 out of 67) the ERβΔ5 mRNA relative levels were higher than 20 – BC group I (median 35.7); in 54% of the analyzed samples (36 out of 67) the ERβΔ5 mRNA expression was lower than 20 – group II (median 8.4). The levels of ERβΔ5 mRNAs in the group II breast cancers (less ERβΔ5 than in group I) significantly differs from the levels in healthy mammary tissue and group I ($p < 0,05$ Kruskal Wallis). In group I of breast cancer samples, the levels of ERβΔ5 mRNAs was in the range specific for healthy mammary tissue (Figure 2).

In the BC group II (with less ERβΔ5 than in group I) the median level of PR protein was

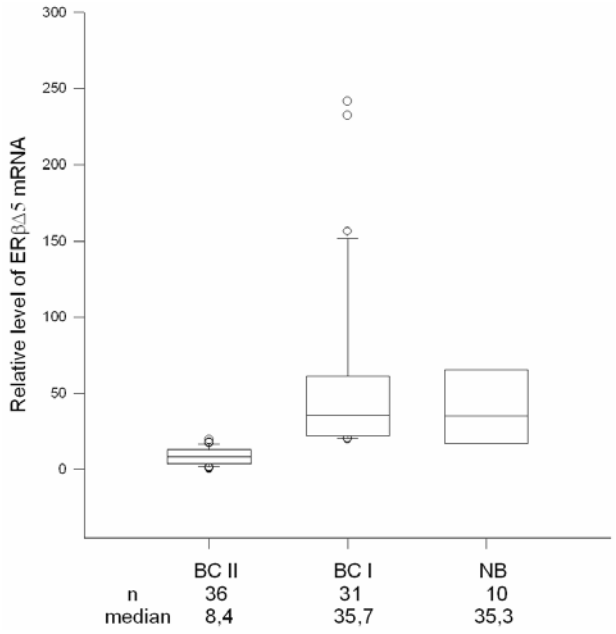

Figure 2. Two groups of BC defined according to ERβΔ5 mRNA relative levels: **BC-I** – group with ratio > 20 of relative expression units, and **BC-II** - group with ratio < 20 of relative expression units. In the analyzed BC-I group of samples, ERβΔ5 mRNA are in the range of those in normal breast tissue (**NB**), but the BC-II group samples significantly differ from NB (Kruskal Wallis, Dunn's Method, $p < 0.05$).

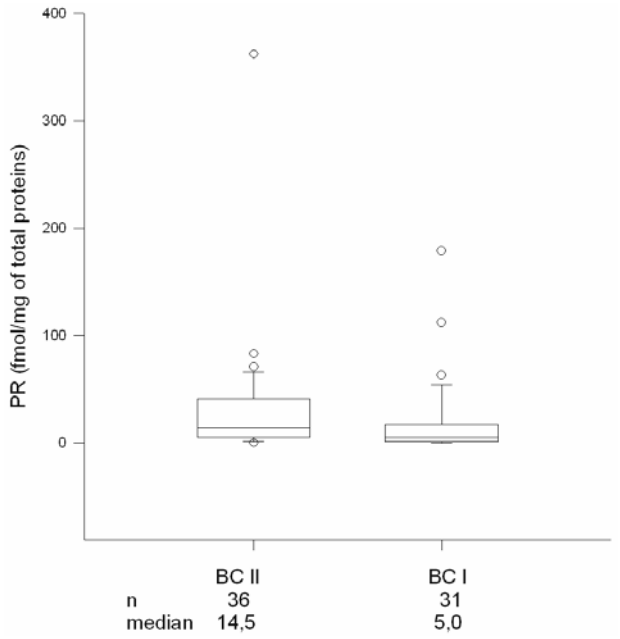

Figure 3. Box plots show different expression level of PR protein in BC I and BC II groups.

significantly higher (14.5 fmol/mg total proteins of cytosol) than in the BC group I (5 fmol/mg total proteins), p=0,013, Mann-Whitney (Figure 3).

DISCUSSION

In this study, we performed the specific and sensitive TaqMan assay for qPCR to quantify the mRNAs of ERβΔ5 as a portion of the ERβ1 (wt isoform) total transcript pool. ERβΔ5 is a splice variant without the fifth exon of ERβ receptor.

ERβΔ5 mRNA encodes truncated protein without ligand binding domain and it poses dose dependent inhibitory activity against the E2 stimulated transcriptional activity of ERα on ERE (Inoue 2000). In spite of the fact that this variant is detected in normal samples of breast tissue as well as in cancer samples, until now reports are scarce about this variant in clinical samples of BC (Poola 2002b). In this study we normalized ERβΔ5 expression to the ERβ1 total transcript pool and obtained a value that represents the portion of this variant. Using this approach, it is not necessary to use the reference gene which is often differentially regulated in individuals, especially in tumors (Bustin 2000; Sabath 1990). In addition, searching for the appropriate reference gene is often time-consuming and expensive, especially when a large number of samples are analyzed. Using this approach we showed that the relative levels of splice variant without the fifth exon were associated with the PR status. This effect may be a direct consequence of the inhibitory activity of the variant protein on the full length ERα and ERβ transcription from the PR promoter.

To recapitulate, ERβΔ5 mRNA levels relative to ERβ1 were analyzed as a continuous variable and an inverse association was found with the PR protein level. The inverse association of ERβΔ5 PR expression may be a consequence of a decreased level of ERβΔ5 transcript rather than an increase of ERβ1, as we showed previously in a similar patient

Table 1. The clinical and histopathological characteristics of patients

Parameter	Number of patients
Age	
(mean: 57.8; range: 32 – 84)	
<45	12
45-59	29
>59	27
Menopausal status	
pre	18
peri	6
post	42
unknown	2
Histological type	
IDC	31
ILC	23
IDC+ILC	5
other	9
Histological grade	
I	3
II	55
III	6
unknown	4
ER	
ER (+)*	41
ER (-)	27
PR	
PR (+)**	21
PR (-)	47
Tumor size	
T1[§]	31
T2+ T3[§]	35
unknown	2
Nodal status	
N0	16
$N+_{(1-3)}$	25
$N+_{(\geq 4)}$	15
unknown	12

*ER-positive ≥ 10 and ER-negative < 10fmol/mg of total cytosol proteins; **PR-positive ≥ 20 and PR-negative< 20 fmol/mg of total cytosol proteins; [§]T1 ≤ 20mm; T2: 20-50mm; T3 ≥50mm.

cohort by analyzing these two variants separately and using the normalization according to the β-actin gene (Mandusic 2007). Consistent with our previous data, we suggest that the increased relative ratio of ERβ1 to ERβΔ5 is connected to the process of malignant transformation. The changed ratio of ERβ1/ERβΔ5 in approximately 50% of the cases mainly comes from a more pronounced decrease of ERβΔ5 mRNA than from ERβ1 mRNA in breast tumors compared to normal breast tissue (Mandusic 2006).

The protein encoded by ERβΔ5 mRNA lacks C terminal domain (responsible for ligand binding), but has retained domains for nuclear localization and heterodimerization. Such a truncated receptor could potentially affect the transcriptional activities of both ERα and ERβ receptors. Using a cell transfection system, Inoue and coworkers showed , that the ERβΔ5 variant protein attenuates E2-stimulated trans-activation by ERβ1 and ERα in a dose dependent manner (Inoue 2000). The inhibition of ERα transcriptional activity by the ERβΔ5 variant protein can cause a decrease in the PR protein level since it is ERα's downstream target. This can be one of the sources of the ER+/PR-phenotype in breast cancers. Consequently, the ERβΔ5 receptor may act as an inhibitor of transcriptional activity of wt isoforms of ERα (which leads to cell proliferation) and ERβ (whose activity suppresses proliferation) (Bardin 2004). The decrease of ERβΔ5 level may lead to a loss of inhibition of estrogen-stimulated proliferation via ERα. In this way, the ERβΔ5 splice variant may be involved in the progression of breast carcinogenesis.

We suggest possible applications of this approach in future analyses of ER isoforms and splice variants in clinical samples of breast cancer. Although this approach was suggested previously, normalization of the splice variant to the wt receptor is not exploited enough. It is proposed that numerous isoforms of the estrogen receptor β, which differ in C terminal region, have different biological functions (Peng 2003). These isoforms are generated using the alternative eighth exon (Hirata 2003) and can be easily analyzed as an RNA ratio in clinical studies on large number of samples without using the reference gene. Such an approach is cheap and not so time-consuming and may be useful for the analyzing of estrogen responsive tissues, where

a subtle balance within the different isoforms of ERα and ERβ is crucial for the response to estrogen.

In conclusion, we stress the necessity for analyzing the complete isoform and splice variants profile of ERβ, as well as ERα and PR, in clinical samples, since it is possible that the pattern of isoform expression might be of prognostic and predictive value in clinical practice.

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