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ORIGINAL ARTICLE

Identification of novel transcript variants of estrogen receptor α, β and progesterone receptor gene in human endometrium

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Abstract The human progesterone receptor (PR) and estrogen receptor genes (ESR1 and ESR2) are known to code for a multitude of transcript variants resulting from alternative splicing. Many of them are translated into nuclear receptor proteins with altered structure and function. Expression of these alternative estrogen and progesterone receptors modulates the cellular response to sexual steroid hormones. Recent studies also suggested their significance in development of hormone-dependent diseases like gynecological cancers. We report identification of 12 new transcript variations of the PR, ESR1, and ESR2 gene in human endometrium which result from differential exon-skipping. We succeeded in cloning of four new double or triple exon-deletion transcript variants of ERα, four single, double or triple exon-skipped mRNA isoforms of ERβ, and four new transcript variations of PR gene. Sequence analysis suggested that at least four of them, ERαΔ5/6, ERαΔ5/6/7, PRΔ7, and PRΔ6/7 are translated into receptor proteins which might exert ligand-independent effects on steroid hormone signalling. Comparison of pre- and post-menopausal endometrium revealed differential expression of PRΔ6/7, ERαΔ5/6/7, ERαΔ3/4/5, and ERβΔ1-0N. We also report differential expression of the exon-skipped isoforms in a panel of human cancer cell lines derived from the breast, ovary, and endometrium. Our identification of additional transcript variations further increases the complexity of steroid hormone receptor gene expression and signalling.

Keywords Estrogen receptor alpha · Estrogen receptor beta · Progesterone receptor · mRNA splicing · Endometrium · Gene expression · Cancer cell line

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Introduction

Estrogens and progesterone are involved in regulation of growth and differentiation of hormone-dependent tissues such as endometrium and mammary gland. The effects of these steroid hormones are mediated both by putative membrane receptors and by nuclear receptors like the estrogen receptors (ER) α and β and the progesterone receptor (PR) [1].

In humans, numerous variant mRNA forms of these nuclear receptors have been described both in normal and neoplastic tissues [2–4]. Splicing variants of these genes result from usage of alternative 3′- or 5′-exons or from the use of different transcription start sites [5–7]. Another important mechanism underlying transcript variation is termed exon-skipping, by which multiple forms of mRNAs are generated from a common pre-mRNA through differential exon use [8]. Due to the fact, that different combinations of exons are joined together to generate a mature protein-coding mRNA transcript, this alternative splicing mechanism contributes to a high proteomic diversity.

Over the years, many mRNA splice variants of ERα, ERβ, and PR with single, double or multiple exon-deletions

have been identified [9–11]. It was demonstrated that based on the loss of particular exons, most of these mRNA variants code for proteins which have different biochemical and structural properties. Some variant proteins are unable to bind DNA or their ligand and thus are incapable of mediating estrogen signals (ERαΔ4, ERβΔ3) [12, 13]. Other variants are not able to activate gene transcription, but exert dominant positive (ERαΔ5) [14, 15] or negative effects on receptor function (ERαΔ7, ERβΔ5) [16, 17].

For some steroid hormone receptor splicing variants, an important role in development and progression of breast cancer has been shown. For example, clinical studies have indicated that the PR isoforms A and B, which result from usage of different transcription start sites, exert different functions in breast cancer. PR-B exhibits an additional N-terminal region and interacts with a different set of coactivators as PR-A [18, 19]. Whereas the ratio of PR-A and B is approximately equal in mammary epithelium, breast cancer cells exhibit a stronger expression of the A-variant exerting proliferative and anti-apoptotic functions [20, 21]. Further examples are ERβ1 and ERβ2, which exert different inhibitory effects on ERα and thereby on estrogen responsiveness [22, 23]. It was reported, that high levels of ERβ2, but not of ERβ1, are associated with a better relapse-free survival (RFS) and overall survival (OS) in women receiving endocrine treatment [24]. Thus, demonstration of the biological significance of splice variants adds another dimension to our understanding of ERα, ERβ, and PR signalling and their role in carcinogenesis.

Here, we report identification of 12 new splicing variants of steroid hormone receptor genes *ESR1*, *ESR2*, and *PR* in human endometrium and analyzed their expression in a panel of cancer cell lines derived from the breast, ovary, and endometrium.

Materials and methods

Endometrial tissue specimen

The 17 postmenopausal and 11 premenopausal endometrial tissue specimens were collected between 2007 and 2009 by the Second Department of Gynecology of the Medical University of Lublin, Poland and by the Clinic of Obstetrics and Gynecology, Medical University of Regensburg, Germany. From the premenopausal patients, aged 43–53 years, five were in the proliferative phase and six in the secretory phase of the menstrual cycle. The postmenopausal women were aged between 46 and 90 years. Endometrial tissue was obtained from women subjected to surgery for reasons other than pathology of the endometrium, mainly cervical cancer. All patients granted consent for the collection and use of biologic material. Tissue samples of patients were collected in accordance with German or Polish regulations and in agreement with the Ethical Committees of the University School of Medicine in Lublin, Poland or the Medical University of Regensburg, Germany. Complete clinical data were available for every patient. Immediately following surgery, tissues were stored in liquid nitrogen until RNA extraction.

Cell lines

MCF-7, T47-D, MDA-MB-231, SKBR-3, and ZR75-1 breast cancer cells, RL95-2, HEC-1A, and HEC-1B endometrial cancer cells and SK-OV-3 and NIH-OVCAR-3 ovarian cancer cells were maintained in DMEM/Ham′s F-12 medium (1:1) (Sigma, Deisenhofen, Germany) supplemented with 10% FCS (PAA, Pasching, Austria). The following supplements were added: 10 ng/ml insulin (NIH-OVCAR-3), 1 nM EGF (MCF-10A) or a combination of 10 ng/ml insulin and 1 mM sodiumpyruvate (MCF-7, T47-D, HEC-1B, and RL95-2). Cells were cultured at 37°C in a humidified CO_2 incubator (5%) prior to RNA isolation.

All cancer cell lines were obtained from American Type Culture Collection (Manassas, USA). Human recombinant epidermal growth factor (EGF) as well as insulin and sodiumpyruvate were purchased from Sigma (Deisenhofen, Germany).

RNA preparation and real-time RT–PCR

Total RNA extraction from cancer cell lines was performed by means of the SV Total RNA Isolation System (Promega, Mannheim, Germany) including DNase treatment, following the manufacturer's instructions. Total RNA from endometrial tissue samples was isolated from 30 to 80 mg frozen tissue using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer's protocol. RNA purity and concentration were analyzed by spectrophotometry. From each sample, 500 ng of total RNA was reverse transcribed to cDNA using 40 units of M-MLV Reverse Transcriptase and RNasin (Promega, Mannheim, Germany) with 80 ng/µl random hexamer primers (Invitrogen, Karlsruhe, Germany) and 10 mM dNTP mixture (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. After reverse transcription, the levels of exon-skipped transcripts of steroid hormone receptor genes *ESR1*, *ESR2*, and *PR* were determined by real-time PCR. For this purpose, 4 µl of cDNA were amplified using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and 5 mM of each primer (Table 1). Oligonucleotides (Metabion, Planegg-Martinsried, Germany) were designed according to the genomic organization of the human steroid hormone receptor genes [25–28].

Table 1 Primer sequences used for real-time RT–PCR amplification

Target	Forward primer	Reverse primer	Amplicon [bp]
pan ERα	CACATGAGTAACAAAGGCATGG	ATGAAGTAGAGCCCGCAGTG	181
ERα Δ5/6	AGCCCCCGATACTCTATTCC	GTACACTCCTGGCACCCTCT	137
ERα Δ2/3/4	TCTACAGGCTTTGTGGATTTG	AGTAGCTTCCCTGGGTGCTC	119
ERα Δ3/4/5	TATTCAAGGAACCAGGGAAA	TCCTCTCCCTGCAGATTCAT	104
ERα Δ5/6/7	GAGGGTGCCAGTAACAAAGG	ATGAAGTAGAGCCCGCAGTG	184
pan ERβ1	GGCATGCGAGTAACAAGGGC	GGGAGCCCTCTTTGCTTTT	177
pan ERβ2	GTTTGGGTGATTGCCAAGAG	TTCTGCCCTCGCATGC	101
ERβ Δ1-0K	TGGTTCTGAAGAGAGACACTGA	CTTCACACGACCAGACTCCA	151
ERβ Δ1-0N	TATCTGCAAGAGAGACACTGAAAAGG	GGCCTTACATCCTTCACACG	161
ERβ Δ1/2//3-0K	CTGAAGGCTCCCGGAGAGAG	CTCCAGGAGGGTGAGCACTA	176
ERβ Δ1/2/3-0N	CTCGCTTTCCTCAACAGGTG	CGGGAGCCTTGCAGATAAAC	120
pan PR	CAACTACCTGAGGCCGGATT	CATTGCCCTCTTAAAGAAGACCT	160
PR Δ7	CTTCTTAATACAACTTGTCAAACAAC	CACCATCCCTGCCAATATCT	142
PR Δ4/5	TCCTTGGAGACAGCGGATG	GGAACTCTTCTTGGCTAACTTGA	107
PR Δ6/7	ATACTAAATGACTTGTCAAACAACTTC	CACCATCCCTGCCAATATCT	140
PR Δ3/4/5	CAATGGAAGACAGCGGATG	GGAACTCTTCTTGGCTAACTTGA	107

Real-time PCRs were carried out in a LightCycler® 1.0 Instrument (Roche, Mannheim, Germany) under the following conditions: initial denaturation at 95°C for 15 min, followed by 45 cycles with 10 s denaturation at 95°C, 5 s annealing at 60°C, and 12 s extension at 72°C. The PCR program was completed by a standard melting curve analysis. Negative controls were prepared by adding distilled water instead of cDNA. To verify the identity of the PCR products, they were also analyzed by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. After size check, each PCR product was then purified using the "QIAquick Gel Extraction Kit" (Qiagen, Hilden, Germany), following the manufacturer's protocol and verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). In all RT–PCR experiments, a 190 bp β-actin fragment was amplified as reference gene using intron-spanning primers actin-2573 and actin-2876. Data were analyzed using the comparative $\Delta\Delta C_T$ method [29] calculating the difference between the threshold cycle (C_T) values of the target and reference gene of each sample and then comparing the resulting ΔC_T values between different samples.

Results

Identification of novel exon-deleted transcript variations of ERα, ERβ, and PR gene

Though a big number of exon-deleted isoforms of ERα, ERβ, and PR mRNAs has been described earlier, this number was smaller than possible because these genes consist of eight exons theoretically allowing an even larger variety of transcripts with single, double, triple, and multiple exon-deletions. We designed PCR primer sets which would be able to detect such additional undescribed mRNA variations. From each primer set, one of the primers was designed to bind at the borders of two distant exons which would be only adjacent in a specific exon-skipped transcript. We screened total RNA from human endometrium for new steroid hormone receptor splicing variants by means of real-time RT–PCR.

We succeeded in identification of 12 new exon-skipped splicing variants of *ESR1*, *ESR2*, and *PR* gene exhibiting one, two or three exons deletions. After standard melting curve analysis, the resulting PCR products were analyzed by agarose gel electrophoresis, eluted and sequenced to verify their identity. We were able to identify four new double or triple exon-deletion transcript variants of ERα (Δ5/6, Δ2/3/4, Δ3/4/5, Δ5/6/7) (Fig. 1), four single or triple exon-skipped mRNA isoforms of ERβ (Δ1-0K, Δ1-0N, Δ1/2//3-0K, Δ1/2/3-0N) representing exon-deletion mRNAs transcribed from the *ESR2* promoter regions 0N or 0K (Fig. 2), and four new transcript variations of *PR* gene (Δ7, Δ4/5, Δ6/7, Δ3/4/5) (Fig. 3). Sequence analysis of the novel isoforms revealed that only one of them, ERαΔ5/6, allowed in-frame translation of the adjacent 3′-region, whereas in all other variants, exon-deletion led to a translational frameshift affecting the C-terminal region of the receptor variants (Table 2). Thus, the majority of the novel ERα- and PR-transcripts only coded for intact N-terminal receptor domains, whereas the ERβ variants lacking 5′-exons do not code for an intact receptor domain at all.

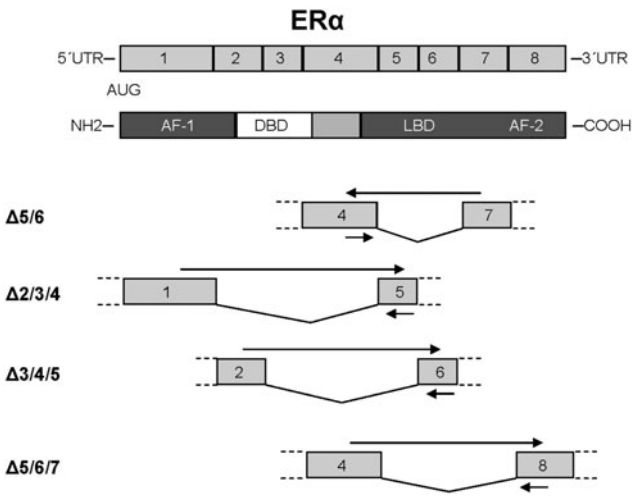

Fig. 1 ER α transcript variations identified in this study. The full-length receptor mRNA consists of eight exons containing an 5′- and 3′-untranslated region (UTR) and codes for a protein with activation function (AF)-1 and 2 domains, a DNA- and a ligand-binding domain (DBD, LBD). *Arrows* indicate position of the PCR primers used for identification of transcript variations

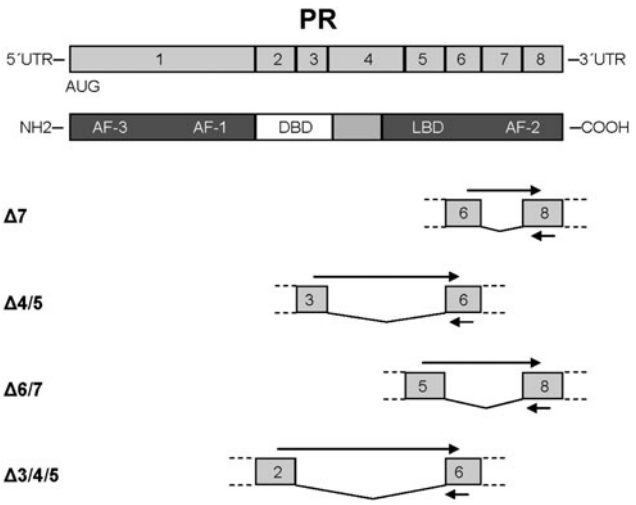

Fig. 3 New PR transcript variations resulting from exon-skipping. The full-length receptor mRNA consists of eight exons and codes for a protein with three activation functions (AF-1, 2, and 3), a DNA- and a ligand-binding domain (DBD, LBD). *Arrows* indicate position of the PCR primers used for identification of transcript variations

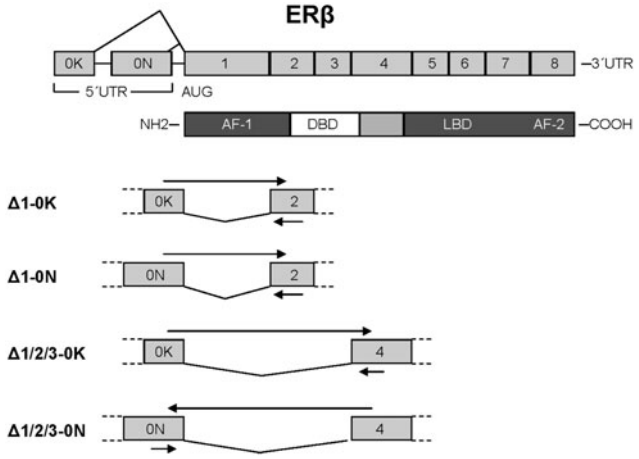

Fig. 2 ERβ mRNA variations reported in this study. The full-length receptor mRNA consists of eight coding exons and alternative untranslated exons 0N and 0K. The full-length ERβ protein exhibits activation function (AF)-1 and 2 domains, a DNA- and a ligand-binding domain (DBD, LBD). *Arrows* indicate position of the PCR primers used for identification of transcript variations

Table 2 Characteristics of the reported exon-deleted variants

Variation	Skipped region (bp)	Affected domains	Frame
ERα			
Δ5/6	273	LBD	In
Δ2/3/4	644	DBD, NLS	Out
Δ3/4/5	592	DBD, NLS, LBD	Out
Δ5/6/7	457	LBD	Out
ERβ			
Δ1-0K	452	AF-1	Out
Δ1-0N	452	AF-1	Out
Δ1/2//3-0K	742	AF-1, DBD	Out
Δ1/2/3-0N	742	AF-1, DBD	Out
PR			
Δ7	158	LBD	Out
Δ4/5	451	NLS, LBD	Out
Δ6/7	289	LBD	Out
Δ3/4/5	568	DBD, NLS, LBD	Out

Differential expression of exon-skipped variants in human pre- and post-menopausal endometrium

We then compared expression of the new ERα, ERβ, and PR mRNA variants in 11 premenopausal and 17 postmenopausal endometrial tissue samples. All 12 novel exon-skipped splicing variants were detected both in pre- and post-menopausal endometrium, with ERα exhibiting the highest mRNA levels, a moderate PR expression and relative low levels of ERβ transcripts (Figs. 4, 5, 6).

Analysis of PR exon-deletion variants generally showed higher mRNA levels of this receptor in premenopausal than in postmenopausal endometrium, but this difference did only reach significance ($P < 0.05$) in case of the PRΔ6/7 isoform (Fig. 4). Using PCR primers binding to the cDNA of all PR transcript isoforms (pan PR), we did not observe a significant difference between both groups. Examining the ERα transcript variants, both ERαΔ5/6/7 and Δ3/4/5 were

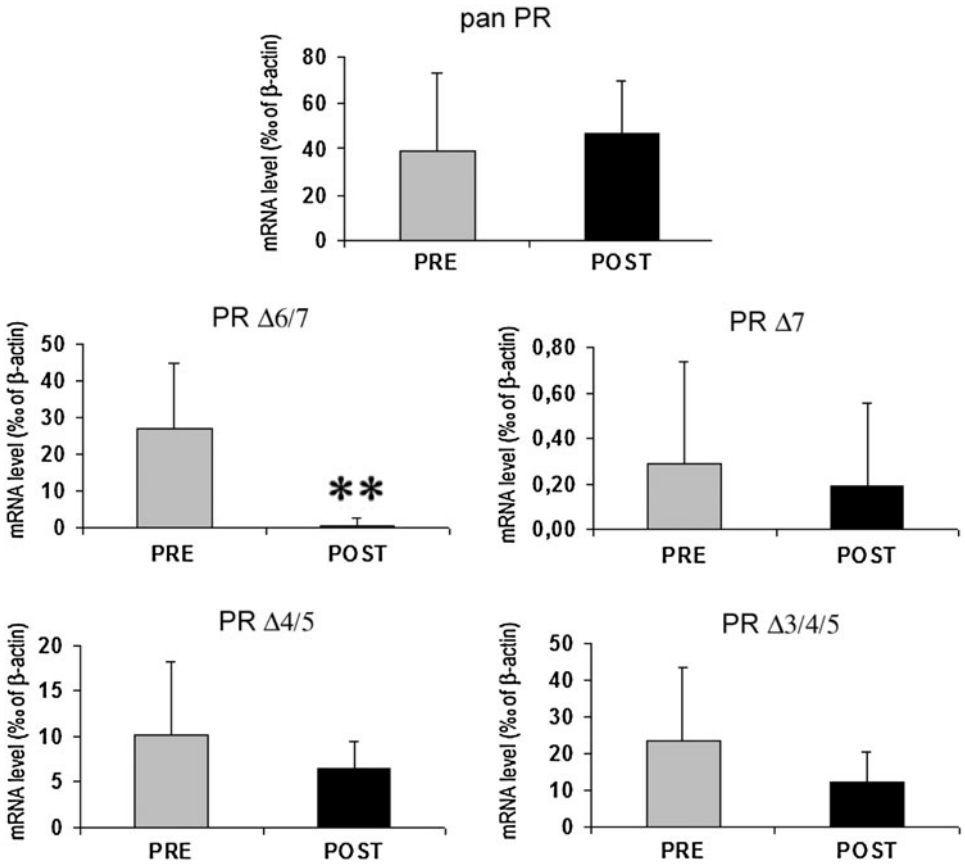

Fig. 4 Expression of transcript variants of PR in human pre- and post-menopausal endometrium as determined by real-time RT–PCR. Only PR isoform Δ6/7 exhibited a significant different expression in both groups (** $P < 0.005$ vs. premenopausal). Total RNA was isolated from 17 premenopausal and 11 postmenopausal endometrium samples. Relative transcript levels are expressed in percent of the corresponding β-actin mRNA level ($n = 3$)

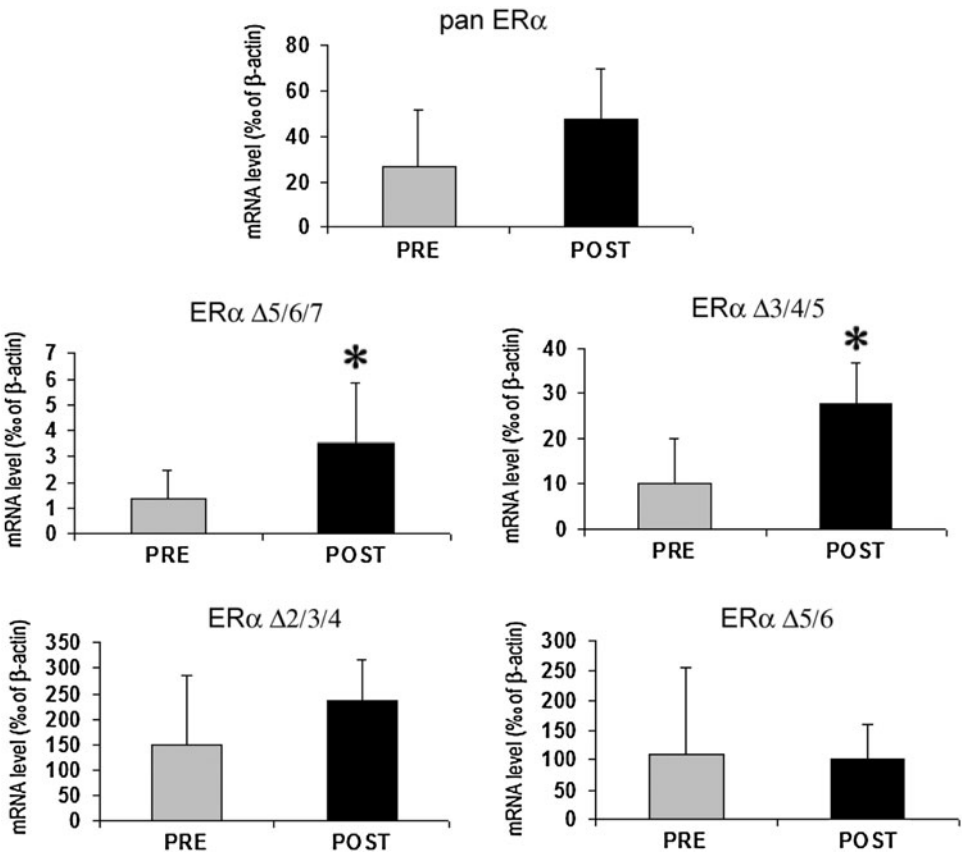

Fig. 5 Expression of transcript variants of ERα in human pre- and post-menopausal endometrium as determined by real-time RT–PCR. Total RNA was isolated from 17 premenopausal and 11 postmenopausal endometrium samples. Relative transcript levels are expressed in percent of the corresponding β-actin mRNA level ($n = 3$).
* $P < 0.05$ vs. premenopausal

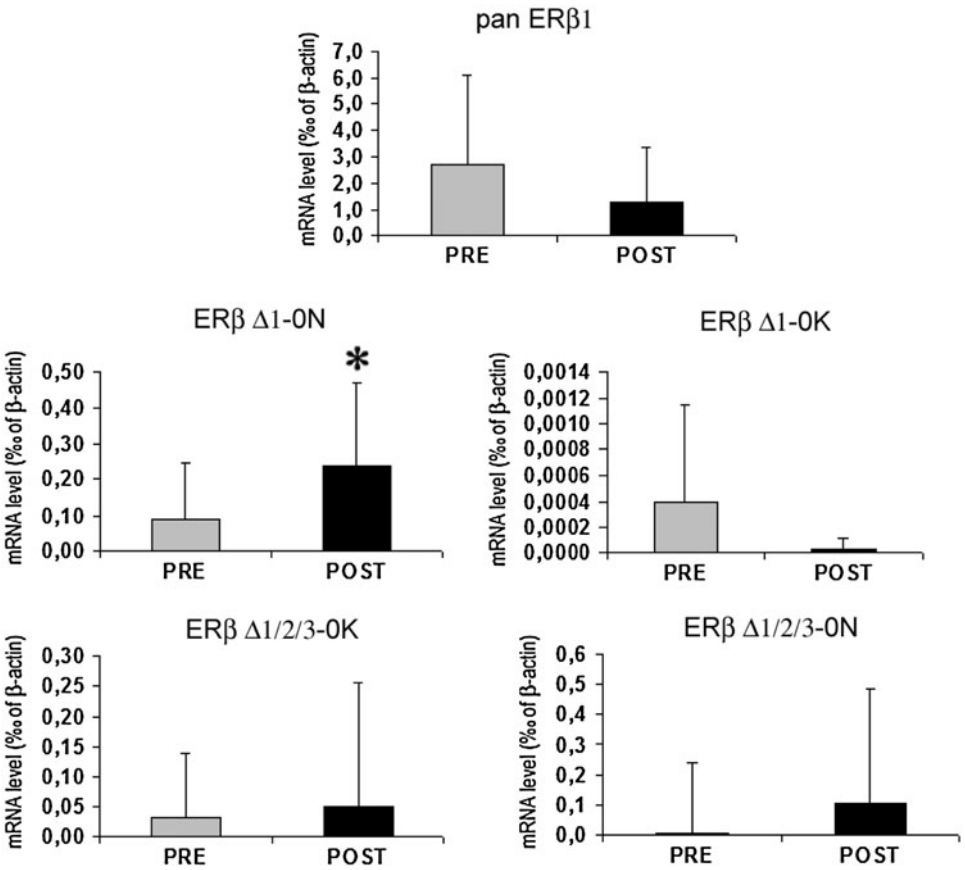

Fig. 6 Expression of transcript variants of ERβ in human pre- and post-menopausal endometrium as determined by real-time RT–PCR. Total RNA was isolated from 17 premenopausal and 11 postmenopausal endometrium samples. Relative transcript levels are expressed in percent of the corresponding β-actin mRNA level ($n = 3$). * $P < 0.05$ vs. premenopausal

significantly stronger expressed in postmenopausal compared to premenopausal endometrium. In contrast, we did not observe a significant difference between post- and premenopausal women after analysis of the other ERα variants or with pan-ERα primers (Fig. 5). The ERβ transcript variant Δ1-ON exhibited a significantly higher expression in postmenopausal than in premenopausal endometrium, whereas we did not observe significant differences between both groups with regard to the other ERβ isoforms tested (Fig. 6). Analysis with PCR primers binding to all ERβ1 cDNAs also did not reveal a significant difference with regard to menopausal status.

Differential expression of exon-skipped variants in human cancer cell lines

Next we analyzed expression of the new ERα, ERβ, and PR mRNA variants in a panel of human cancer cell lines derived from the breast, ovary or endometrium.

ERα transcripts were detected in six of 10 cancer cell lines, with the highest expression in SK-OV-3 cells, moderate mRNA levels in MCF-7 and T47-D cells, and low expression in ZR75-1 and SKBR-3 breast cancer cells. Three cell lines (SK-OV-3, MCF-7, and T47-D) exhibited expression of all novel ERα exon-deletion variants, whereas three other cell lines (ZR75-1, SKBR-3, and NIH-OVCAR-3) showed only partial expression of those variants. Four of the screened cell lines (MDA-MB-231, HEC-1A, HEC-1B, and RL95-2) were completely ERα-negative.

Expression of ERβ1 was detected in all cell lines tested. Whereas SK-OV-3 ovarian cancer cells exhibited high ERβ1 mRNA levels, expression was moderate in HEC-1A and RL95-2 cells, and low in the other endometrial cell line and in all breast cancer cell lines tested. A similar expression pattern was observed for ERβ2. In this study, we identified an ER$\beta\Delta$1 and an ER$\beta\Delta$1/2/3 mRNA variation and characterized the *ESR2* gene promoter (0N or 0K) used for their expression. Whereas both promoters were used for transcription of these exon-skipped variations in normal endometrium, only the 0N promoter was used in the cancer cell lines tested (Table 3). Expression levels of ER$\beta\Delta$1-0N were highest in MDA-MB-231 and T47-D breast cancer cells, moderate in NIH-OVCAR-3 cells, and low in SK-BR-3, ZR75-1, and HEC-1B cells. In contrast, expression of ER$\beta\Delta$1/2/3-0N was only detected in SK-BR-3 and HEC-1A cells. Three cell lines did not express any of the new ERβ transcripts (MCF-7, RL95-2, and SK-OV-3).

Expression of PR was detected in four ERα-positive cancer cell lines, with the highest expression in T47-D cells and only low levels in MCF-7, ZR75-1, and SK-OV-3

Table 3 Relative expression of total estrogen receptor (ER) α, β and progesterone receptor (PR) transcripts and their exon-deleted variations in breast, endometrial, and ovarian cancer cell lines as well as in normal endometrial tissue

	Endometrium	Endometrial cancer			Breast cancer					Ovarian cancer	
		RL95-2	HEC-1B	HEC-1A	MCF-7	MDA-MB-231	SKBR-3	T47-D	ZR75-1	OVCAR-3	SK-OV-3
ERα	✔	−	−	−	++	−	+	++	+	−	+++
Δ5/6	✔	−	−	−	+++	−	+	++	+	+	++
Δ2/3/4	✔	−	−	−	+++	−	+	++	+	+	+++
Δ3/4/5	✔	−	−	−	++	−	−	++	+	+	+++
Δ5/6/7	✔	−	−	−	++	−	−	++	−	−	+++
ERβ1	✔	++	+	++	+	+	+	+	+	+	+++
ERβ2	✔	++	+	+	+	+	−	+	+	+	++
Δ1-0K	✔	−	−	−	−	−	−	−	−	−	−
Δ1-0N	✔	−	+	−	−	+++	+	+++	+	++	−
Δ1/2//3-0K	✔	−	−	−	−	−	−	−	−	−	−
Δ1/2/3-0N	✔	−	−	+++	−	−	++	−	−	−	−
PR	✔	−	−	−	+	−	−	+++	+	−	+
Δ7	✔	−	+	+	+	+	−	+++	−	−	−
Δ4/5	✔	−	−	−	+	−	−	+++	+	+	−
Δ6/7	✔	−	−	−	+	+	−	+++	+	−	+
Δ3/4/5	✔	−	−	−	++	−	−	+++	+	−	−

cells. All four PR exon-deletion variants were strongly expressed in T47-D and weakly expressed in MCF-7 breast cancer cells. Whereas six cell lines expressed at least one of the novel variants, three cell lines were totally negative for PR (MCF-10A, SKBR-3, RL95-2).

Discussion

We report identification of 12 so far undescribed transcript variants of steroid hormone receptor genes *ESR1*, *ESR2*, and *PR* in endometrial tissue which result from exon-skipping. We also report differential expression of these variants in pre- and post-menopausal endometrium and in cancer cell lines from the breast, endometrium, and ovary.

Alternative splicing is the major source of protein diversity for higher eukaryotic organisms and is frequently regulated in a developmental stage-specific or tissue-specific manner [30]. Alternative splicing results from the action of numerous protein factors recognizing specific sequences within immature messenger RNAs [31]. Exon-deleted transcripts of *ESR1*, *ESR2*, and *PR* gene have been previously reported to be expressed not only in cancer, but also in normal tissue. Thus, exon-skipping seems to be a normal feature of steroid hormone receptor expression. Generally, the exon/intron architecture of genes determines splice sites recognition by the spliceosome [32]. The intron size can profoundly influence the likelihood that an exon is constitutively or alternatively spliced. Exon-skipping is more likely to occur when exons are flanked by long introns, which is also often the case in steroid hormone receptor genes [33]. However, misregulation of alternative splicing, resulting in altered expression of splicing variants, has been reported to be implicated in many human diseases like certain types of cancer or neurological disorders.

We succeeded in identification of four new exon-skipped transcripts of *ESR1* gene expressed in human endometrium. Deletion of ERα exons 5 and 6 (Δ5/6) allows in-frame translation of the following exons, such transcript is supposed to code for a receptor protein without intact ligand binding domain (LBD). However, all other essential ER domains like the ligand-independent activation function (AF-1), the DNA-binding domain (DBD), and a nuclear localization signal (NLS) are intact. An ERα protein lacking the LBD would lose its ligand-dependent functions, but could exert a constitutive, AF-1-mediated transcriptional activity [34, 35] like it was reported from ERαΔ5 [14, 36]. This variant was described as predominate ERα-mRNA in ER-negative but PR-positive tumors, suggesting that ERαΔ5 activates estrogen-independent PR gene expression [37, 38].

We also identified the three triple exon-deleted ERα transcript variations Δ2/3/4, Δ3/4/5, and Δ5/6/7, which all result in loss of the correct reading frame. The Δ2/3/4 variation exhibits a stop-codon at the beginning of exon five, the Δ3/4/5 variation has a stop-codon in exon six. Thus, translation of both transcripts would result in short proteins not only lacking the deletion area (DBD, NLS), but also the C-terminal part of the full-length protein. The exon-skipped variant Δ3/4/5 contains a functional AF-1

domain, but loses its ability to dimerize. Thus, it can be supposed that such variants are without any importance in transcription regulation of estrogen-related genes. In contrast, the receptor coded by the Δ5/6/7 variant would only lack the complete C-terminal domain and might, like discussed above, exert similar constitutive actions as ERαΔ5 [36–38].

In addition to these new findings, we compared the expression of these ERα transcripts in pre- and post-menopausal endometrium. Expression of steroid hormone receptors is commonly regulated by their ligands to create autoregulatory feedback loops [39]. Whereas previous studies on ERα expression in human endometrium performed by means of immunohistochemistry (IHC) reported a decline of this receptor in absence of estrogens like in postmenopausal endometrium [40–42], we did not observe a differential expression of total ERα mRNA in pre- and post-menopausal endometrium. This fact could be explained by the different methodological approach of our study, allowing the detection of all ERα transcripts exhibiting exons 7 and 8, partially coding for proteins which are not recognized by standard IHC antibodies. However, PCR primers specifically amplifying the ERα transcript variants Δ5/6/7 and Δ3/4/5 revealed a significantly higher expression of these mRNAs in postmenopausal endometrium. Interestingly, both variants code for proteins not being able to bind estrogens, suggesting that their expression might be hormone-independent because they cannot mediate autocrine regulation.

In contrast to pre- and post-menopausal endometrium, neither total ERα nor the exon-skipped splicing variants were expressed in the endometrial cancer cell lines tested; supporting previous observations that loss of ERα expression is a frequent step in endometrial carcinogenesis [43]. In the breast and ovarian cancer cell lines previously described to be ERα-positive and estrogen-responsive [44, 45], the novel exon-skipped ERα transcripts were also expressed, suggesting that they are not subject to a specific regulation in these cancer cells.

We also report identification of four novel ERβ transcripts with single or triple exon-skips. ERβ gene expression is regulated in a tissue-specific manner by multiple untranslated first exons and promoter systems (e.g., 0N and 0K) [6]. Thus, we examined which of these alternative 5′-untranslated regions were used for synthesis of the new transcripts. In human endometrium we identified the exon-skipped variants ERβΔ1 and ERβΔ1/2/3 which exhibited an untranslated 5′- 0K- or a 0N-exon. In contrast, we could only detect transcripts generated from the 0N promoter in the breast, endometrial, and ovarian cancer cell lines tested, encouraging further studies on ERβ promoter usage in cancer.

Exon-skipped transcripts ERβΔ1-0N and -0K identified in this study both are out-of-frame deletion variants. The translation initiation codon presumably is the first ATG triplet in exon 2, leading to a protein without functional domains. The new variations ERβΔ1/2/3-0N and -0K also exhibit a frame-shift leading to a non-functional receptor. As translation initiation codon, the first ATG triplet in exon 4 could be used. However, given that neither exon 2 nor exon 4 contains a consensus Kozak sequence, which would facilitate the initial mRNA binding to the small subunit of the ribosome and thus determines the efficiency of translation, both the Δ1 and the Δ1/2/3 transcript would only be translated at low levels [46].

In pre- and post-menopausal endometrium we could not detect a significant difference in the expression neither of total ERβ nor the exon-skipped transcripts, except ERβΔ1-0N. The ERβΔ1 transcript generated from the 0N promoter showed a significantly higher expression in postmenopausal than in premenopausal endometrium, which is in contrast to single IHC-based studies reporting a decline of endometrial ERβ expression after menopause [40]. This finding again could be explained by the different methodological approach of our study detecting all ERβ transcripts containing exons 7 and 8, which is more than 95% of all ERβ transcripts. It is noticeable that the transcripts of ERβ1 and β2 were expressed nearly in all cancer cell lines tested (with a low expression level in breast cancer cell lines), but the new exon-skipped transcripts were rarely expressed in these cell lines. Given that they were expressed in normal endometrium, our findings support the hypothesis of ERβ being a tumor suppressor, but suggest that particularly reduced expression of splicing variants might be associated with cellular transformation [47, 48].

In this study, we also identified new PR transcripts, lacking one, two or three exons. All of these splicing variants exhibit a translational frameshift. Two transcripts, lacking exon 7 or exons 6/7, exhibit premature stop-codons in exon 8 and code for proteins without functional LBD but containing domains such as AF-3, AF-1, DBD, and NLS. Such proteins could possibly influence progesterone action in a hormone-independent manner or by formation of heterodimers with the full-length receptor. Transcript PRΔ4/5 is suggested to code for a truncated amino-terminal receptor due to the fact that it exhibits a stop-codon in exon 6, but the deduced protein still contains the AF-3 and AF-1 domain as well as the DBD. Given that the truncation notably would reduce the ability of this protein to dimerize, it is unlikely that it could affect progesterone signaling by heterodimerization with functional PR proteins. Considering that the exon-deleted PR mRNA Δ3/4/5 additionally lacks the region coding for the second DBD zinc finger, it is reasonable to hypothesize, that such a translated protein

is also without any function mediating progesterone signals.

We could detect all described PR transcripts both in human pre- and post-menopausal endometrium, and expression of PR transcript variant Δ6/7 was significantly higher in premenopausal women. This is in line with previous studies reporting a decline of PR expression in postmenopausal endometrium [40]. In breast cancer cell lines we could detect broad expression of exon-skipped PR transcripts, whereas in some ovarian and endometrial cancer cell lines single PR splicing variants were expressed. Because total ERα and the examined ERα splice variants were missing in endometrial cancer cells, the expression of these PR exon-skipped variants might be activated by other ERα isoforms not being examined in this study or might be regulated by ERβ. Compared to normal endometrium, where all PR variants could be detected, the low number of PR transcripts observed in endometrial cancer cells is in line with previous reports on loss of receptor expression during endometrial tumorigenesis.

In summary, we identified 12 new transcript variants of the *ESR1*, *ESR2*, and *PR* gene in human endometrium. Comparison of pre- and post-menopausal endometrium revealed differential expression of PRΔ6/7, ERαΔ5/6/7, ERαΔ3/4/5, and ERβΔ1-0N. We also report differential expression of the exon-skipped isoforms in human cancer cell lines derived from the breast, ovary, and endometrium. However, a limitation of this study is the fact that we were not able to demonstrate expression of the at least four expected corresponding proteins, because no specific antibodies are available. Our report increases the complexity of steroid hormone receptor gene expression and signalling, but further studies are required to clarify the biological roles of splicing variants in human endometrium.

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