Estrogen receptor β transcript variants associate with oncogene expression in endometrial cancer

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Abstract. The human ESR2 gene codes for estrogen receptor β1 and for multiple splice variants, which are suggested to exert distinct functions in the cellular estrogen response. Given that the function of ERβ in endometrial cancer remains unclear, we examined the expression of ERβ1, ERβ2 and various further ERβ transcript variants and their association with selected cancer-related genes in 74 human endometrium samples and endometrial cancer specimens by means of RT-qPCR. Additionally, we knocked down ERβ expression in HEC-1A endometrial adenocarcinoma cells by means of siRNA transfection. Expression of four ERβ transcript variants was significantly elevated in cancer tissue or in G3 tumors compared to postmenopausal endometrium. Expression of ERβ1, ERβ2, ERβ5 and five further variants was associated with the oncogenes MYBL2 or HER2 in endometrial cancer. In addition, siRNA-triggered knockdown of ERβ expression led to a significant decline of MYBL2 mRNA and protein levels in endometrial cancer cells. Our observation of increased ERβ transcript levels in cancer tissue and particularly their correlation with the expression of oncogenes, as well as the results of our knockdown studies, suggest a role of ERβ in endometrial carcinogenesis.

Introduction

Endometrial cancer is known to be a hormone-dependent neoplasia which is caused by a stepwise accumulation of molecular alterations in cellular pathways regarding proliferation and differentiation. Expression of steroid hormone receptors like estrogen receptors (ER) α and β and progesterone receptor (PR) plays an important role in regulation of normal endometrial function. Changes of the ER and PR expression patterns may play an important role in the pathogenesis of endometrial cancer. Loss of steroid receptors during tumorigenesis is often associated with an aggressive clinical course and a poorer survival in endometrial cancer patients. Whereas ERα is thought to primarily mediate the proliferative effect of estrogens in endometrial tissue, the function of ERβ in this tissue remains unclear. In other hormone-dependent tissues like the breast, ERβ is known to exert antagonistic effects on ERα action, resulting for example in reduction of cellular proliferation. The growth inhibitory action of ERβ and the observed decline of ERβ expression during carcinogenesis have raised the hypothesis that this receptor may act as a tumor suppressor in hormone-dependent tissues like the breast or prostate (1-3).

The ERβ gene (ESR2) codes for a multitude of different transcripts resulting from alternative splicing. The ERβ splice variants 1, 2 and 5 are characterized by a specific C-terminal region coded by ESR2 exon 8, whereas other ERβ splice variants result from exon skipping. Previous studies clearly suggested that ERβ variants differ in their ability to bind to ligands, DNA or cofactors, and thus exert distinct functions and have a specific significance in hormone-dependent cancer (4,5).

Recent studies have revealed that molecular alterations such as oncogene activation and tumor suppressor inactivation are characteristic steps during endometrial carcinogenesis. Loss or mutation of tumor suppressor PTEN is seen in up to 80% of endometrioid tumors, whereas overexpression of HER2 receptor tyrosine kinase is present in about 60% of endometrial adenocarcinoma (6). Another molecular alteration in endometrial cancer is overexpression of growth promoting genes like cyclins, which is associated with an undifferentiated phenotype and an unfavorable prognosis (7).

In the present study, we further evaluated the function of ERβ, represented by its most frequent variants ERβ1, 2 and 5 and various exon-skipped isoforms, in endometrial cancer. For this purpose, we compared expression of these ERβ variants and of two oncogenes in normal and malignant tissues. Because no antibodies were available for the 15 exon-skipped ERβ isoforms tested, this study had to be performed at the mRNA level by means of RT-qPCR.

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Patients and methods

Patients. In this study, we examined a total of 74 endometrial samples including 28 samples from normal endometrium and 46 cases of endometrioid endometrial carcinoma. The 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 11 premenopausal patients, aged 43-53 years, 5 were in the proliferative phase and 6 in the secretory phase of the menstrual cycle. The 17 postmenopausal women were aged between 46 and 90 years. Normal endometrial tissue was obtained from women subjected to surgery for reasons other than pathology of the endometrium, mainly cervical cancer. The group of endometrial cancer patients (endometrioid subtype), ranging from 54 to 82 years of age, underwent curative resection and could be divided into grading subgroups (G1, 15 cases; G2, 16 cases; G3, 15 cases). Informed consent for their participation in the studies was obtained from all patients. Tissue samples of patients were collected in accordance with German or Polish regulations and in agreement with the Ethics 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.

Materials. DMEM/F12 culture medium was obtained from Invitrogen (Karlsruhe, Germany) and FCS was purchased from PAA (Pasching, Austria). HEC-1A endometrial adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). M-MLV-P reverse transcriptase was purchased from Promega (Mannheim, Germany). RNeasy mini kit, RNase-free DNase Set and the QuantiTect SYBR-Green PCR kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). The transfectin reagent was obtained from Bio-Rad (Hercules, CA, USA). Platinum Pfx polymerase and OptiMEM medium were purchased from Invitrogen. ERβ siRNAs were from Ambion (Austin, TX, USA). Serum replacement 2 (SR2) was from Sigma (Deisenhofen, Germany).

Cell culture and cell proliferation assay. HEC-1A and HEC-1B endometrial adenocarcinoma cells obtained from the American Type Culture Collection were maintained in phenol red-free DMEM/F12 medium supplemented with 10% FCS or with 1X serum replacement 2 (SR2, Sigma). Cells were cultured with 5% CO_2 at 37˚C in a humidified incubator. Cells were seeded in 96-well plates (MDA-MB-231 200 cells/well; BT-474 800 cells/well; SK-BR-3 800 cells/well). The next day cells were treated with the GPR30 agonist G-1 (10, 100 nM or 1 μM), herceptin (10 μg/ml) or the combinations of both agents in triplicate. On Days 0, 3 and 5 relative numbers of viable cells were measured in comparison to solvent controls by means of the fluorimetric, resazurin-based CellTiter-Blue assay (Promega) according to the manufacturer's instructions at 560Ex/590Em nm in a Victor3 multilabel counter (Perkin-Elmer, Germany). Cell growth was expressed either as percentage of Day 0 or as percentage of the solvent controls. For statistical analysis we performed a two-way ANOVA analysis and Bonferroni post-tests, using the GraphPad Prism Version 5.01 Software (GraphPad Software, San Diego, CA, USA). We considered data as significant at $P<0.05$.

Transient transfection assay. HEC-1A cells ($4x10^5$ per well) were seeded in DMEM/F12 containing 10% FCS in a 6-well dish. After 24 h, cells were transfected with 300 nM siRNA in OptiMEM reduced serum medium using 8 μl of Transfectin reagent (Bio-Rad). For knockdown of ERβ expression, we used an equimolar mixture of three different pre-designed Silencer siRNAs (#4148, #202031 and #145911; Ambion) targeting different regions of ERβ mRNA. The siRNA sense sequences (5'→3') were GGAAUUCUGGAAAUCUUUGtt, GCAUUCAAGGACAUAAUGAtt and CCUUACCUGUA AACAGAGAtt, respectively. As a negative control siRNA verified to not interact with any human RNA, the Silencer Negative Control #1 siRNA (AM4611, Ambion) was used. At 3-4 days after siRNA treatment, cells were harvested and total-RNA was isolated.

RNA preparation and real-time RT-PCR. Total-RNA from endometrial tissue samples was isolated from 30 to 80 mg frozen tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA purity and concentration was analyzed by spectrophotometry. From each sample, 300 ng of total-RNA was reverse transcribed to cDNA using 40 units of M-MLV reverse transcriptase and RNasin (Promega) with 80 ng/μl random hexamer primers (Invitrogen) and 10 mM dNTP mixture (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. After reverse transcription, the mRNA levels of ERβ variants and marker genes were determined by real-time PCR. For this purpose, 4 μl of cDNA were amplified using the LightCycler® FastStart DNA Master^{PLUS} SYBR-Green I (Roche Diagnostics GmbH, Mannheim, Germany) and 5 mM of each primer (Table I). Oligonucleotides (Metabion, Planegg-Martinsried, Germany) were designed to be intron-spanning to avoid genomic contaminations.

Real-time PCRs were carried out in a LightCycler® 1.0 Instrument (Roche) under the following conditions: initial denaturation at 95˚C for 15 min, followed by 45 cycles with 10 sec denaturation at 95˚C, 5 sec annealing at 60˚C and 12 sec 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), following the manufacturer's protocol and verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Generally, PCR primers were designed to be intron-spanning. In all RT-PCR experiments, a 190 bp β-actin fragment was amplified as a reference gene using the intron-spanning primers actin-2573 and actin-2876. Data were analyzed using the comparative $\Delta\Delta C_T$ method (8) 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.

Table I. PCR primer sequences used for qPCR.

Target/splice variant	Primer (5'→3')	Amplicon (bp)
ERβ1	GGCATGCGAGTAACAAGGGC GGGAGCCCTCTTTGCTTTT	177
ERβ2	GTTTGGGTGATTGCCAAGAG TCTGCCCTCGCATG	101
ERβ5	GTTTGGGTGATTGCCAAGAG CGTACCTCGCATGCCTGA	146
ERβΔ1	TGGTTCTGAAGAGAGACACTGA CTTCACACGACCAGACTCCA	151
ERβΔ2	ACCACAAGCCCAAATGTGTT ATCATTATGTCCTGTTTACAGGTAAG	157
ERβΔ3	TCAAGGCTCCCGGAGAGAG CAGGAGGGTGAGCACTAGC	172
ERβΔ4	TTTGTCCAGCTACAAATCAGTG TCCACAAAGCCACACTTCAC	112
ERβΔ6	TTCTGGACAGGTATGTACCCTCTG GGGACAGGAGCATCAGGAG	175
ERβΔ1/2 (0K)	AATATGGTTCTGAAGGACATAATGA CTTCACCATTCCCACTTCGT	128
ERβΔ1/2 (0N)	CTCGCTTTCCTCAACAGGTG CATTATGTCCTTGCAGATAAACAC	122
ERβΔ2/3	AAACAGGCTCCCGGAGAGAG CTCCAGGAGGGTGAGCACTA	176
ERβΔ3/4	AGCATTCAAGGCTTTGTGGA CCAGAACAAGATCTGGAGCA	145
ERβΔ4/5	AAGTGTGGGATGAGGGGAAA GATCATGGCCTTGACACAGA	128
ERβΔ1/2/3 (0K)	CTGAAGGCTCCCGGAGAGAG CTCCAGGAGGGTGAGCACTA	176
ERβΔ1/2/3 (0N)	CTCGCTTTCCTCAACAGGTG CGGGAGCCTTGCAGATAAAC	120
ERβΔ3/4/5	ATTCAAGGGATGAGGGGAAA GATCATGGCCTTGACACAGA	128
ERβΔ4/5/6	AAGTGTGGTATGTACCCTCTGG GACAGGAGCATCAGGAGGTT	170
ERα	CACATGAGTAACAAAGGCATGG ATGAAGTAGAGCCCGCAGTG	181
PR	CAACTACCTGAGGCCGGATT CATTGCCCTCTTAAAGAAGACCT	160
MYBL2	GGAAGACCAAAAAGTCATCGAG CTCAGGGTTGAGGTGGTTGT	130
HER2	CATGGTCAAATGTTGGATGATT CCTCATTCTGGATGACCACA	116

Antibodies and western blot analysis. HEC-1A endometrial adenocarcinoma cells were lysed in RIPA buffer [(1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS) in phosphate-buffered solution (PBS) containing aprotonin and sodium orthovanadate]. Aliquots containing 15 μg of protein were resolved by 10% (w/v) SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to a PVDF Hybond (Amersham, Buckinghamshire, UK) membrane. Immunodetection was carried out using monoclonal MYBL2 (M02) antibody (clone

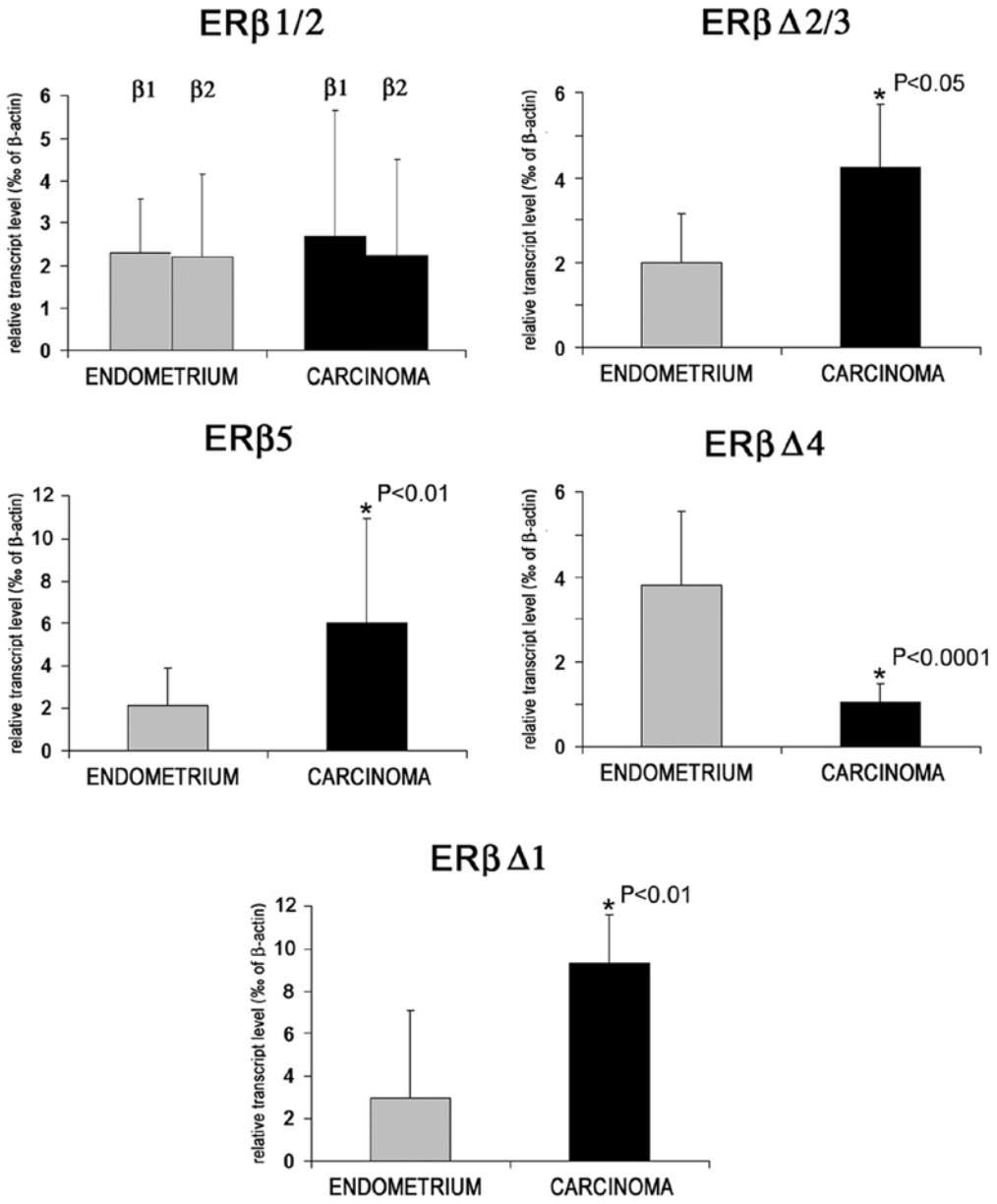

Figure 1. ERβ expression in postmenopausal endometrium and in endometrioid endometrial carcinoma as assessed by RT-qPCR-based relative quantification of the indicated ERβ splice variants. *Significant differences vs. the postmenopausal endometrium.

1C7, antikörper-online.de, Germany) or β-actin antibody (8226, Abcam, Germany) diluted 1:5,000 in PBS containing 5% skim milk (w/v) followed by horseradish peroxidase-conjugated secondary antibody which was detected using a chemiluminescence (ECL) system (Amersham).

Statistical analysis. Statistical analysis of the data was carried out using the InStat software (GraphPad Software). The statistical significance of the molecular gene expression changes in the endometrial carcinoma and the normal endometrium control group was assessed using the nonparametric Mann-Whitney test. Furthermore, we compared the gene expression of separate subgroups (G1, G2, G3) with the nonparametric Mann-Whitney test. The Spearman's rank correlation was used to evaluate the correlation between the expressions of two target genes. P-values <0.05 were considered statistically significant.

Results

Differential gene expression in human endometrium and endometrial cancer. We analyzed expression of ERβ1, ERβ2, ERβ5, the further exon-skipped ERβ-isoforms, ERα, PR, and the cancer-related genes MYBL2, PTEN, Cyclin A2, Cyclin B1, Cyclin D1 and HER2 in a total of 74 endometrial tissue samples by means of RT-qPCR.

Transcript levels of ERβ1 and 2 did not differ between the cancer- and the control group (Fig. 1) or between the grading subgroups (data not shown). Expression of ERβ5 was about 3-fold elevated in endometrioid endometrial cancer, particularly in the G3 subgroup when compared to the postmenopausal endometrium (P<0.01) (Fig. 2). The exon-skipped splice variant ERβΔ1, transcribed from the 0N promoter of ESR2 gene, also was 3-fold elevated in endometrial cancer (P<0.01) (Fig. 1), particularly in the G1 and G2 subgroup

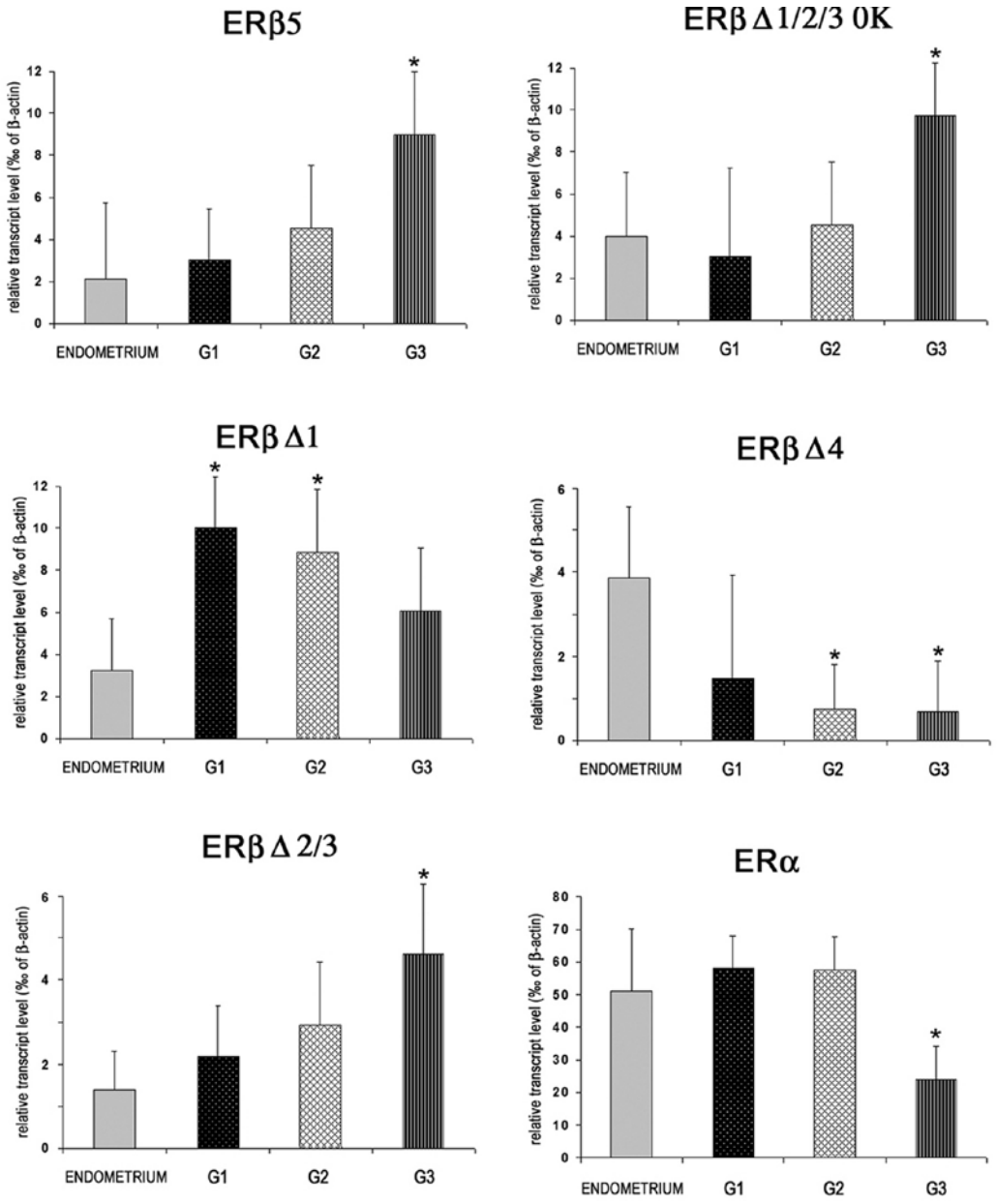

Figure 2. Expression of the indicated estrogen receptor transcripts in postmenopausal endometrium and in G1, G2 and G3 graded endometrial carcinoma. Data were assessed at the mRNA level by means of RT-qPCR. *P<0.01 vs. postmenopausal endometrium.

(Fig. 2). Increased transcript levels in cancer tissue were also detected analyzing the exon-skipped isoform ERβΔ2/3 (P<0.05) (Fig. 1).

In contrast, mRNA levels of the exon-deletion variant ERβΔ4 were significantly, about 3-fold decreased in endometrial cancer tissue (P<0.0001). Particularly the G2 and G3 subgroup exhibited a lower expression of this variant when compared to postmenopausal endometrium (both P<0.001). Expression of the other ERβ splice variants tested did not significantly differ between the compared groups or subgroups.

When we analyzed ERα transcript levels for comparison, we did not observe a difference between the cancer and the control group, but a significantly smaller expression was noted in the G3 subgroup compared to the normal endometrium (P<0.01) or the G1 (P<0.001) or G2 tumors (P<0.05).

Analysis of the ERβ/ERα ratio revealed a significant increase in G3 tumors when compared to the postmenopausal endometrium. This was true for the ratios ERβ1/ERα, ERβ2/ERα (both P<0.01) and particularly for the ERβ5/ERα ratio (P<0.001) (Fig. 3).

Our data also revealed differences in the expression patterns of cancer-related genes. Expression of the oncogene MYBL2 was about 4.5-fold higher in endometrial cancer than in pre- or postmenopausal endometrium (P<0.0001) (Fig. 4). We observed this significant increase of MYBL2 expression in all grading subgroups (P<0.0001). The cell cycle gene cyclin B1 exhibited a 3.9-fold elevated expression in endometrial cancer tissue compared to normal endometrium (P<0.001) (Fig. 4), particularly in G2 tumors (P<0.01) and G3 tumors (P<0.0001). Cyclin D1 was 4-fold elevated in the cancer group (P<0.001), whereas cyclin A2 mRNA levels were doubled in endometrial

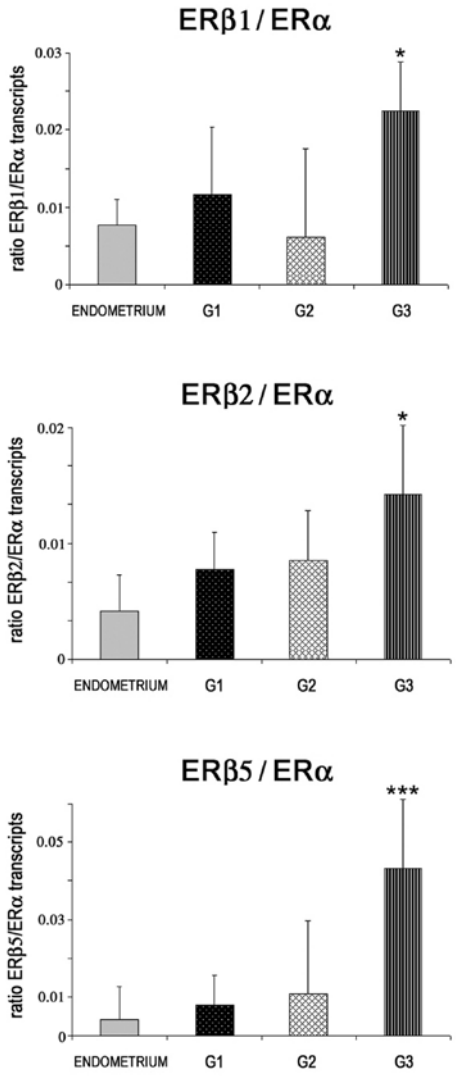

Figure 3. Ratio between expression of ERβ splice variants 1, 2 or 5 and ERα transcripts in postmenopausal endometrium and in G1, G2 and G3 graded endometrial carcinoma as assessed per means of *RT-qPCR*. *P<0.01 vs. postmenopausal endometrium; ***P<0.001 vs. postmenopausal endometrium.

tumors (P<0.001) (Fig. 4) and particularly were elevated in the G3 subgroup (data not shown). As expected, expression of the tumor suppressor PTEN was reduced in endometrial cancer tissue (P<0.05) (Fig. 4), however, loss of PTEN expression did not differ between the grading subgroups (data not shown). In comparison to postmenopausal endometrium, slightly higher HER2 transcript levels were detected in the cancer group (P<0.05) (Fig. 4).

Association of ERβ1 and its splice variants with oncogenes and cancer related genes. To examine the potential significance of ERβ expression in endometrioid endometrial cancer, we first correlated the levels of ERβ1 and its variants with expression of ERα, PR and other cancer-related genes and oncogenes using the nonparametric Spearman test.

First, this test showed the expected strong positive correlation of ERa transcript levels with PR expression (rho=0.55, P<0.0001). Expression of ERβ1 and ERβ2 was strongly associated (rho=0.79) and also correlated with transcript levels of ERα (rho=0.38), but not PR. In contrast, ERβ5 expression was

Table II. Correlation of ERβ splice variant expression with the indicated genes in endometrial cancer, as indicated by the Spearman's rank correlation coefficient (rho), corrected for ties.

	MYBL2	HER2
ERβ1	-	0.310[a]
ERβ2	-	0.308[a]
ERβ5	0.350[a]	-
ERβΔ1	0.415[a]	0.337[a]
ERβΔ3	0.500[a]	0.501[a]
ERβΔ2/3	0.536[a]	0.477[a]
ERβΔ1/2/3 (0K)	0.488[a]	0.431[a]
ERβΔ1/2/3 (0N)	0.411[a]	0.400[a]

[a]Considered to be significant with P<0.001 to P<0.0001 (multiple comparison testing). -, no significant correlation observed; 0N, transcribed from the 0N promoter of the ESR2 gene; 0K, transcribed from the 0K promoter of the ESR2 gene; ERβΔ, splice variant with the indicated exon deletion but undefined exon 8.

weakly associated with ERβ2 expression (rho=0.36), but not with ERα or ERβ1 (data not shown).

Transcript levels of ERβ1 and 2 were found to be associated with the expression of the HER2 oncogene at the mRNA level. Additionally, 5 exon-deleted ERβ variants were observed to positively correlate with HER2 transcript levels (Table II). The expression of ERβ5 mRNA and of five exon-skipped variants was associated with the transcript levels of the oncogene MYBL2 (Table II).

Expression of ERβΔ1, Δ3 and Δ2/3 variants was associated with cyclin D1 mRNA levels, whereas ERβΔ3, Δ2/3 and Δ1/2/3 (0K, transcribed from the 0K promoter of ESR2 gene), positively correlated with cyclin B1 expression (P<0.01, data not shown).

Knockdown of ERβ expression in HEC-1A endometrial adenocarcinoma cells. To examine the functional significance of the observed correlation between ERβ and expression of oncogenes, we then knocked down expression of ERβ by means of transient siRNA transfection in the endometrial adenocarcinoma cell line HEC-1A. Three days after transfection with ERβ-specific siRNAs, a maximum suppression of total ERβ transcript levels by about 72% was observed. In these cells, we also observed downregulation of MYBL2 mRNA by about 60%. When we performed an additional western blot analysis of MYBL2 protein expression, we also observed downregulation of this gene in HEC-1A cells treated with ERβ siRNA (Fig. 5). In contrast, HER2 expression was not altered after knockdown of ERβ, at the mRNA or at the protein level (data not shown).

Discussion

Estrogens have been shown to play a significant role in endometrial physiology and tumorigenesis (9). Both ERs are expressed in normal endometrium, but levels of ERβ have been reported to be lower than those of ERα (10-12).

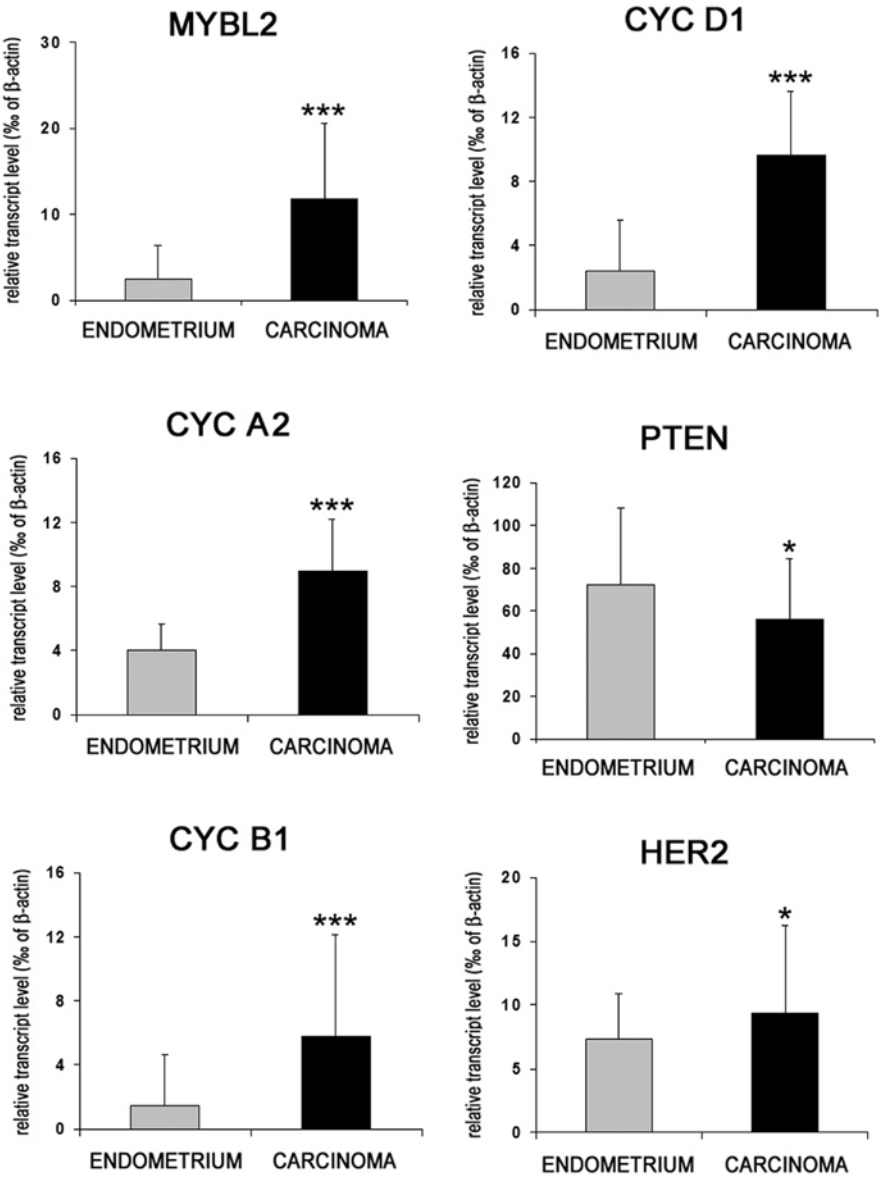

Figure 4. Expression of the indicated genes in postmenopausal endometrium and in endometrial carcinoma. Data were assessed at the mRNA level by means of RT-qPCR. *P<0.05 and ***P<0.001 vs. postmenopausal endometrium.

ERα is thought to primarily mediate the proliferative effect of estrogens both in cyclic endometrium and in pathogenesis of endometrial proliferative diseases, but the function of ERβ in this tissue is not quite clear (13). Particularly the role of ERβ, coded by the human ESR2 gene, in endometrial carcinogenesis remains nebulous. The ESR2 gene codes for the estrogen receptor β1 and for multiple splice variants resulting from alternative exon usage or exon skipping, which are able to exert different functions in the cellular estrogen response (1,5,14). Thus, to elucidate the whole picture of ERβ action in endometrial cancer, we decided to examine expression of all relevant ERβ transcript variants.

Estrogen receptor β previously has been suggested to act as a tumor suppressor in endocrine responsive tissues like the breast and prostate (14-16). This hypothesis primarily arose from the observed decline of ERβ expression in cancer tissue and from *in vitro* data demonstrating a growth-inhibitory action of this receptor (17-19). Whereas some studies supported the idea that ERβ might also act as a tumor-suppressor in the endometrium, primarily due to observation of decreased receptor levels in cancer tissue (20-22), other studies did not (22-26).

The results of our study do not suggest the presence of an antitumoral effect of ERβ1 or of its splice variants in human endometrium. Transcript levels of 18 ERβ variants tested were found to be unchanged or even elevated in endometroid endometrial carcinoma. From these ERβ variants, four were significantly overexpressed in endometrial cancer or in G3 tumors. Eight ERβ isoforms were positively correlated with the expression of the oncogenes MYBL2 or HER2 and six associated with the cyclins B1 or D1. Supporting the results of our correlation study, and establishing a direct functional connection between ERβ and MYBL2, knockdown of ERβ in HEC-1A endometrial adenocarcinoma cells by means of RNAi led to downregulation of MYBL2 in this cell line. Only one ERβ isoform, ERβΔ4, was decreased in cancer tissue. Though being limited by the fact that we had to perform this study at

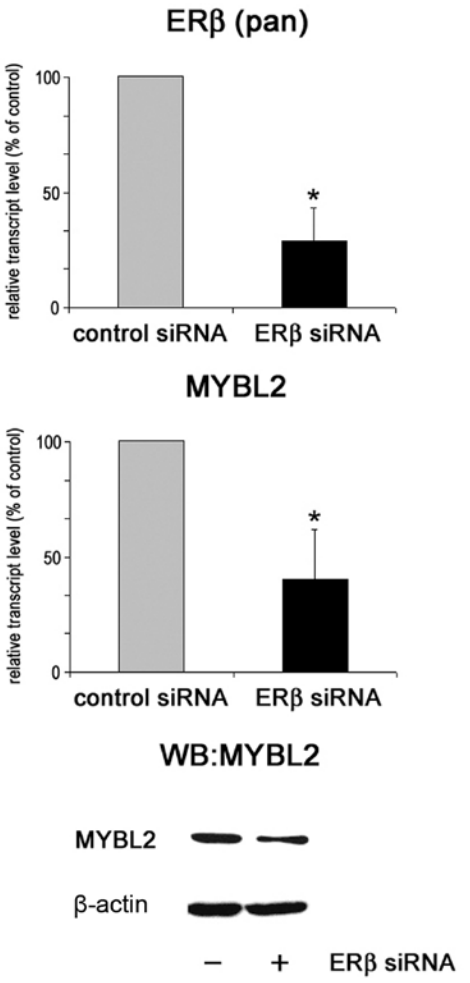

Figure 5. RNAi-mediated knockdown of ERβ expression in HEC-1A endometrial adenocarcinoma cells decreases MYBL2 expression both at the mRNA and the protein level. Data were obtained by means of RT-qPCR (upper panels) or by western blot analysis as described in Materials and methods.

the mRNA level because no specific antibodies existed for the 15 exon-deleted ERβ splice variant tested, the results of our study clearly suggest that ERβ and most of its splice variants do not act as tumor suppressors in human endometrium, but may have tumor-promoting properties.

ERβ1, 2 and 5 are known to be differentially expressed in hormone-dependent cancer (1-4). Whereas ERβ1 expression has been described to decline in breast and prostate cancer tissue, this effect seems not to be present in endometrial carcinoma (27-29). In line with previous studies, we did not observe altered ERβ1 transcript levels in endometrial cancer tissue (30,31). ERβ1 was also previously found not to be associated with the depth of myometrial invasion (31). With regard to ERβ2, some reports demonstrated a decline of this variant in endometrial cancer (31), whereas others found this receptor variant not to be altered in endometroid cancer tissue (30). Our results are in line with the latter report and these findings may be supported by the fact that, in contrast to ERβ5, transcript levels of ERβ2 were not associated with proliferation markers. However, we found an unexpected, significant association of ERβ1, ERβ2 and 7 exon-skipped variants with HER2 receptor tyrosine kinase. Increased HER2 levels in breast and ovarian cancer as well as in different other malignancies have been reported previously (32) and are associated with accelerated tumor progression (32). Accordingly, we detected an overexpression of HER2 in endometrial cancer tissue compared to normal endometrial tissue. Given that expression of HER2 is associated with an aggressive tumor phenotype (33,34), its association with ERβ1, ERβ2 and 7 further variants might be another argument against a protective role of ERβ in endometrioid cancer (35). Our observation is in accordance with previous studies reporting association of ERβ1 with HER2 in breast cancer (36-38).

However, in other studies, expression of ERβ5 was reported to be unchanged or to be elevated in endometrial cancer tissue (30,39). Our study observing elevated ERβ5 mRNA levels in endometrial cancer, particularly in G3 tumors, is in line with the latter study and with reports from breast and prostate tissue (28,30,40). The demonstrated association of ERβ5 and 5 exon-skipped isoforms with the oncogene and cell cycle promoter MYBL2 (B-MYB) suggests that these ERβ variants may be involved in tumor growth. The results of our ERβ knockdown study employing the endometrial adenocarcinoma cell line HEC-1A not only corroborate these correlation data, but clearly suggest that MYBL2 transcript levels may be regulated by ERβ variants in this cell line. MYBL2 is a transcription factor of the MYB family, which is amplified and overexpressed in many tumor types like breast or prostate cancer (41,42). It is proposed that MYBL2 is involved in controlling cell proliferation and differentiation (43-46). Our data showing increased levels of the MYBL2 gene in premenopausal tissue support the relevance of this gene in endometrial proliferation.

Transcript levels of 6 exon-skipped ERβ variants were positively correlated with cyclin expression, again suggesting that ERβ isoforms are associated with proliferation of endometrial cancer. Upregulated expression of cyclin B1 (47), cyclin D1 (48-54) and cyclin A2 (55) in endometrial carcinomas as well as various other cancer types have been previously reported (56). Furthermore, cyclin B1, D1 and A2 have been found to be associated with high levels of Ki-67, indicating their implication in cell proliferation and progression of endometrial cancer (57-59).

Our data suggest a role of ERβ and its splice variants in endometrioid endometrial cancer. Whereas levels of ERβ1 and 2 were not altered in endometrial cancer tissue, the common ERβ variant 5 was overexpressed in endometrial cancer. The significant increase of four ERβ splice variants in cancer tissue and in G3 tumors and particularly the association of ERβ isoforms with the oncogenes MYBL2 or HER2 suggest that ERβ may be involved in the growth of endometrial cancer. These correlation data were corroborated by the results of our RNAi study establishing a direct link between ERβ and MYBL2 expression.

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