Arch Gynecol Obstet
DOI 10.1007/s00404-017-4405-x

GENERAL GYNECOLOGY

Promotor analysis of ESR1 in endometrial cancer cell lines, endometrial and endometriotic tissue

Vanessa Toderow[1] · Martina Rahmeh[1] · Simone Hofmann[1] · Verena Kirn[2] · Sven Mahner[1] · Udo Jeschke[1,3] · Viktoria von Schönfeldt[1]

Received: 21 March 2017 / Accepted: 16 May 2017

Abstract
Purpose The nuclear hormone receptor estrogen receptor α (ERα) is pivotal for numerous processes in the cell. As a transcription factor, it regulates eukaryotic gene expression and affects cellular proliferation and differentiation in target tissues. Moreover, ERα is known for its influence on various gynecological diseases and carcinogenesis. Since its expression is often altered in diseased tissues and this alteration was found to be caused by hypermethylation of the ESR1 promotor region in cancer, including breast and colorectal cancer, the aim of this study is to elucidate if the expression of ERα is also regulated epigenetically in endometriosis and endometrial cancer.
Methods Using real-time methylation-specific PCR (rt-MSP), we examined endometrial and endometriotic tissues as well as five endometrial cancer cell lines and compared the methylation status with the actual expression of ERα.
Results The results of our study indicate that, though its expression is altered in endometrial and endometriotic tissue, ERα is not regulated by methylation of the promotor region in endometriosis. In contrast, three of the five endometrial cancer cell lines are methylated in the promotor region of ESR1.
Conclusions Thus, further investigation of the connection between ERα and endometrial cancer will be the next step.

Keywords Estrogen receptor α (ERα) · Real-time methylation-specific PCR (rt-MSP) · ESR1 promotor · Endometrium · Endometriosis

Introduction

The expression of ERα plays a diverse and not fully understood role in different types of gynecological cancer like breast cancer and ovarian cancer. Previous studies revealed that patients with ER expression have a lower risk of recurrence and a better overall chance of survival leading to the clinical routine of ER expression analysis [2, 22]. In contrary, several studies pointed out that ER promoting proliferation increases the risk of tumorigenesis [13, 25, 34]. In case of estrogen sensitive breast cancer, Ellegde et al. showed that a positive ER expression supports a hormone therapy with tamoxifen which is an anti-estrogen hormone and binds to ER impairing the functionality of the receptor [8]. Nevertheless, the exact mechanisms of ER and its regulation in cancer remain unresolved. Additionally, it is necessary to differentiate the role of estrogen and its receptor regarding expression and abundance in non sex-specific tissues compared to gynecological tissues and with that in gynecological cancer. In 1994, ER expression was first described to be regulated by methylation of its promotor region in colorectal cancer which resulted in examination of the methylation status of several malignant und non-malignant tumors [16]. This revealed that the promotor of the estrogen receptor gene (ESR) is not methylated in non-malignant tissues from thyroid, breast, lung, bronchial epithelium, cervix and

✉ Udo Jeschke
udo.jeschke@med.uni-muenchen.de

[1] Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Klinikum der Universität München, Munich, Germany

[2] Klinik und Poliklinik für Frauenheilkunde, Universitätsklinikum Köln, Cologne, Germany

[3] Department of Obstetrics and Gynecology, Ludwig-Maximilians-University, Maistrasse 11, 80377 Munich, Germany

Published online: 03 June 2017

prostate and, consequently, the ERα expression is positive in these tumors [10]. In contrast, ESR promotor methylation seems to play a role in early carcinogenesis of lymphoma, esophageal and colorectal cancer. Eventually, Lapidus et al. characterized breast cancer cell lines which are positive (MDA-MB-231 and MDA-MB-435) and negative (MCF-7) regarding their ESR promotor methylation status. This was the first step to establish a methylation-specific real-time PCR (rt-MSP) for the ESR locus which was also used in this study [6, 11, 17, 23, 24].

Since endometriosis is another estrogen influenced gynecological disease and it was suggested that the ERα expression is altered in ectopic endometrium compared to eutopic endometrium, this study shall elucidate if ESR1 promotor methylation plays a role in this disease as well [9]. Endometriosis is one of the most common diseases in premenopausal women and it is estimated that around 10% of all women in their reproductive years suffer from this illness [31]. It is characterized by the growth, adhesion and progression of endometrial glands and stroma outside the uterine cavity which can lead to dysmenorrhea, pelvic pain and infertility [3].

Ectopic endometrium behaves similarly to eutopic endometrium due to the influence of estrogen and progesterone. If the interaction of these hormones is not tuned accurately, the result is a phase delay or a so-called luteal phase defect (LPD). A common symptom of LPD is the failure of implantation of the blastocyst caused by the endometrium not being prepared for this step due to the hormonal phase delay. LPD is often associated with endometriosis and thus may be a cause of endometriosis related infertility [4, 5, 27, 28]. To reveal a potential correlation between these estrogen influenced processes and a promotor methylation of ESR1, DNA of different tissues, namely proliferative, early secretory and ectopic endometrium, was analyzed regarding their methylation status of ERα. Only few samples were examined because the results should help to decide if an examination on a grand scale was worth doing. In addition, the promotor methylation status was compared to actual ERα expression in the tissues by immunohistochemical staining.

In the second part of this study, we performed rt-MSP of the ESR1 locus of five endometrial cancer cell lines. Endometrial cancer is one of the most common cancers of the reproductive system in industrialized countries [18]. Ali et al. state that ERα has a similar effect on carcinogenesis of endometrial carcinoma as on breast cancer, since an overexpression of ER inhibits the cell growth [1]. Moreover, treatment of endometrial cancer with tamoxifen, which obtains good results in breast cancer, has a very limited effect [21, 29, 30, 33]. This emphasizes once more the complexity and diversity of the effect of the estrogen receptor in the different diseases. Nonetheless, the importance of ERα is unchallenged. Therefore, more information has to be obtained about both function and regulation. To contribute to this aim, we examined the methylation status of the CpG islands in the promotor region of ESR1 in all five cell lines and compare the result to the mRNA level of ERα in the same cell population.

Materials and methods

Examination of endometrial and endometriotic tissue

All in all, four endometrial tissues, two proliferative and two early secretory, and three endometriotic ovarian tissues were analyzed regarding their ERα expression and ESR1 promotor methylation.

Immunhistochemical staining

Serial sections of surgically resected and archived endometrium and endometriosis tissue were prepared after having been completely processed by formalin fixation and paraffin embedding. For examination, two staining methods were used. Hematoxylin and eosin stain (HE stain) helps to localize the relevant areas, whereas the immunohistological avidin–biotin-complex staining method (ABC stain) was used to reveal the expression of ERα in the areas previously identified. The pretreatment is identical and consists of deparaffinizing by incubating in xylene, subsequently blocking of endogenous peroxidase by incubating in 3% hydrogen peroxide (H_2O_2) and demasking of antigenes by cooking in citrate buffer. For HE stain, one section of each sample was rehydrated using ethanol in decreasing concentrations and incubated for 8 min in hematoxylin for staining of the nuclei. After the plasma had been stained by eosin, the sections were analyzed by light microscopy and the endometriosis lesions were marked. The primary antibody for the ABC staining was diluted 1:400 (Abcam) and dripped on the sections which then were incubated for 16 h at 4 °C. Subsequently, the biotinylated secondary antibody was applied for 30 min. By addition of 3,3-diaminobenzidine (DAB), the peroxidase coupled to the ABC-complex starts an enzymatic reaction which results in coloration of the spot the complex is binding. Uterine tissue served as positive control whereas the negative control was an isotype control, i.e., the primary antibody was replaced by immunoglobulins that do not bind to human tissue. The staining of nuclei and plasma was examined separately and valued with an immunoreactive score (IRS) which allowed a comparison of the receptor expression in the different tissues and

considers the staining intensity (proportionate to receptor expression) and the percentage of positively stained cells.

DNA extraction and bisulfite treatment

After immunohistochemical analysis, the samples of eutopic endometrium and the identified endometriotic lesions were processed molecular-biologically. To reveal a DNA methylation of ERα, a methylation-specific polymerase chain reaction (MSP) was performed. Bisulfite treatment of isolated DNA results in conversion of unmethylated cytosine to uracil, whereas methylated cytosine (5-methylcytosine) remains unmodified. This way, after bisulfite treatment the DNA sequence is changed due to its methylation status and can now be used for MSP. Therefore, special primer pairs are needed which are themselves methylation specific, i.e., they are only hybridizing with unconverted 5-methylcytosine but not with DNA containing uracil. Hence, there is an amplification product only if the DNA segment in question is actually methylated.

Genomic DNA was isolated using a commercially available DNA extraction kit (Qiagen; DNA FFPE Tissue) which is used for purification of DNA from formalin-fixed and paraffin-embedded tissues. The endometrial sections were used as a whole, whereas the marked endometriotic lesions were scraped off with a scalpel whilst keeping the contamination of ovarian DNA as low as possible. Consequently, the amount of DNA in these samples was lower.

ESR1 promotor analysis

To reveal the methylation status of the ESR1 promotor region, a real-time methylation-specific PCR (rt-MSP) was performed. The following oligonucleotides were used: forward primer, 5′-ggcgttcgttttgggattg-3′; reverse primer, 5′-gccgacacgcgaactctaa-3′; TaqMans probe, FAM 5′-cgataaaaccgaacgacccgacga-3′ TAMRA (custom synthesized by Applied Biosystems, Foster City, USA). The concentration of primer and probe was 30 mM. Two microliters of the eluate containing 6 ng of bisulfite-treated DNA were used for each MSP reaction. Amplifications were carried out in a 96-well plate in a 7500 Taqman real-time PCR cycler (Applied Biosystems) in two replicates using Universal Mastermix (Applied Biosystems). The reaction volume was 20 µl. Thermal cycling was initiated by denaturation at 95 °C for 20 s. The PCR profile was 95 °C for 3 s and 60 °C for 30 s for a total of 40 cycles.

Moreover, water blanks as well as positive (MDA-MB-231 cell line) and negative (MCF-7 cell line) controls were used. The quality of the isolated DNA was checked by amplifying keratin 19 (CK 19, located on chromosome 17—36937606—36938071).

ESR1 promotor analysis of endometrial cancer cell lines

Five human endometrial cancer cell lines were examined regarding their ESR1 methylation status. The Ishikawa^{+} cell line is known to be positive for the estrogen receptors (ER) which are also the case for the cell line RL95-2, whereas Ishikawa^{-} and HEC-1-A have a lower expression of ER. Moreover, HEC-1-A is known to be positive for the receptor platelet activating factor (PAF) which increases the expression of the oncogene c-fos. HEC-1-B is a substrain of HEC-1-A which exhibited a stationary growth period between day 135 and 190 of culturing and appeared on recovery to be flattened and more pavements patterned than the parent line.

The cells were cultured in RPMI-1640 media (ThermoFisher Scientific, Invitrogen, Cat. No. 61870-010) containing glutamax and 10% FCS (FBS South American ThermoFisher Scientific, Invitrogen, Cat. No. 10270-106) without antibiotics. For passaging, the cells were treated with Trypsin/EDTA (Biochrom, Cat. No. L2143). After having been counted using a hemocytometer, the cells were diluted to a final cell number of 5 million cells in 200 µl PBS. Subsequently, a commercially available DNA extraction kit (QIAamp DNA mini and blood mini, Qiagen, Cat. No. 51304) was used to isolate the DNA of the cells. The DNA concentration was measured by photometer in µg/µl. Further, 500 ng DNA was used for the sodium bisulfite treatment (Epitect Fast DNA Bisulfite Kit, Qiagen) to convert unmethylated cytosine to uracil. 2 µl of each sample containing 50 ng converted DNA was analyzed via rt-MSP using the same methylation-specific primer and probe as mentioned above (Applied Biosystems, TaqMan®) in a final concentration of 30 mM, TaqMan Universal PCR Master Mix 2× and DEPC-treated DI water. DNA of the breast cancer cell lines MDA-MB-231 and MCF7 served as positive and negative control, respectively. The amplification reaction was performed using an ABI PRISM 7500 Fast Real-Time PCR machine. To compare the methylation status of the ESR1 promotor region to the actual expression of ERα, RNA was extracted using a commercially available kit (RNeasy mini, Qiagen). Extracted RNA was converted to cDNA (MMLV-Kit Biozym, Cat. No. 150772) of which 350 ng was analyzed via rt-PCR.

Results

Immunohistochemical staining

The H&E stained sections were analyzed via a light-microscope attached to a digital camera, so micrographs of typical endometrial and endometriotic tissue could be

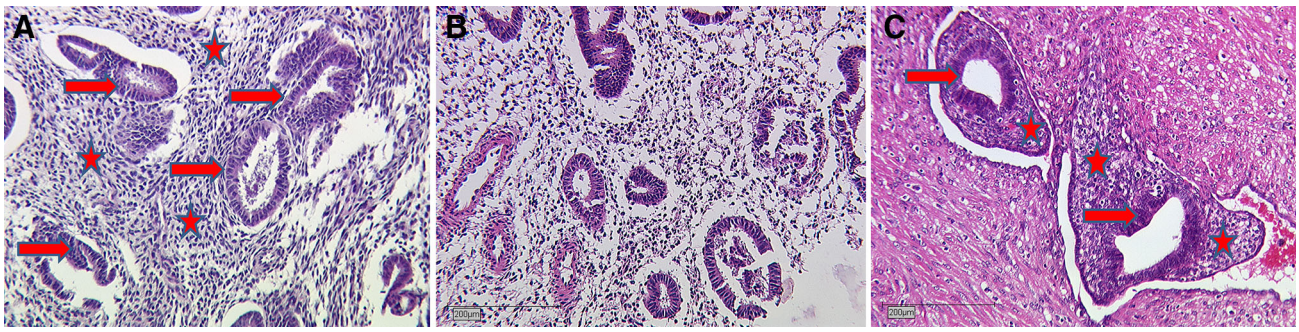

Fig. 1 Hematoxylin and eosin stain of eutopic (**a**, **b**) and ectopic (**c**) endometrium. **a** Proliferative endometrium with tubular glands (*arrowhead*) and dense stroma (*asterisk*), ×10 magnification. **b** Early secretory endometrium with less dense stroma, ×25 magnification. **c** Ovarian endometriosis characterized by its epithelium (*arrowhead*) surrounding a cyst like area as well as the accumulation of nuclei around it (*asterisk*), ×10 magnification

taken which are presented in Fig. 1. In Fig. 1a, tubular glands of a proliferative endometrium are shown; the characteristic dense stroma is well recognizable. In contrast, early secretory endometrium consists of less dense stroma and prominent sub-nuclear vacuoles appear in the cells forming the glands (Fig. 1b). Figure 1c finally shows ovarian endometriotic lesions which were marked with a pencil and scraped off for DNA extraction and bisulfite treatment.

The immunohistochemical stained sections were analyzed by microscoping and images were taken of which some characteristic ones are shown below (Fig. 2). In Fig. 2a, b, we show uterine tissue which served as positive and negative control. Figure 2c, d shows images of proliferative and early secretory endometrium, respectively. It is noticeable that the proliferative endometrial tissue is mainly stained in the nuclei, whereas the secretory endometrial tissue is primarily stained in the cytoplasm. ERα is a nuclear receptor protein which acts as a transcription factor and has its main influence during the proliferative phase and thus is found in the nuclei at this stage. Moreover, it is built in the cytoplasm during early secretory phase and, thus, accumulates in this cell part. The staining was analyzed by scoring the IRS which is summarized in the box plot graphs (Fig. 2g, h).

ESR1 promotor analysis

rt-MSP of bisulfite-treated DNA of all tissue samples showed that only the positive control MDA-MB-231 is methylated in the promotor region of ESR1 (Fig. 3). The remaining samples of endometrial and endometriotic tissue as well as the negative control MCF-7 yielded no product which is demonstrated by the unsteady graphs beneath the threshold. The quality of the extracted DNA was tested by amplifying the CK19 locus. Here, a product could be detected for all samples except the negative control.

The analysis of rt-MSP of the endometrial cancer cell lines indicates that three of five of the examined cell lines are methylated in the promotor region of ERα. Ishikawa^{-}, HEC-1-A as well as RL95-2 yielded a product in the methylation-specific amplification which was not the case for Ishikawa^{+} and HEC-1-B. The extracted mRNA which was converted to cDNA was analyzed using rt-PCR. The amplification plots show that no product could be detected for HEC-1-A, whereas the amplification of the ESR1 locus in both Ishikawa^{-} and RL95-2 yielded a product in a late cycle (34th and 32th cycle, respectively). In contrast, amplification of ESR1 in Ishikawa^{+} and HEC-1-B yielded a product in an earlier cycle.

Discussion

The positive and negative control of uterine tissue indicates that the immunohistological staining of ERα was successful (Fig. 2a, b). The sections were analyzed by scoring the IRS for nuclei and cytoplasm in several relevant areas (glands in endometrium, lesions in endometriotic tissue). The box plots shown in Fig. 2 summarize the results which were then used for statistical tests to reveal significant differences in ERα expression in various tissues. Since this is a matter of independent samples, a non-parametric Mann–Whitney *U* ranked test was performed using statistics software program (SPSS). All three types of tissues were compared with each other. The null hypothesis was that there is no difference in IRS, and was rejected for all three cases on a high level of significance ($p < 0.001$). This indicates that ERα is indeed expressed on a different level in endometriotic tissue compared to endometrial tissue, which is consistent with previous studies. Nevertheless, these results should rather be interpreted as a hint in the same direction than a confirmation since the amount of samples was small.

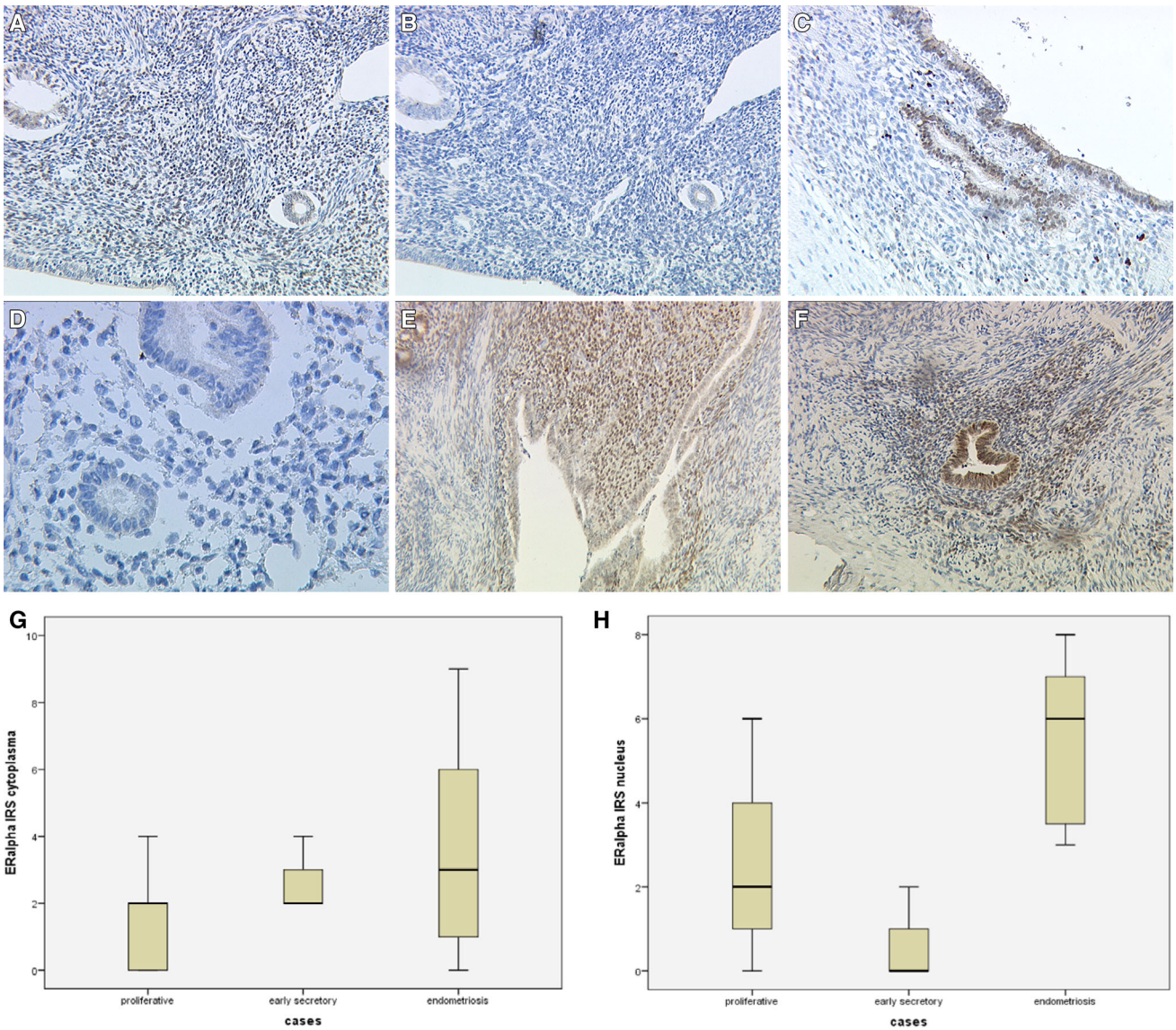

Fig. 2 ERα antibody staining of **a** uterine tissue as positive control, high percentage of stained nuclei is well recognizable, ×10 magnification a positive control, high intensity of stained nuclei, ×25 magnification, **b** uterine tissue as isotope control, ×10 magnification, **c** characteristic gland of proliferative endometrium, ×10 magnification, **d** early secretory endometrium, ×25 magnification, **e** and **f** endometriosis lesion, ×10 magnification, **g** results of IRS regarding several characteristic spots of proliferative, early secretory and endometriotic tissue, box plots of IRS of stained plasma, **h** box plots of IRS of stained nuclei

Samples of endometrial and endometriotic DNA yielded no methylation-specific amplification product which suggests contrarily that the analyzed promotor region is not methylated either in endometrial tissue or endometriosis, that is to say the expression of ERα seems not to be regulated by DNA methylation. Since the positive and negative controls were successful, it can be assumed that the concentrations chosen were appropriate for the experiment. Moreover, the quality check of the isolated unconverted DNA using CK19 primers yielded products in all samples except the water control so the success of DNA isolation is highly probable. Only the success of bisulfite conversion cannot be ensured completely, but since there is no amplification product of converted DNA in combination with CK19-primers (not methylated-specific) this is quite probable. The only alternative is that the DNA was mostly destroyed during the conversion process which is, on the other hand, not very likely.

Ishikawa^{-} is known to have a low expression of ESR1 so the result of our study supports the assumption that the expression pattern is caused by epigenetic modification. The methylation status is compatible with the result of cDNA-PCR which pointed out a C_t value of approximately 33, i.e., in the 34th cycle of 40 an amplification product

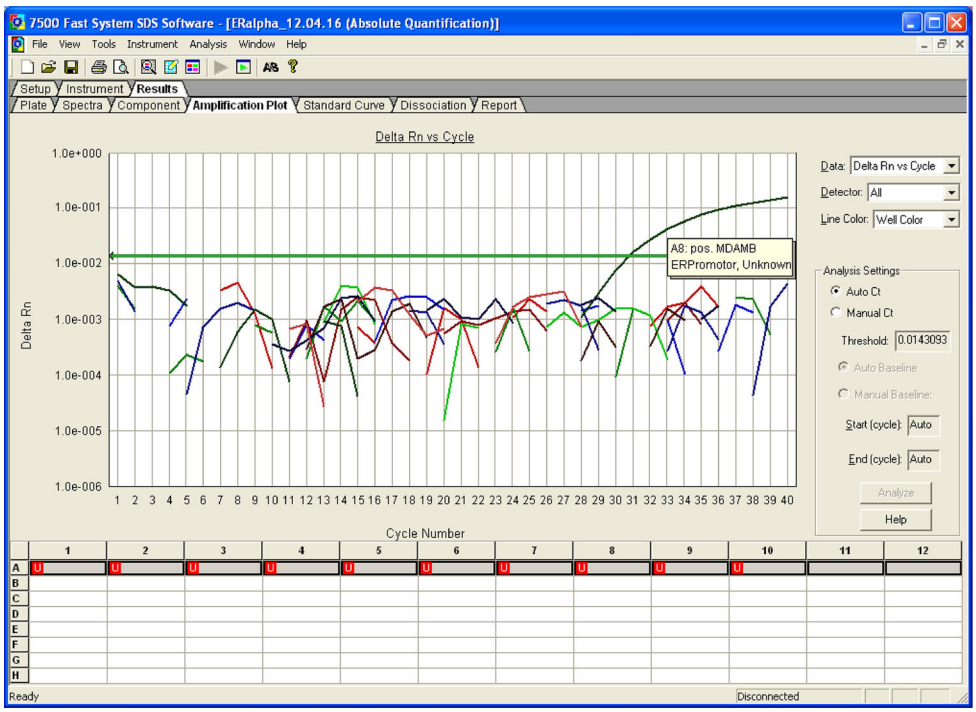

Fig. 3 rt-MSP amplification plots of all samples after bisulfite conversion, only the positive control MDA-MB-231 yielded a product (graph similar to log function), all other samples were negative (graphs unsteady); in *green*: threshold, automatically set, The crossing of a positive amplification plot and the threshold states the C_t value. This is the cycle in which the product was amplified enough to be detected. The lower the C_t value, the more DNA with the gene of interest was in the reaction sample. Here negligible since this is a qualitative experiment

could be measured which is comparatively late. The C_t value is indirectly proportional to the amount of transcripts. In the case of HEC-1-A, the examination of cDNA indicates that the amount of the ESR1 transcript is very small in this cell line, since no product could be detected in 40 cycles of amplification. Again, this matches the results of MSP which is also the case for the results of cDNA-PCR and MSP of the RL95-2 cell line (C_t-value 32). In contrast, the examination of the cDNA revealed that the expression of ERα is definitely higher in the Ishikawa$^+$ and HEC-1-B cell lines compared to the remaining three cell lines, which can be concluded by a C_t value of 23 and 25.

The causes of endometriosis related infertility are still unresolved. In cases of severe endometriosis including extensive cysts in the pelvic cavity, there might be a mechanical impairment of folliculogenesis and ovulation or occlusion of the fallopian tubes. But infertility does not seem to correlate with the severity of the disease, since women with little anatomic distortion can also suffer from infertility. Apparently, it is not even certain that endometriosis actually causes infertility, or if there is a so far unknown dysfunction that causes both endometriosis and infertility. But since endometriosis, infertility and LPD are moderately or even highly correlated it is extremely promising to examine the hormonal conditions and alterations in affected patients in comparison to healthy persons. In doing so, the regulatory mechanisms are of particular interest. The expression of ERα is altered in various diseases, for example in breast and ovarian cancer but also in endometriosis and endometriotic cancer. It is well known that this alteration can occur due to epigenetic modifications like promotor methylation in cancer cells [20]. Since our study did not suggest a regulation of ERα expression via methylation either in proliferative and early secretory endometrium or in endometriotic tissue, the next step would be to examine other regulatory mechanisms such as histone modifications. Moreover, it will be interesting to focus on patients with a high correlation between LPD, endometriosis and infertility and compare tissues from patients with severe symptoms with those of patients with mild symptoms since the link between all this could be the alteration of ERα expression. This was not considered in this study, since it should reveal differences between the tissues and their expression pattern in general. Moreover, there is no difference in the methylation status of the ESR1 promotor in proliferative and early secretory endometrial tissue. These two phases are regulated by different sex steroid hormones that are estrogen in proliferative and progesterone in secretory tissue. The histological differences are attributed to the associated hormonal receptors (estrogen receptors and progesterone receptors). Their expression differs in the various phases of the endometrium as it is shown in immunohistological staining of the tissue sections, but this, again, seems not to be regulated by DNA methylation.

The revelation of three endometrial cancer cell lines which are methylated in the CpG islands of the ESR1 promotor region is highly promising. Several studies suggest that a high content of ERα in primary endometrial carcinomas affects prognosis favorably [19]. The overexpression of

ERα in the Ishikawa cell line leads to inhibition of cell growth in culture as well as in the chicken chorioallantoic membrane (CAM) model which confirms the previously reported significance of ERα in carcinogenesis. Knowing that ERs promote proliferation and thus are seen as cancer risk factors, this seems surprising. Furthermore, contradictory studies exist about the effect of hormonal therapy in endometrial cancer. Tamoxifen therapy of breast cancer patients even seems to increase the risk of endometrial cancer. This emphasizes the diverse and critical role of estrogen receptors and how poorly we understand its complex activity patterns. The results of our study suggest that promotor methylation is an important regulation mechanism of ERα in endometrial cancer and should be examined further. It is important to reveal the mechanisms by which ERα acts in the different stages of endometrial cancer as well as in other gynecological diseases, especially in comparison to non-sex-related diseases. The same applies to the diverse regulatory mechanisms which determine the ERα content in the cell. While ESR1 is regulated by DNA methylation in non-malignant tissues and several cases of tumors, it can be regulated positively or negatively by circulating estrogen levels depending on sex, type of tissue and developing stage and this regulation can occur on mRNA or protein level since not every transcript is translated into a protein [26]. For example, it was shown that the expression of ERα is regulated in a coordinated fashion by numerous transcription factors including retinoic acid receptor-α, PAX2, GATA3, NKX3.1, LEF1 and FOXA1 which turned out to be of particular interest in the attempt to increase tamoxifen response in cells with low ERα amount [7, 12, 14, 15, 32]. However, the knowledge about these processes is superficial and the mechanisms are poorly understood. Hence, future examination of both function and regulation of ERα will eventually lead to an improved understanding of cancer and epigenetics and hopefully reveal new ways of fighting endometrial cancer.

Author contributions VT: Performed the experiments, wrote the first draft of the manuscript. MR: Performed the experiments, supervised the methodology. SH: Performed the experiments, supervised the methodology. VK: Developed the Methodology of the methylation analyses. SM: Organized and Supervised Funding and final manuscript preparation. UJ: Supervised the Methodology and the final draft of the manuscript. VvS: Development of the project idea, principal supervision of the investigators.

Compliance with ethical standards

Funding This study was funded by the Medical Faculty of the Ludwig Maximilians University of Munich.

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Ali SH, O'donnell AL, Mohamed S et al (2004) Overexpression of estrogen receptor-alpha in the endometrial carcinoma cell line Ishikawa: inhibition of growth and angiogenic factors. Gynecol Oncol 95:637–645
2. Berger U, Mcclelland RA, Wilson P et al (1991) Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis. Can Res 51:239–244
3. Bloski T, Pierson R (2008) Endometriosis and chronic pelvic pain: unraveling the mystery behind this complex condition. Nurs Women's Health 12:382–395
4. Bopp B, Shoupe D (1993) Luteal phase defects. J Reprod Med 38:348–356
5. Cunha-Filho JS, Gross JL, Bastos De Souza CA et al (2003) Physiopathological aspects of corpus luteum defect in infertile patients with mild/minimal endometriosis. J Assist Reprod Genet 20:117–121
6. Eads CA, Lord RV, Kurumboor SK et al (2000) Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Can Res 60:5021–5026
7. Eeckhoute J, Keeton EK, Lupien M et al (2007) Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. Can Res 67:6477–6483
8. Elledge RM, Green S, Pugh R et al (2000) Estrogen receptor (ER) and progesterone receptor (PgR), by ligand-binding assay compared with ER, PgR and pS2, by immuno-histochemistry in predicting response to tamoxifen in metastatic breast cancer: a Southwest Oncology Group Study. Int J Cancer 89:111–117
9. Engemise SL, Willets JM, Taylor AH et al (2011) Changes in glandular and stromal estrogen and progesterone receptor isoform expression in eutopic and ectopic endometrium following treatment with the levonorgestrel-releasing intrauterine system. Eur J Obstet Gynecol Reprod Biol 157:101–106
10. Esteller M, Corn PG, Baylin SB et al (2001) A gene hypermethylation profile of human cancer. Can Res 61:3225–3229
11. Harder J, Engelstaedter V, Usadel H et al (2009) CpG-island methylation of the ER promoter in colorectal cancer: analysis of micrometastases in lymph nodes from UICC stage I and II patients. Br J Cancer 100:360–365
12. Holmes KA, Song JS, Liu XS et al (2008) Nk3–1 and LEF-1 function as transcriptional inhibitors of estrogen receptor activity. Can Res 68:7380–7385
13. Holst F, Hoivik EA, Gibson WJ et al (2016) Recurrent hormone-binding domain truncated ESR1 amplifications in primary endometrial cancers suggest their implication in hormone independent growth. Sci Rep 6:25521
14. Hua S, Kittler R, White KP (2009) Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. Cell 137:1259–1271
15. Hurtado A, Holmes KA, Geistlinger TR et al (2008) Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. Nature 456:663–666

16. Issa JP, Ottaviano YL, Celano P et al (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 7:536–540
17. Issa JP, Zehnbauer BA, Civin CI et al (1996) The estrogen receptor CpG island is methylated in most hematopoietic neoplasms. Can Res 56:973–977
18. Jemal A, Bray F, Center MM et al (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
19. Jongen V, Briet J, De Jong R et al (2009) Expression of estrogen receptor-alpha and -beta and progesterone receptor-A and -B in a large cohort of patients with endometrioid endometrial cancer. Gynecol Oncol 112:537–542
20. Kanwal R, Gupta S (2012) Epigenetic modifications in cancer. Clin Genet 81:303–311
21. Kokka F, Brockbank E, Oram D et al (2010) Hormonal therapy in advanced or recurrent endometrial cancer. Cochrane Database Syst Rev. doi:10.1002/14651858.CD007926.pub2
22. Lapidus RG, Ferguson AT, Ottaviano YL et al (1996) Methylation of estrogen and progesterone receptor gene 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res Off J Am Assoc Cancer Res 2:805–810
23. Lapidus RG, Nass SJ, Butash KA et al (1998) Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Can Res 58:2515–2519
24. Lapidus RG, Nass SJ, Davidson NE (1998) The loss of estrogen and progesterone receptor gene expression in human breast cancer. J Mamm Gland Biol Neoplasia 3:85–94
25. Li Q, Seo JH, Stranger B et al (2013) Integrative eQTL-based analyses reveal the biology of breast cancer risk loci. Cell 152:633–641
26. Liu X, Shi H (2015) Regulation of estrogen receptor α expression in the hypothalamus by sex steroids: implication in the regulation of energy homeostasis. Int J Endocrinol 2015:949085
27. Matorras R, Rodriguez F, Perez C et al (1996) Infertile women with and without endometriosis: a case control study of luteal phase and other infertility conditions. Acta Obstet Gynecol Scand 75:826–831
28. Moeloek FA, Moegny E (1993) Endometriosis and luteal phase defect. Asia Ocean J Obstetr Gynaecol 19:171–176
29. Pandya KJ, Yeap BY, Weiner LM et al (2001) Megestrol and tamoxifen in patients with advanced endometrial cancer: an Eastern Cooperative Oncology Group Study (E4882). Am J Clin Oncol 24:43–46
30. Rendina GM, Donadio C, Fabri M et al (1984) Tamoxifen and medroxyprogesterone therapy for advanced endometrial carcinoma. Eur J Obstet Gynecol Reprod Biol 17:285–291
31. Rogers PA, D'hooghe TM, Fazleabas A et al (2009) Priorities for endometriosis research: recommendations from an international consensus workshop. Reprod Sci (Thousand Oaks, Calif) 16:335–346
32. Ross-Innes CS, Stark R, Holmes KA et al (2010) Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. Genes Dev 24:171–182
33. Thigpen T, Brady MF, Homesley HD et al (2001) Tamoxifen in the treatment of advanced or recurrent endometrial carcinoma: a Gynecologic Oncology Group study. J Clin Oncol 19:364–367
34. Yue W, Yager JD, Wang JP et al (2013) Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. Steroids 78:161–170