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TRANSCRIPTIONAL RATIO OF ESTROGEN RECEPTOR β mRNAs IN CARCINOMAS AND IN NORMAL TISSUES. Vesna Mandušić¹, Koviljka Krtolica-Žikić¹, Dragica Nikolić-Vukosavljević², D. Popov-Čeleketić^{1,3}, D. Plećaš⁴, I. Boričić⁵, B. Dimitrijević¹, and N. Tanić⁶ ¹ Vinča Institute of Nuclear Sciences, Laboratory of Radiobiology and Molecular Genetics, 11000 Belgrade, Serbia; ² Institute of Oncology and Radiology of Serbia, 11000 Belgrade, Serbia; ³ present adress: Adolf-Butenandt-Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, München, Germany; ⁴ Institute of Obstetrics and Gynecology, Clinical Center of Serbia, 11000 Belgrade, Serbia; ⁵ Institute of Pathology, School of Medicine, University of Belgrade, 11000 Belgrade, Serbia; ⁶ Siniša Stanković Institute for Biological Research, 11000 Belgrade, Serbia

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The existence of two receptors for estrogen, ER α and ER β , encoded by two different genes (ESR1 and ESR2), together with the existence of its isoforms and splice variants, imposes the need to clarify their function in estrogen signaling. In order to investigate if the weight ratio of estrogen receptor beta (ER β 1) and its splice variant (ER β Δ 5) is different in malignant tissue compared to healthy tissue we analyzed their expression by the method of quantitative RT-PCR and showed that ratio ER β 1/ER β Δ 5 in breast cancer and cell line MDA MB 361 is increased compared to healthy tissues. This finding suggests that decreasing of ER β Δ 5 may be one of the phenomena related to tumorigenesis in estrogen responsive tissues and points to possible application of this type of analysis in future standard clinical practice

The status of estrogen receptor alpha (ER α) and that of progesterone receptor (PR) are routinely used in breast cancer treatment to select optimal therapy. Overall, approximately 60% of ER α -positive tumors respond to endocrine therapies. Although combination of ER α with PR level determination offers some improvement in the prediction of endocrine response, it still does not serve as an absolute predictor of responsiveness to endocrine therapy. Identification of the second estrogen receptor, named estrogen receptor beta (ER β), as well as the existence of numerous isoforms/splice variants of both ER α and ER β , suggests that complex regulation of estrogen function exists. After the cloning and characterization of ER β , several isoforms and splice variants at the mRNA level were described but their biological significance has not yet been resolved. Deletion of one or more coding exons was among the first recognized mechanisms for generation of splice variants (Vlačić et al., 1998; Lu et al., 1998; Poola et al., 2002). The splice variant without the fifth exon (ER β Δ 5), which encodes truncated protein, was one of first recognized deletion variants of the ER β transcripts, but little is known about its expression level in cancer. We propose that two mRNA transcripts of ER β , viz., β 1 encoding wild type receptor and Δ 5, are differentially expressed in malignant and normal cells. In light of the fact that inhibito-

ry activity of ER β Δ 5 seems to be dose-dependent, we quantified the mRNA level of these two transcripts (ER β 1 and ER β Δ 5) in normal and malignant tissues and in malignant cell lines. In this study, we analyzed the weight ratio isoform ER β 1 and ER β Δ 5 mRNA. Here analyzed normal and breast carcinoma tissue (BC) and MDA MB 361 (a cell line from human breast adenocarcinoma), as well as normal testicular and uterine tissue. Expression of ER β 1 and ER β Δ 5 was measured using sensitive and specific TaqMan probes by the method of quantitative RT-PCR.

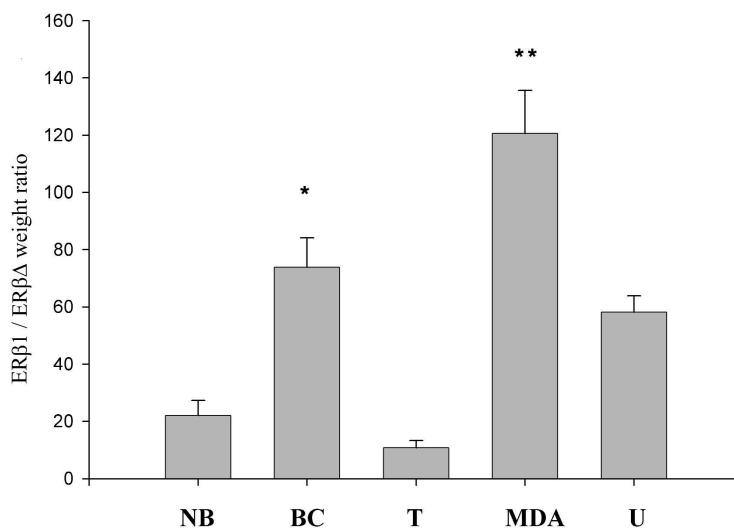
Analysis was performed on 34 samples obtained after surgery from patients with primary breast tumors hospitalized at the Institute of Oncology and Radiology of Serbia, Belgrade. These samples were chosen as positive for ER β 1 mRNA expression based on the cut-off value established in our previous study (Mandušić et al., 2006). Samples of adjacent normal tissues were obtained after total mastectomy. Samples of testicular tissue were obtained from the Institute of Pathology, Belgrade, while samples of uterus were obtained from patients who underwent hysterectomy at the Institute of Obstetrics and Gynecology in Belgrade. Tissue was stored on liquid nitrogen until RNA isolation. All patients met the following criterion: primary operable unilateral invasive breast carcinomas without previous treatment. The study was approved by the Institutional Review Board and adhered to the National Health Regulation Guidelines. Approximately 50 – 100 mg of tissue frozen in liquid nitrogen was pulverized in a cold mortar and extraction of total RNA was performed by the acid-phenol guanidine method according to Chomczynski and Sacchi (1987). Quality and concentration of the RNA preparation were verified on 0,8% agarose gels stained with ethidium bromide. One μ g of total RNA was reverse-transcribed over a period of 60 min at 37°C in a reaction volume of 20 μ l with the Omniscript RT Kit (Qiagen, Hilden, Germany) using the 10- μ M random hexamer and 1- μ M oligodT(15) primer according to manufacturer instructions. All PCR reactions were performed using a 7000 Sequence Detection System (Applied Biosystems, Foster City,

CA). PCR was carried out in 25- μ l reaction volume containing the 1x TaqMan Universal PCR Master Mix, 1x TaqMan Pre-Designed Gene Expression Assay (Applied Biosystems, Foster City, CA) and 10 μ l of cDNA diluted with water (1:10). Assays were specific for target transcripts: assay ID Hs01100359_m1 for ER β 1 and assay ID Hs01105521_m1 for ER β Δ 5. The ratio of examined transcripts ER β 1 and ER β Δ 5 in each sample was expressed as N-fold differences relative to each other according to the equation: $N = 2^{-(Ct_{ER\beta 1} - Ct_{ER\beta \Delta 5})}$, where Ct is the threshold cycle. Differences in expression of ratio ER β 1/ER β Δ 5 between healthy breast tissue and either BC or the MDA MB 361 cell line were analyzed by the Mann-Whitney and t- test, respectively.

The ratio of ER β 1 and ER β Δ 5 mRNA was expressed as the N-fold difference in the relation to each other in examined samples. Expression of ratio ER β 1/ER β Δ 5 in normal breast tissue is significantly lower than in BC ($p = 0,016$, Mann-Whitney, Fig. 1). In addition, expression of ratio ER β 1/ER β Δ 5 in the adeno-

pression in breast tumors compared to normal breast tissue. We have shown (Mandušić et al., 2007) decrease of both ER β 1 and ER β Δ 5 mRNA expression in breast tumors compared to normal breast tissue. Protein encoded by ER β Δ 5 mRNA lacks the C terminal domain (responsible for ligand binding) but retains the domains for nuclear localization and heterodimerization. Such a receptor could, potentially, have an effect on transcriptional activities of both estrogen receptors, ER α and ER β . It has been shown that ER β Δ 5 protein in a cell transfection system attenuates E2-stimulated transactivation by ER β 1 and ER α in a dose-dependant manner (Inoue et al., 2000). In contrast, ER β Δ 5 alone lacks transcriptional activity even in the presence of a ligand (Inoue et al., 2000). It follows that the ER β Δ 5 receptor acts as an inhibitor of transcriptional activity of weight isoforms of ER α and ER β . This means that decrease in inhibitory activity of ER β Δ 5 during estrogen-stimulated proliferation may be involved in tumorigenesis. In conclusion, we point out the need to analyze the complete isoform profiles of ER β , ER α , and PR in clinical samples, since it is possible that the pattern

Fig. 1. Ratio of ER β 1/ER β Δ 5 in normal tissue (NB - breast, T - testis, U - uterus), malignant tissue (BC - breast carcinomas), and an adenocarcinoma cell line (MDA mb 361). The Y axis represents relative expression ratio of two transcripts, ER β 1 and ER β Δ 5, obtained from Ct values as described in the text.



carcinoma cell line significantly differs from that in normal breast tissue ($p < 0,001$, t-test) and is similar to that found in BC. Normal uterine and testicular tissue as a control did not significantly differ from normal breast tissue regarding the ER β 1/ER β Δ 5 ratio (Fig. 1).

These data suggest that an increased relative weight ratio of receptor ER β 1 and truncated receptor ER β Δ 5 is connected with the process of malignant transformation. Increase of ratio ER β 1/ER β Δ 5 in BC results mainly from more pronounced decrease of ER β Δ 5 mRNA expression than of ER β 1 mRNA ex-

pression of isoform expression might be of prognostic and predictive value in clinics.

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