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Molecular profiling of estrogen receptor α and progesterone receptor transcript variants in endometrial cancer

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ABSTRACT

The human genes coding for estrogen receptor alpha (ERα) and progesterone receptor (PR) express multiple receptor splice variants. Some of these receptor variants previously have been shown to exert distinct functions in cancer cells and might therefore differentially affect individual prognosis or therapy response. To examine the role of ERα- and PR-isoforms in endometrial cancer, we compared the expression of 19 ERα transcripts and 15 PR mRNA isoforms in human endometrium and in endometrioid endometrial cancer. Expression of seven ERα splice variants, total PR and of five PR transcript isoforms was found to be significantly decreased in endometrial cancer. In endometrioid G3 tumors, expression of 17 ERα and 10 PR splice variants was reduced when compared to normal tissue. Notably, only 13% of G3 tumors did not express any ERα variant and only in 25% of G3 samples no PR transcripts were expressed. Seven splice variants were preferentially expressed in G1 and G2 tumors. In G1 tumors, a higher number of different ERα and PR splice variants was expressed than in normal endometrium, G2 or G3 tumors. Expression of total PR and of single PR splice variants was found to be positively associated with PTEN. Our results encourage further studies to elucidate to what extent the heterogeneous co-expression profiles we found in endometrial cancer patients differentially affect both individual prognosis and therapy response.

1. 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 sex 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 might play an important role in pathogenesis of endometrial cancer and loss of steroid receptors during tumorigenesis is often associated with an aggressive clinical course and a poorer survival in endometrial cancer patients.

Type 1 endometrial tumors mostly express ERα and PR, they are associated with hyperestrogenism and are characterized by low tumor grade, young age at diagnosis, endometrioid histology, and a good prognosis. Type 2 tumors generally occur in older women, are high-grade and have a relatively poor prognosis. Endometrioid tumors with G3 grading have been suggested to be type 2 tumors, because they have similar clinical data, poor survival and are reported to have negative ERα- and PR-status [1,2]. The genes coding for ERα and PR are known to be alternatively spliced by exon-skipping or exon duplication, resulting in a variety of transcript variants also being expressed in human endometrium [3–6].

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 K-Ras, CTNNBI and PTEN is seen in most of type 1 tumors, whereas changes in PT53 and HER2 receptor tyrosine kinase often are present in type 2 endometrial adenocarcinoma [7]. 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 [8].

The identification of a variety of splice variants of ERα and PR raised the question, to what extent these isoforms might be co-expressed and might affect endometrial carcinogenesis. To

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approach the role of these variants in endometrial cancer, we performed molecular profiling of 19 ERα- and 15 PR-transcript variants in 74 tissue samples of normal and malignant endometrium. Because no antibodies were available to differentiate the large number of existing ERα and PR isoforms, this study had to be performed on the mRNA level by means of qPCR.

2. Patients and methods

2.1. Patients

In this study, we examined a total of 74 endometrial samples including 28 samples from normal endometrium and 46 cases of endometrial carcinoma. Tissue samples from normal and malignant endometrium were collected between 2007 and 2012 by the Second Department of Gynecology of the Medical University of Lublin, Poland. From the 28 samples from women without carcinoma, 11 were from premenopausal women, aged 43–53 years (five in the proliferative phase and six in the secretory phase of the menstrual cycle), and 17 were from postmenopausal women aged between 46 and 90 years. Normal endometrial tissue was obtained from women examined and treated for reasons other than pathology of the endometrium, mainly cervical cancer. The group of endometrioid endometrial cancer patients, ranging from 54 to 82 years of age, underwent curative resection and could be divided 15 G1, 16 G2 and 15 G3 carcinoma (Table 1). Consent for the following studies was obtained from all patients. Tissue samples of patients were collected in accordance with Polish regulations and in agreement with the Ethical Committee of the University School of Medicine in Lublin, Poland. Complete clinical data were available for every patient. Immediately following surgery, tissues were stored in liquid nitrogen until RNA extraction.

2.2. RNA preparation and RT-qPCR

Total RNA from endometrial tissue samples was isolated from 30 to 80 mg frozen tissue using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to manufactureŕs 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, 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 mRNA levels of steroid hormone receptor genes and marker genes 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. Oligonucleotides (Metabion, Planegg-Martinsried, Germany) were designed intron-spanning to avoid genomic contaminations.

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 manufactureŕs protocol and verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). Generally, PCR primers were designed intron-spanning.

The sequences of the 19 ERα and 15 PR variants tested were taken from previous publications reporting their structure [3–6]. On the basis of these sequences, isoform-specific primer pairs were designed (S1). In this study, we used two different PR primer pairs – the pan-PR primers were able to bind to all PR mRNA isoforms. Additionally we used specific primers binding to PR-B, whereas specific PR-A primer could not be designed due to transcript structure. Thus, we determined PR-A transcript levels by usage of pan PR primers binding both to PR-A and B, followed by subtraction of the PR-B transcript level from these results. In all RT-qPCR 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 [9] 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. Expression values of each splice variant were divided into three tertiles, the upper tertile was characterized as strong expression, the middle tertile as moderate expression, and the last tertile contained samples with weak or negative expression.

2.3. Statistical analysis

Statistical analysis of the data was carried out using the InStat software (GraphPad, San Diego, USA). 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. Spearman's rank correlation was used to evaluate the correlation between the expressions of two target genes. *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Differential expression of splice variants in human endometrium and endometrial cancer

For comparison with the cancer group (or grading subgroups), we have chosen (1) a normal endometrium control group containing both pre- and postmenopausal women, (2) a normal premenopausal and (3) a normal postmenopausal control group. We never observed a significant difference between receptor expression in the cancer group (or the grading subgroups) and the normal endometrium or premenopausal group. Comparison of splice variant expression in the small premenopausal subgroups of proliferative ($n = 5$) vs. secretory phases ($n = 6$) did not reveal significant differences.

Only splice variant expression in the postmenopausal control group often differed from the one in cancer in a statistically significant way.

Total PR transcript levels were generally lower in cancer tissue than in postmenopausal endometrium ($p < 0.01$). This difference

Table 1
Characteristics of the endometrioid endometrial tumor samples ($n = 46$).

FIGO	G1	G2	G3
IA	4	–	–
IB	7	9	–
IC	4	6	–
IIA	–	1	–
IIB	–	–	4
IIIA	–	–	11