

Functional characterization of somatic point mutations of the human estrogen receptor α (hER α) in adenomyosis uteri

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Endometriosis and adenomyosis uteri are chronic, benign diseases caused by the presence of endometrial tissue in ectopic locations, e.g. peritoneal or deep inside the myometrial wall of the uterus and/or in the rectovaginal septum. Although adenomyosis might be considered as a special form of endometriosis, both conditions differ with respect to clinical symptoms and treatment. Induction of a hypo-estrogenic state alone or in combination with surgical removal of the extra-uterine lesion is mostly sufficient for treatment of peritoneal endometriosis. By contrast, adenomyosis uteri rarely responds to hormonal therapy and usually requires a hysterectomy for cure. Consequently, the role of steroid hormone receptors with respect to the aetiology of either condition is still a matter of discussion. Using PCR/single strand conformation polymorphism analysis, we identified somatic estrogen receptor (ER) α gene mutations in three out of 55 samples from adenomyosis uteri. Functional characterization revealed that two of the mutant ER α proteins display severely impaired DNA-binding and transactivation properties secondary to an altered response to estrogens or changes in epidermal growth factor-mediated ligand-independent activation. Although the exact mechanism remains unknown, we suggest that mutation-related silencing of estrogen responsiveness might render endometriotic cells resistant to hypo-estrogenic conditions thereby accounting for failure of estrogen-ablative therapy in adenomyosis.

Key words: adenomyosis uteri/endometriosis/human estrogen receptor alpha/somatic mutation/steroid receptor

Introduction

Endometriosis is a benign disease characterized by the presence of functional endometrial tissue outside the uterine cavity. In about 30% of patients this is accompanied by adenomyosis, i.e. the presence of endometrial glands and stroma deep inside the myometrium and/or the rectovaginal septum (Nisolle and Donnez, 1997; Ferenczy, 1998; Mataliotakis *et al.*, 2003). The predominant clinical symptoms of both conditions are chronic pelvic pain, dysmenorrhoea and infertility. Although aetiology and pathogenic mechanisms are still a matter of discussion, it is now generally accepted that endometriosis and adenomyosis have to be regarded as distinct diseases requiring different therapeutic approaches (Nisolle and Donnez, 1997). Whereas ablation of ovarian steroids and induction of hypo-estrogenic conditions by means of GnRH analogues is effective to treat endometriosis and/or to prevent recurrence after surgery, hysterectomy is required for definitive treatment of adenomyosis uteri (Ferenczy, 1998; Winkel, 2003).

The predominant biological effects of estrogens are mediated through two distinct intracellular receptors, estrogen receptor (ER) α and ER β , which are both expressed in normal endometrium and endometriotic lesions (Matsuzaki *et al.*, 2001). ERs are members of

the nuclear receptor superfamily and act as ligand-regulated transcription factors (Beato, 1989; Beato and Klug, 2000). They are modular proteins consisting of several functional domains including a N-terminal domain (NTD), a DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Renaud and Moras, 2000; Steinmetz *et al.*, 2001; McDonnell and Norris, 2002). ER-mediated transcriptional activation is controlled by the binding of its natural agonist estradiol (E₂) to the LBD, which harbours the activation function 2 (AF2). The LBD is basically composed of α -helices (H1–H12) and a short β -sheet inserted between H5 and H6 (Renaud and Moras, 2000; Steinmetz *et al.*, 2001). H12 is essential for the functioning of the AF2. The NTD also contains an activation function (the AF1), which is regulated by phosphorylation (Ali *et al.*, 1993; Kato *et al.*, 1995; Bunone *et al.*, 1996). Typically, extracellular stimuli (e.g. growth factors) trigger the activation of protein kinase cascades, which ultimately results in phosphorylation of the NTD and stimulation of AF1 activity. In certain cell types, the AF1 and AF2 functionally co-operate to control the transcription of target genes (Beato and Klug, 2000).

Crystal structures of the ER LBD complexed with its natural agonist E₂, or the synthetic AF2 antagonists 4-hydroxytamoxifen (TAM), raloxifene (RAL), or the pure anti-estrogen ICI 164384 revealed the basic mechanisms controlling the activity of the AF2

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(Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Pike *et al.*, 2000, 2001). Upon binding of E₂, the ER LBD undergoes significant conformational changes and H12 is precisely positioned to permit the recruitment of transcriptional co-activators, such as the steroid receptor co-activator-1 (SRC-1). By contrast, binding of antagonists either induces re-positioning of H12 (TAM, RAL) or destabilizes its conformation (ICI compounds) thereby precluding interaction with SRC-1 (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Pike *et al.*, 2001).

TAM and RAL are AF2 antagonists, which also display agonist activity in certain cell types and/or promoter contexts. While TAM and RAL are referred to as (tissue-specific) selective estrogen receptor modulators (SERMs), ICI 164384 or ICI 182780 (referred to as ICI) are classified as full ER antagonists blocking ER activity in all tissues (Lonard and Smith, 2002; McDonnell *et al.*, 2002).

Several ER gene mutations resulting in receptors with altered response to E₂ and/or SERMs have been reported previously (Murphy *et al.*, 1997). We hypothesized that the limited response of adenomyosis to hormonal ablation therapy might be secondary to mutated ER proteins. In a PCR/single strand conformation polymorphism analysis (SSCP) mutation screening of ER α , we were able to identify somatic mutations in three out of 55 adenomyosis samples. These ER α mutations were functionally characterized to define their possible role in adenomyosis uteri.

Materials and methods

Tissue specimens

Fifty-five adenomyosis specimens of women who underwent hysterectomy at the Department of Obstetrics and Gynaecology, University of Beijing, China were included in the study. Diagnosis of adenomyosis was confirmed by two independent gynaecologic pathologists. Adenomyotic lesions were dissected from uterine specimens and embedded in paraffin. This archival material together with peripheral blood leukocyte (PBL) DNA from corresponding patients was provided by the Department of Pathology, University of Beijing, China for the purpose of this study. All patients consented to the use of tissue and blood samples for research purposes.

DNA extraction

DNA was extracted from paraffin-embedded tissue samples essentially as previously described (Wright and Manos, 1990). Briefly, sections (5 μ m) were deparaffinized with octane (4 \times), washed (2 \times) with ethanol (95%),

dried and incubated with proteinase K (200 mg/ml; 12 h, 37°C) in 50 mM Tris-HCl (pH 8.5), containing 1 mM EDTA and 0.5% (v/v) Tween 20. Finally, the protease was inactivated (95°C; 10 min), cell debris was removed by centrifugation and the supernatants were submitted to PCR analysis.

PCR/SSCP

PCR/SSCP analysis covering exons 1–8 of the hER α gene was performed as previously described (Orita *et al.*, 1989). 1 μ l of DNA solution was subjected to PCR amplification (primers are given in Table I) in a total volume of 25 μ l. Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 pM of each primer, 125 pM dNTPs (each), 0.8 pCi of [α -³²P]dCTP (specific activity 3000 Ci/mmol; Amersham, Arlington Heights, USA), and 0.5 U of *Taq* DNA polymerase (Perkin Elmer, Norwalk, USA). A total of 34 amplification cycles (denaturation: 1 min at 94°C; annealing: 1 min at 56°C (exon 1), 58°C (exon 2) or 60°C (exons 3–8); extension: 1 min at 72°C) was followed by 5 min of final extension at 72°C. For SSCP analysis, 5 μ l of the PCR product was diluted with 45 μ l of stop-solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured (5 min; 95°C) and immediately loaded on a 6% native polyacrylamide gel. Electrophoresis was performed at 8 W for 13–14 h at room temperature. The gel was transferred onto Whatman 3MM paper, vacuum-dried, and exposed on a phosphorimager plate (Fuji Photo Film Co. Ltd., Paramus, USA) for 6 h.

DNA sequencing

DNA displaying an abnormal SSCP migration pattern was amplified and isolated by electrophoresis on a 2% agarose gel. Specific bands were dissected and the DNA was extracted by centrifugation using Micropure Separator columns according to the instructions of the manufacturer (Amicon, Beverly, USA). Purified PCR products were subjected to cycle sequencing (Amersham, Arlington Heights, USA). Both sense and antisense DNA strands were sequenced with the primers previously used for PCR amplification. The sequencing reaction was resolved on a 6% denaturing urea/polyacrylamide gel. Gels were dried and exposed on a phosphorimager plate for 8 h. Identification of mutations was based on their presence in the sense and antisense DNA strands and confirmed by an independent round of PCR amplification and sequencing. Corresponding samples of DNA isolated from PBL were used to discriminate between somatic and germline mutations.

Recombinant plasmids

The full-length wild-type hER α cDNA was cloned into the eukaryotic expression vector pCMX leading to pCMX-ER α (Umesono *et al.*, 1991). Site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, USA) was used to generate the plasmids pCMX-ER α (P129R), pCMX-ER α (K231R), and pCMX-ER α (M4271/L429M).

Table I. Sequences of primers used for exon-specific amplification of hER α .

Specificity (size of amplicon)	Sequence of sense and antisense primer 5'–3'	Annealing temperature (°C)
exon 1 (474 bp)	CCC TGC CCC GCG GCC ACG GAC C CTG TAG AAT GCC GGC GGG CCG	56
exon 2 (191 bp)	GCC AAA TTC AGA TAA TCG ACG CTT GAA TAC TTC TCT TGA AGA	58
exon 3 (116 bp)	GAC ATA ACG ACT ATA TGT GTC CAC CTT TCA TCA TTC CCA CTT	60
exon 4 (337 bp)	GGA TAC GAA AAG ACC GAA GAG CTG GCA CCC TCT TCG CCC AGT	60
exon 5 (139 bp)	GCT TTG TGG ATT TGA CCC TCC CTG TCC AAG AGC AAG TTA GGA	60
exon 6 (134 bp)	GAA CCA GGG AAA ATG TGT AGA CAG AAT TAA GCA AAA TAA TAG	60
exon 7 (182 bp)	GAG TGT ACA CAT TTC TGT CCA CAT GTG CCT GAT GTG GGA GAG	60
exon 8 (258 bp)	AGT AAC AAA GGC ATG GAG CAT CGT GTG GGA GCC AGG GAG CTC	60

Primers are given together with the size of the amplicons and the respective annealing temperatures. (Gene bank accession number of hER α .M12674.)

In addition, the LBD of wild-type and mutant (M427I/L429M) receptor was PCR-amplified and cloned at the EcoRI/BamHI sites of the eukaryotic expression vector pCMX-Gal, thereby generating the plasmids pCMX-GalER α -LBD and pCMX-GalER α -LBD(M427I/L429M). All plasmids were verified by sequencing.

The reporter plasmid p(vit-ERE)₂-TK-Luc contains two copies of the estrogen response element (ERE) from the *Xenopus vitellogenin A2* gene (VitA2) (nucleotides -334 to -289) fused upstream of the herpes simplex virus thymidine kinase (TK) promoter region (nucleotides -109 to +52) and linked to the firefly luciferase coding sequence (Klein-Hitpass *et al.*, 1986). The related reporter plasmid p(S2-ERE)₃-TK-Luc contains three copies of the ERE from the human pS2 gene (Berry *et al.*, 1989), while p(GAL4)₃-TK-Luc contains three copies of the DNA binding site of the yeast activator GAL4.

DNA binding assays

A double-stranded oligonucleotide (vitERE) containing the ERE from the vitellogenin A2 promoter (Klein-Hitpass *et al.*, 1986) was used for electrophoretic mobility shift assays (EMSAs).

Wild-type and mutant ER α proteins were *in vitro* translated using the respective expression plasmid and TNT-coupled reticulocyte lysate (Promega, Madison, USA). A volume of 1.5 μ l of primed lysate was incubated in buffer containing 10 mM HEPES (pH 7.9), 1 mM MgCl₂, 50 mM KCl, 5% glycerol, and 1 μ g poly(dI-dC) in the absence or presence of different concentrations of E₂, TAM, RAL or ICI. Approximately 1 ng of the ³²P-labelled oligonucleotide probe was added and samples were incubated for 20 min at room temperature, followed by 5 min incubation on ice. Subsequently, the reactions were resolved on a 5% non-denaturing polyacrylamide gel in 0.5 \times TBE running buffer at 4°C.

Cell culture and transient transfection assays

Cell lines (COS-7 cells: African green monkey kidney; HepG2 cells: human hepatocellular carcinoma; Hec-1A, Hec-1B cells: human endometrial adenocarcinoma) were purchased from American Type Culture Collection (ATCC) (Manassas, USA) and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Gibco BRL, Eggenstein, Germany). Forty-eight hours prior to transfection, cells were cultured in medium containing 10% of charcoal double-stripped fetal calf serum. Transient transfection assays were carried out using the standard calcium phosphate co-precipitation technique as described (Greiner *et al.*, 2000). Briefly, cells were seeded in 12-well plates (10⁵ cells per well) and transfected with 500 ng of reporter plasmid [p(vit-ERE)₂-TK-Luc, p(S2-ERE)₃-TK-Luc or p(Gal4)₃-TK-Luc], 100 ng of receptor expression plasmid (pCMX-ER α or pCMX-Gal-ER α -LBD encoding wild-type or mutant ER α), and 1.4 μ g of pUC18 carrier DNA per well. The empty pCMX or pCMX-Gal expression plasmids served as controls. In some experiments, cells were co-transfected with steroid receptor co-activators-1 (SRC-1) expression plasmid (pSG5-SRC-1; Mueller *et al.*, 2000). At 8–16 h after transfection (depending on the cell line), cells were washed with PBS, and fresh culture medium, containing either no additives, hormones, and/or epidermal growth factor (EGF) (Roche, Mannheim, Germany) was added. After a further 16 h of incubation, cells were washed with PBS, lysed, and assayed for luciferase activity as recommended by the manufacturer (Promega, Madison, USA) using a Luminometer ML3000 (Dynatech, Roseville, USA). Protein concentrations were determined with the Bradford dye assay (BioRad, Munich, Germany). Each experiment was repeated independently at least three times.

Statistical analysis

Statistics were performed using the Statistics Package for Social Sciences (SPSS) 5.0 for Windows (SPSS Inc, Chicago, USA). Comparison between groups were made applying the Mann-Whitney *U*-test.

Crystal structure analysis

PDB accession numbers of ER α LBD/ligand complexes are: 1ERE (ER α LBD/E₂), 3ERT (ER α LBD/TAM), 1ERR (ER α LBD/RAL), and 1HJ1 (ER β LBD/ICI 164384). The proteins were superimposed using the lsq commands of the program 'O' (Jones *et al.*, 1991) and visually analysed for

protein–ligand interactions and conformational changes. Figure 6A and B was generated with SETOR (Evans, 1993).

Results

Identification of mutations in the hER α gene in endometrial tissue

Adenomyosis specimens ($n = 55$) were screened for mutations in the hER α gene by PCR/SSCP analysis. Three samples, for which the SSCP analysis revealed migration shifts, were subjected to sequence analysis and were found to contain point mutations in coding regions of the hER α gene (one of the migration shifts is shown in Figure 1A). These mutations appear to be heterozygous because they were found in association with a wild-type receptor DNA sequence (data not shown). All mutations cause single or double amino acid exchanges in the corresponding protein sequences (Figure 1B) and result in the mutant receptors ER α (P129R), ER α (K231R), and ER α (M427I/L429M). The mutations are located in different receptor domains, ER α (P129R) in the NTD, ER α (K231R) in the DBD, and ER α (M427I/L429M) in H7 of the LBD (Figure 1B). They were characterized in functional assays in order to evaluate whether the ER α mutants exhibit properties distinct from the wild-type receptor. None of the mutations was detectable in the corresponding genomic DNA, indicating that these mutations are somatic (data not shown).

ER α (P129R) and ER α (M427I/L429M) display altered DNA binding properties

The DNA-binding properties of wild-type and mutant ER α were compared in EMSAs (Figure 2A) using a double-stranded oligonucleotide containing the estrogen responsive element from the vitellogenin A2 promoter (vitERE) (Klein-Hitpass *et al.*, 1986). As observed previously (Beato and Klug, 2000), DNA binding of ER α is strongly induced in the presence of E₂ (Figure 2A, lanes 2 and 6). Compared to the wild-type receptor, ER α (P129R) migrates with a higher mobility in the absence as well as in the presence of E₂ (Figure 2A, lanes 3 and 7). This suggests that the replacement of a proline residue by arginine in the NTD significantly changes the structure of this mutant, thereby altering not only the shape of the receptor but also its interaction with other molecules.

No significant change in the DNA-binding properties of ER α (K231R) was observed (Figure 2A, lanes 4 and 8). While DNA binding of ER α , ER α (P129R), and ER α (K231R) was induced to similar levels at 10⁻⁸ M E₂, binding of ER α (M427I/L429M) to the vitERE was not inducible under these conditions (Figure 2A, lanes 5 and 9). Thus, the double point mutation in H7 of the ER α LBD may either affect binding of E₂ or indirectly influence DNA binding, e.g. by changing the dimerization properties of the LBD.

Effects of ER agonist and antagonists on DNA binding of ER α and ER α (M427I/L429M)

While in EMSAs DNA binding of the wild-type receptor is already detectable at 10⁻⁹ M E₂, binding of ER α (M427I/L429M) is induced to comparable levels only at about three orders of magnitude higher concentration of E₂ (10⁻⁶ M) (Figure 2B). A similarly dramatic difference between ER α and ER α (M427I/L429M) is found in the presence of TAM. DNA binding of the wild-type receptor is already significantly induced at about 10⁻⁷ M TAM, whereas only a slight induction of ER α (M427I/L429M) is observed at 10⁻⁵ M TAM (Figure 2B). In comparison, the excess of ligand required to induce DNA binding of mutant receptor is slightly lower for RAL and ICI

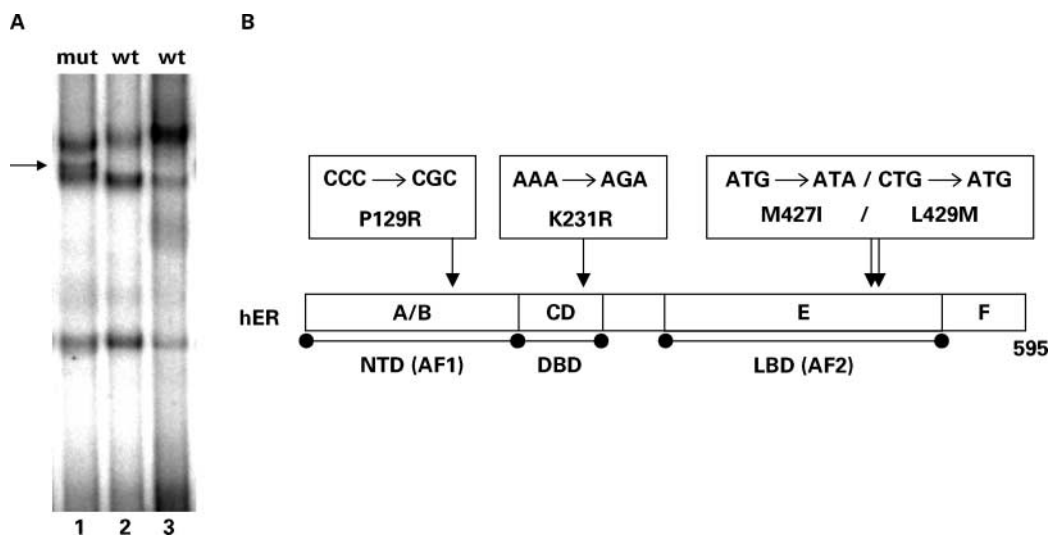


Figure 1. Identification of ER α gene mutations by PCR/SSCP analysis. (A) Mobility shift of a fragment from a PCR/SSCP reaction containing exon 6 of the ER α gene (lane 1) compared to the wild-type receptor control (lanes 2 and 3). (B) Schematic representation of the domain organization of ER α and location of the identified mutations P129R, K231R, and M427I/L429M.

(Figure 2B). About two orders of magnitude higher concentration of these compounds induces DNA binding of ER α (M427I/L429M) to levels comparable to the wild-type receptor. These observations indicate that DNA binding of ER α (M427I/L429M) is impaired for a variety of ligands and establish gradual effects of these ligands on the DNA-binding properties of the mutated receptor protein.

ER α mutants display altered transcriptional activities

The transcriptional activities of wild-type and mutant ER α were analysed in transient transfection assays using different cell lines

(COS-7, HepG2, Hec1A, and Hec1B). Cells were co-transfected with the respective receptor expression plasmid and the reporter plasmid p(vit-ERE)₂-TK-Luc, which contains two copies of the vitERE fused to the TK promoter and the luciferase (Luc) cDNA (Klein-Hitpass *et al.*, 1986). In addition, the transcriptional properties of ER α proteins were tested on a pS2 gene promoter construct (Berry *et al.*, 1989).

In COS-7 and HepG2 cells, ER α activates transcription in response to E₂ in a dose-dependent manner, reaching maximal activation levels already at about 10⁻⁹ M of E₂ (Figure 3). By comparison, ER α (P129R) activates transcription to lower levels (*P* < 0.05;

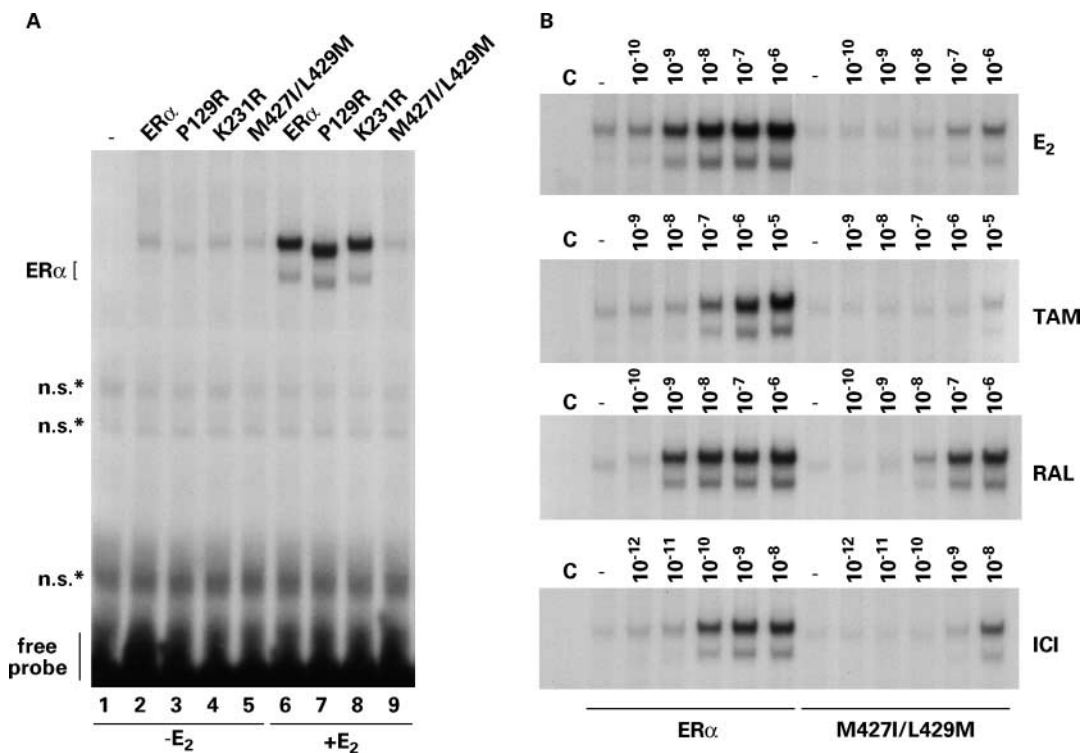


Figure 2. DNA binding of wild-type and mutant ER α . (A) EMSA of ER α , ER α (P129R), ER α (K231R), and ER α (M427I/L429M) in the absence and presence of 10⁻⁸ M E₂. (B) Comparison of DNA binding of ER α and ER α (M427I/L429M) in response to increasing concentrations of E₂, TAM, RAL and ICI (*non-specific).

Mann–Whitney *U*-test). However, wild-type ER α and ER α (P129R) display similar response profiles upon stimulation with E₂. These observations suggest that the P129R mutation in the NTD may affect the activity of the AF1, thereby accounting for the reduced overall activity of the mutant receptor. ER α (M427I/L429M), on the other hand, only weakly activates transcription at low E₂ concentrations (10⁻¹⁰ or 10⁻⁹ M) when compared with wtER α ($P < 0.01$; Mann–Whitney *U*-test), but reaches activation levels similar to those of the wild-type receptor at 10⁻⁸ or 10⁻⁷ M E₂. No significant differences were observed between the transcriptional activities of ER α (K231R) and the wild-type receptor (data not shown). Comparable transcriptional activities of the receptor mutants and wild-type ER α were also observed with the p(S2-ERE)₃-TK-Luc reporter in COS-7 and HepG2 cells and with p(vit-ERE)₂-TK-Luc in Hec1A and Hec1B cells (data not shown). These results demonstrate that two of the three ER α mutants show defects in transcriptional activation. While the mutation in ER α (P129R) possibly affects the AF1 located in the NTD, either DNA binding or the AF2 located in the LBD is affected in ER α (M427I/L429M).

The activation function in the NTD of ER α (P129R) is impaired

Polypeptide growth factors such as the EGF have been shown to stimulate ER α secondary to interaction with AF1, which is located in the NTD (Bunone *et al.*, 1996). In transient transfection assays the activity of wild-type ER can be either stimulated by E₂ or EGF, and co-administration of both substances further increases the activity of the receptor in an additive manner (Figure 4). ER α (P129R) shows a decreased response to EGF alone as well as to the combination of E₂ and EGF ($P < 0.01$, Mann–Whitney *U*-test) (Figure 4). However, the response of ER α (P129R) to E₂ is comparable to that of wild-type ER α .

The reduced transcriptional activity of ER α (M427I/L429M) appears to result from altered ligand binding

The reduced transcriptional activity of ER α (M427I/L429M) at low hormone concentrations may result from reduced DNA binding in cells similar to the weak binding in EMSAs. Alternatively, a reduced binding of E₂ to the mutant receptor LBD or an impaired

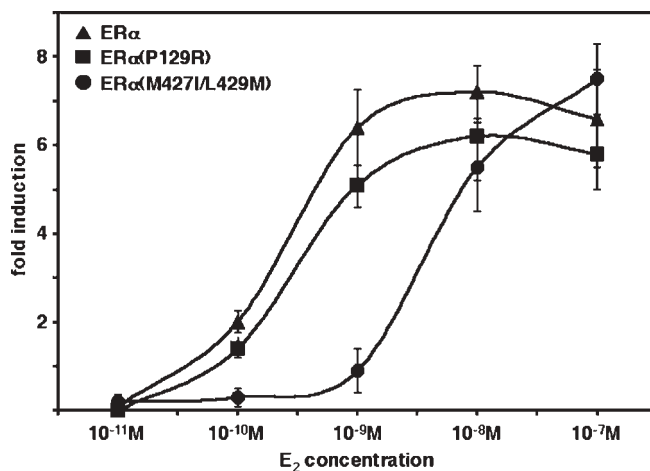


Figure 3. Transcriptional activities of wild-type and mutant ER α in transient transfection assays. Transcriptional activation by ER α , ER α (P129R), and ER α (M427I/L429M) on a p(vit-ERE)₂-TK-Luc reporter in response to increasing concentrations of E₂ in COS-7 cells. Mean and standard deviations are shown.

LBD–co-activators interaction could account for the lower transcriptional activity. Fusion proteins between the Gal4 DBD and the wild-type or mutant ER α LBD were generated to further investigate these possibilities. The use of the heterologous Gal4 DBD is expected to suppress specific allosteric interactions between wild-type or mutant ER α LBD and ER α DBD. In transient transfection assays GalER α -LBD modestly activates transcription in response to low concentrations of E₂ and reaches maximum activity at about 10⁻⁸ to 10⁻⁷ M E₂ (Figure 5A). GalER α -LBD(M427I/L429M) is transcriptionally inactive at low E₂ concentrations, but activates to wild-type receptor levels at 10⁻⁷ M E₂. This behaviour of the wild-type and mutant Gal fusion proteins closely resembles that of the full-length receptor. Next, we co-transfected an expression plasmid for the co-activator SRC-1, which is known to enhance ER α activity (Onate *et al.*, 1995; Shibata *et al.*, 1997). At saturating E₂ concentration, the activities of GalER α -LBD and GalER α -LBD(M427I/L429M) are equally stimulated by SRC-1 (Figure 5B). These observations support the idea that the low activity of ER α (M427I/L429M) at low E₂ concentrations initially results from reduced ligand binding and can be overcome by saturating ligand concentrations. As a consequence, the reduced ligand binding of the mutant receptor results in compromised DNA binding and/or transcriptional properties. However, at saturating E₂ concentrations, the ‘active’ conformation of the LBD does not seem to differ in wild-type and mutant receptor since their activity is equally well stimulated by SRC-1.

Discussion

We have identified different somatic point mutations in the ER α gene in three out of 55 adenomyosis samples using a PCR-SSCP approach. It is likely that the frequency of somatic mutations in adenomyosis is even higher since SSCP analysis is estimated to detect only about 90% of gene mutations (Condie *et al.*, 1993; Hayashi and Yandell, 1993). The prevalence of somatic ER α mutations in our samples (about 5%) suggests that genetic changes could be of relevance for the pathophysiology of adenomyosis. While other groups have reported on the association of ER α polymorphisms and splice variants with endometriosis (Fujimoto *et al.*, 1997; Georgiou *et al.*, 1999; Kitawaki *et al.*, 2001), we are the first describing somatic ER α point mutations and their functional characteristics in adenomyosis uteri.

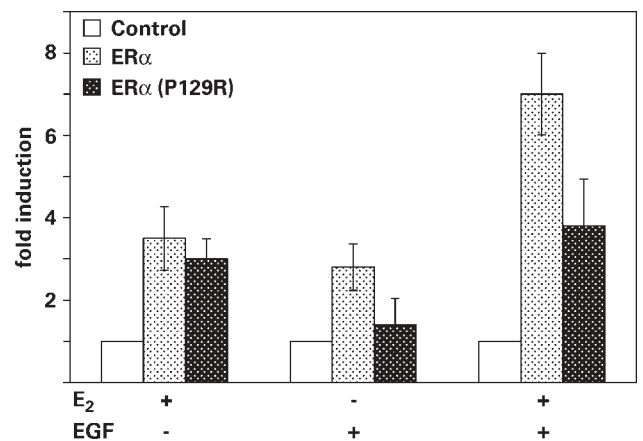


Figure 4. Stimulation of different activation functions (AF1 or AF2) of wild-type and mutant ER α in transient transfection assays. Transcriptional activities of ER α and ER α (P129R) on a p(vit-ERE)₂-TK-Luc reporter upon stimulation of the AF1 with EGF (100 ng/ml) and/or of the AF2 with E₂ (10⁻⁸ M) in HepG2 cells. Mean and standard deviations are shown.

ER α (K231R) contains a point mutation in the DBD that is located at the tip of the second zinc-finger adjacent to the D-box dimerization motif (Schwabe *et al.*, 1993). However, this mutation apparently does not affect DNA binding and transactivation properties of the receptor. By contrast, the mutants ER α (P129R) and ER α (M427I/L429M) display significantly altered DNA binding and transcriptional properties compared to wild-type ER α .

The P129R mutation is located in the NTD in the proximity of serine 118, which can be phosphorylated by mitogen-activated protein kinases in response to mitogenic stimuli such as EGF treatment. Moreover, phosphorylation of serine 118 is essential for AF1 activity (Ignar-Trowbridge *et al.*, 1992; Ali *et al.*, 1993). Importantly, in P129R a rigid and hydrophobic proline residue is replaced by a large basic arginine. Due to the distinct chemical and architectural characteristics of these amino acids, we anticipated that this mutation could affect the structure and/or the function of the NTD. Indeed, EMSAs revealed a significantly higher mobility of ER α (P129R) compared to wild-type ER α (Figure 2A), suggesting that the overall structure of the mutant NTD is strongly perturbed. Similarly, the reduced transactivation potential of ER α (P129R) (Figure 3) most likely results from mutation-related structural

changes. These findings indicate that either the mutant NTD lacks transcriptional activity itself, or transcriptional cooperativity between the NTD and the LBD is affected as has been observed in certain cellular environments (Gandini *et al.*, 1997). To address this issue, we chose EGF for stimulation of AF1 of wild-type and mutant ER α . As expected, ER α (P129R) can be activated by E₂, but is resistant to EGF stimulation (Figure 4). The transcriptional activity of wild-type ER α , however, can be stimulated by either EGF or E₂, or by both substances in an additive fashion (Figure 4) (Ignar-Trowbridge *et al.*, 1996). Thus, the reduced overall activity of ER α (P129R) most likely results from an impaired AF1. It is conceivable that the perturbed structure of the NTD affects the phosphorylation of the AF1 and/or compromises co-activator recruitment.

The double point mutation of ER α (M427I/L429M) is located in H7 of the receptor LBD. Crystal structures of agonist- and antagonist-bound ER α LBDs (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998) show that M427 and L429 are involved in the packing of H7 against neighbouring structural elements (H10/H11 on one side and the loop preceding the β -sheet on the other side). M427 primarily forms contacts with H516 and M517, while L429 interacts with the main chain and/or the side-chain atoms of G400, K401, L402, and R412 (Figure 6A). Importantly, M427 and L429 are neighbouring residues of L428 that forms part of the hydrophobic pocket involved in the binding of E₂, TAM, RAL, and ICI (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Pike *et al.*, 2001; Nettles *et al.*, 2004). Other residues contributing to the ligand-binding pocket are F404 (β -sheet), M421 (loop H6/H7), I424 (H7), and indirectly F425 (H7) (Figure 6A). It therefore appears reasonable to suppose that mutation of M427 and L429 perturbs the local architecture of the LBD, which subsequently affects ligand binding, transcriptional and/or DNA-binding properties of the receptor. Our transfection experiments indicate that the effects of the mutations can be compensated by very high concentrations of the ligand.

The positions occupied by E₂, TAM or RAL in the ligand-binding pocket of ER α are distinct due to the different ligand skeletons (Figure 6B and C). The wild-type receptor adapts to different ligands by adopting slightly changed side-chain or loop conformations as exemplified by movements of the H6/H7 loop or of the side-chains of M421 and F425 and, to a lesser extent, of F404 (Figure 6B and C). This observation suggests that the region around the β -sheet, H6, and H7 is relatively flexible and possibly sensitive to mutation-induced structural changes. In addition, this implies distinct and ligand-dependent effects and may thus explain why a smaller excess of RAL (relative to E₂ or TAM) is required to induce maximal DNA binding of ER α (M427I/L429M) in EMSAs (Figure 2B).

Other mutations documenting the importance of the LBD for function of ER α have been identified previously. The mutations ER α (M421T) and ER α (D426Y) were generated by site-directed random mutagenesis, regardless of their occurrence *in vivo* (Wrenn and Katzenellenbogen, 1993; Miller and Whelan, 1998). They showed functional properties (such as decreased transactivation potency) similar to ER α (M427I/L429M), thereby confirming our findings.

Two of the somatic ER α gene mutations (ER α (P129R) and ER α (M427I/L429M)) identified in this study result in proteins with impaired functions at physiological E₂ levels. The presence of loss-of-function mutations in adenomyotic lesions is consistent with the observation that proliferation of these lesions can occur under hypoestrogenic conditions and estrogen ablation therapy has only little influence on the course of the disease. To date, the complex mechanisms that govern the delicate regulatory balance of the ER signaling network are only partially understood (McDonnell and Norris,

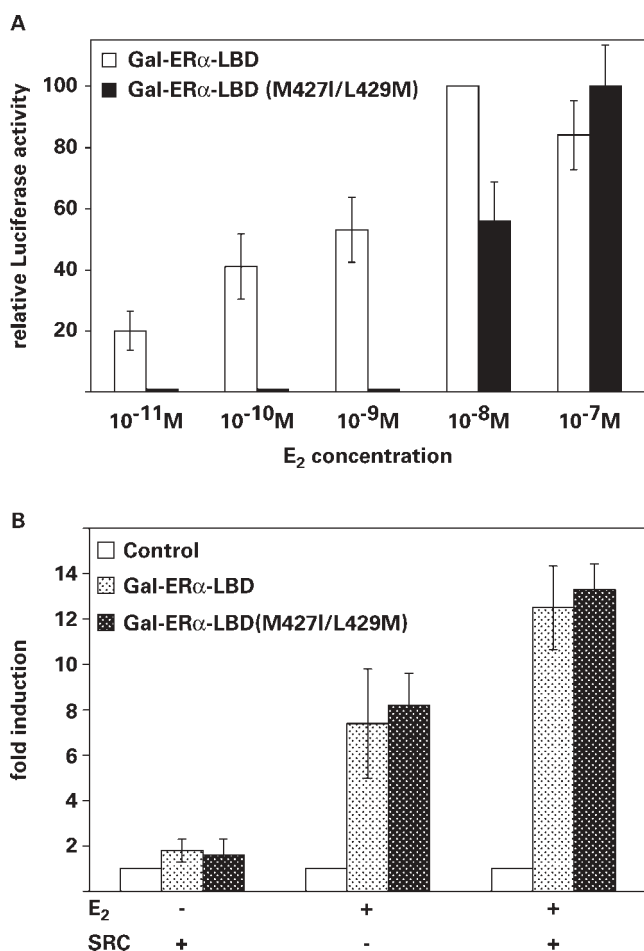


Figure 5. Transcriptional activities of wild-type and mutant Gal-ER α -LBD fusion proteins in transient transfection assays. (A) Transcriptional activation by Gal-ER α -LBD and Gal-ER α -LBD(M427I/L429M) on a p(Gal4)₃-TK-Luc reporter in response to increasing concentrations of E₂ in Hec1B cells. Luciferase activity was calculated relative to the one achieved by stimulation of Gal-ER α -LBD with 10⁻⁸M E₂. (B) Stimulation of the activity of Gal-ER α -LBD and Gal-ER α -LBD(M427I/L429M) by the co-activator SRC-1 in the presence of E₂ (10⁻⁸M) in HepG2 cells. Mean and standard deviations are shown.

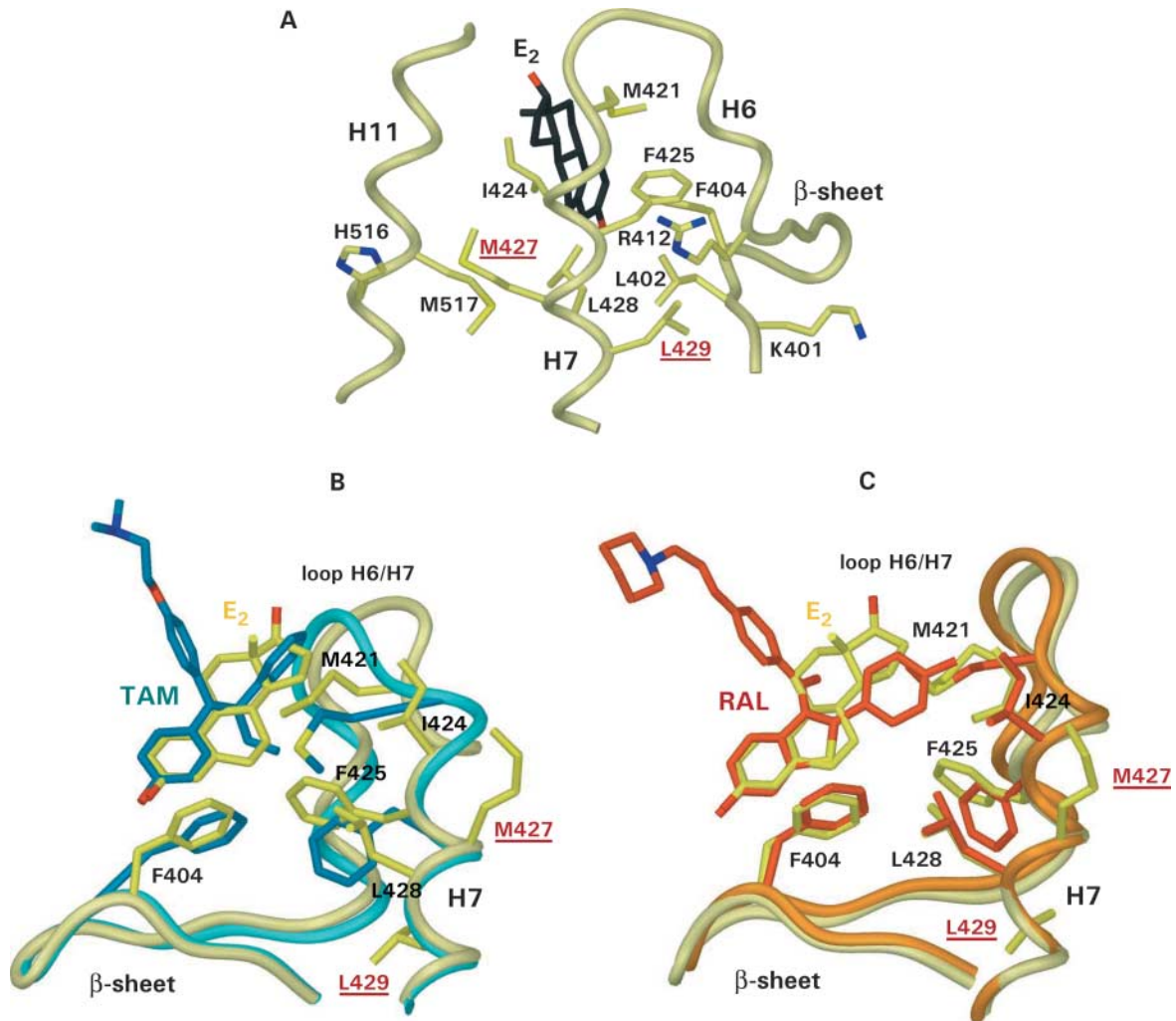


Figure 6. Analysis of the protein environment of M427 and L429 mutated in ER α (M427I/L429M). (A) Schematic representation of selected parts of the ER α LBD bound to E₂ as observed in the crystal structure [PDB: 1ERE]. (B) Superimposition of selected parts of the ER α LBD bound to E₂ with the corresponding region of the ER α LBD/TAM complex [PDB: 3ERT] or (C) of the ER α LBD/RAL complex [PDB: 1ERR].

2002). We are therefore left to speculate on potential mechanisms that might lead to the occurrence of defective ER in endometrium-derived cells. We hypothesize that cells with variant ERs as described in our study represent a subgroup which has acquired the ability to activate novel ER signalling pathways for its proliferation and survival even in the absence of physiological E₂ levels and full ER signalling. Estrogen deprivation might therefore promote the selection and/or even proliferation of those cells. Consequently, any therapeutic approach targeting ER-mediated signalling via reduction or even complete withdrawal of estrogen would have no beneficial, but rather a negative effect on the course of adenomyosis uteri.

It would be interesting to know whether mutated ER α with either constitutive activity and/or increased response to estrogen stimulation are present in endometriotic lesions. However, previous mutational analyses on various estrogen-dependent tissues have shown that ER α mutations resulting in hypersensitive or constitutively active receptor proteins are seldom encountered. By contrast, impaired ER function or decreased ER expression is a common finding in various steroid hormone-dependent diseases including endometrial and breast malignancies (Fuqua *et al.*, 1992; Schodin *et al.*, 1995; Ohlsson *et al.*, 1998). To further elucidate the role of aberrant ER or other steroid receptors in adenomyosis and endome-

triosis, it is mandatory to prospectively screen larger cohorts of patients for the presence of gene mutations affecting steroid receptor function and to correlate these findings with the course of the disease. If patients with somatic ER mutations would represent a subgroup with distinct clinical characteristics this might lead to a molecular sub-classification of endometriosis and potentially also to different treatment options.

Acknowledgements

We are grateful to Prof S.L.Woo, Department of Obstetrics and Gynaecology, University of Beijing, China for providing us with the tissue and DNA specimens.

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Submitted on June 28, 2004; revised on August 30, 2004; accepted on September 8, 2004