HNRNP G and HTRA2-BETA1 regulate estrogen receptor alpha expression with potential impact on endometrial cancer

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Abstract

**Background:** Estrogen receptor alpha (ERα/ESR1) expression is regulated by alternative splicing. Its most frequently detectable exon7 skipping isoform (ERαD7) is a dominant negative variant. Elevated expression of ERαD7 was already detected in endometrial cancer (EC), while its potential prognostic significance has not been characterized so far. Exon7 contains potential binding sites for the two functional splicing regulatory opponents, HNRNPG and HTRA2-BETA1 known to trigger opposite effects on EC outcome. This study served to elucidate the influence of HNRNPG and HTRA2-BETA1 on ERα exon7 splicing regulation and the impact of ERαD7 concentration on type 1 EC outcome.

**Methods:** Functional in vitro experiments for HNRNPG and HTRA2-BETA1 in regard to the regulatory impact on endogenous and exogenous ERαD7 splicing were performed. Additionally, real-time PCR determined mRNA levels of ERαD7, HNRNPG and HTRA2-BETA1 in 116 type 1 EC patients.

**Results:** HNRNPG and HTRA2-BETA1 were found to be specific regulators of ERα exon7 splicing. While HTRA2-BETA1 promoted exon7 inclusion, HNRNPG antagonized this effect by inducing exon7 skipping (p = 0.004). ERαD7 was detected in 71 out of 116 type 1 EC specimens. Statistical analyses revealed an inverse correlation between ERαD7 mRNA levels and tumor grading (p = 0.029), FIGO stage (p = 0.033) as well as lymph node metastases (p = 0.032), respectively. Furthermore, higher ERαD7 expression could be correlated to an improved disease-specific survival (p = 0.034).

**Conclusions:** Our study demonstrates antagonistic regulatory effects of HNRNPG and HTRA2-BETA1 on ERα exon7 splicing with potential impact on type 1 EC clinical outcome due to the consecutively variable expression levels of the ERα isoform D7.

**Keywords:** HNRNPG, HTRA2-BETA1, Estrogen receptor alpha, Endometrial carcinoma, Prognostic significance, Alternative splicing

Background

Endometrial cancer (EC) is the most common gynecological malignancy in the western world and accounts for 6% of all cancers in females [1]. The incidence is estimated at 15–20 per 100,000 women per year and it mainly affects peri- and postmenopausal women, with 89% of cases occurring between 65–69 years of age [2,3]. EC is classified into two subtypes: the estrogen-dependent type 1 with a background of excessive exposure to estrogen unopposed by progesterone and the estrogen-independent type 2 [4]. The lack of expression of estrogen receptor alpha (ERα/ESR1) in type 1 EC was found to be associated with poor differentiation of cancer tissues and poor survival rates of EC patients, respectively [5,6], supporting the hypothesis of a direct involvement of ERα in EC tumorigenesis and progression. The expression of ERα in normal or malignant endometrial tissue is subjected to alternative splicing modulating its biological function [7]. Several ERα splice variants with varying functional differences were described. ERα isoform skipping...
exon7 (ERαD7) is isoform has been identified as the most common phenotype in EC and estrogen receptor and encodes for a protein lacking a portion of the hormone binding domain [7,10]. This isoform represents a dominant negative variant for both ERα and ER β [7,10]. Induced ERαD7 expression has been detected in the proliferative compared to the secretory phase of endometrial tissue [11] and also in well to moderately differentiated EC in comparison to poorly differentiated EC [12]. Besides these findings and an influence on estrogen therapy sensitivity in schizophrenic patients [13], the clinical significance of ERαD7 in estrogen related cancer has not been elucidated yet. Particularly the regulation of ERα mRNA processing is not well understood, despite ERα exon 7 contains potential binding sites for the two antagonistic splicing factors HTRA2-BETA1 and HNRNPG (Figure 1). Recently our group was able to link alternative splicing regulation to EC tumor biology and clinical outcome [14] and identified HNRNPG and HTRA2-BETA1 as independent prognosticators for EC type I progression-free survival. Their antagonizing effects on alternative splicing processes were directly reflected by their opposite effects on EC biology.

Since alternative splicing is a pertinent control mechanism of gene expression with consecutive impact on cellular processes like growth, apoptosis, invasion and metastasis, respectively [15], we intended to elucidate the potential regulatory influence of HNRNPG and HTRA2-BETA1 on ERαD7 isoform expression profile in type 1 EC, as well as its potential impact on clinicopathological characteristics and clinical outcome.

Methods

Patients and tissue samples

One hundred and sixteen consecutive patients with type 1 EC, who were treated at the Gynecological Hospital of University Medical Center Freiburg between November 1997 and December 2005, were included in this study. Median age of patients at the time of diagnosis was 65. Patients receiving hormone replacement therapy prior to surgery were excluded from the study. All patients underwent hysterectomy, salpingo-oophorectomy and pelvic lymphadenectomy (according to the current national guidelines), and were properly staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification at the time. Tissue samples were obtained at the time of surgery and collected in the tumor tissue bank of Comprehensive Cancer Center Freiburg (CCCF), Germany. The institutional review board of CCCF and the local ethical committee of the University Medical Center Freiburg approved and licensed the investigation protocol of this study (#32409).

All patients involved gave their informed consent prior to inclusion in this study.

Paraffin embedded tissue specimen from hysterectomies were obtained from the Institute of Pathology in University Medical Center Freiburg. All hematoxylin-eosin stained slides were reviewed by specially trained pathologists (AzH, MOV). Histological classification was performed according to the World Health Organization 2003 system [16] into well differentiated (G1; n = 33), moderately differentiated (G2; n = 59), and poorly differentiated (G3; n = 24), respectively. Most patients neither had regional lymph node metastases (81.9%) nor distant organ metastases (68.1%). Cancer relapse was found in 17 patients during follow up (14.7%). The time to relapse ranged from 10–101 months after surgery. During follow up nine patients with recurrence died from EC and one from other cause. Seven recurrent patients were under further follow up for an additional median time of 17 months (range 0.3–42 months, Table 1).

RNA extraction from paraffin embedded tissue and cDNA synthesis

Each paraffin block used for RNA extraction was histologically assessed with regard to tumor homogeneity to guarantee a tumor cell content of more than 90%. Total tissue RNA was extracted by using the High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. RNA quality was controlled by densitometry and accepted with A260/280 > 1.7. RNA integrity was controlled on a 2100 Accessory & Spare Parts system (Agilent Technologies, Waldbronn, Germany). Prior to RT-PCR, each RNA sample was digested with 2.0 U DNase I (Roche, Mannheim, Germany) at 37°C for 45 min to eliminate genomic DNA (gDNA) contamination. Four µg of purified RNA were transcribed into cDNA using M-MLV reverse transcriptase (Promega, Mannheim, Germany) and 10 pM random hexamer primers (New England Biolabs GmbH, Frankfurt, Germany) in a total volume of 50 µl.

Real-time quantitative PCR

Primers used for real time PCR were all designed in an exon flanking way, except for ERα exon1 (Additional file 1: Table S1). Since ERα exon1 is constitutively transcribed in all ERα mRNA isoforms, we used this amplicon to
represent the total \( ERa \) transcript level. \( ERaD7 \) sense primer was located in conjunction part of \( ERa \) exon6 and 8 and the antisense primer in \( ERa \) exon8. This primer pair was designed to exclusively detect the \( ERaD7 \) isoform.

Samples of cDNA were heated to 95°C for 5 min followed by 45 cycles of 95°C 20 s, 60°C 20 s, 72°C 20 s. Expression of each gene was aggregated and then normalized against housekeeping gene (HKG) RPS18. Relative expression levels were calculated using the following formula: \( \text{Ratio} = \frac{E_{\text{target}}^{\Delta C_{\text{target}}} \cdot \text{control}}{E_{\text{HKG}}^{\Delta C_{\text{HKG}}} \cdot \text{control}} \) [17]. All PCR analyses were performed in triplicates, while arithmetic mean of data served as base for subsequent statistical analysis.

**Plasmid construction**

Full length of \( HNRNPG \) cDNA (NCBI Reference Sequence: NM_001164803.1) was subcloned into the mammalian expression vector pCMV-Script (Stratagene, Agilent Technologies, Waldbronn, Germany). The pCMV-Script
vector was digested by EcoRV restriction enzyme (Fermentas, St. Leon-Rot, Germany) at 37°C for 1 hour. After digestion, blunt vector ends were dephosphorylated by Antarctic phosphatase (New England Biolabs, Frankfurt, Germany). HNRNPG full length cDNA was phosphorylated by Polynucleotide Kinase (New England Biolabs, Frankfurt, Germany). Dephosphorylated vector and phosphorylated insert were purified with High Pure PCR product purification Kit (Roche, Mannheim, Germany) prior to ligation. Blunt end ligation was achieved by using T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) at 4°C overnight.

HTRA2-BETA1 expression plasmid was kindly provided by Prof. Stefan Stamm (Stamm’s Lab at Department of Molecular & Cellular Biochemistry, University of Kentucky, Lexington, U.S.A.). HNRNPG and HTRA2-BETA1 shRNA plasmids were obtained from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, U.S.A.).

**ERα exon7 minigene construction**

ERα exon7 together with part of its upstream and downstream intron sequence was subcloned into pCMV-INS plasmid. pCMV-INS plasmid contained insulin (INS) gene exon2 and exon3. Vector plasmid was digested between INS exon2 and exon3 with Pfl23II restriction enzyme (Fermentas, St. Leon-Rot, Germany). Vector dephosphorylation, insert phosphorylation and ligation were conducted as described above. The amount of insert for ligation was calculated according to following formula: Insert mass (ng) = 6 × Insert length (bp) / Vector length (bp) × Vector mass (ng). Plasmids were subsequently verified by sequence analyses (GATC BIOTECH, Konstanz, Germany). Plasmid-relevant PCR products are shown in Figure 2D.

**Cell culture and transfection**

Human EC cell line Ishikawa and human cervical cancer cell line HeLa were maintained in GIBCO® DMEM media (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany). Cells (150 × 10^6/well) were seeded in 6-well plates (Ø 30 mm) 24 hours before transfection, leading to a cell confluency of 60-70%. Plasmid DNA was transfected into cells via polyethylenimine (PEI) transfection. Empty pCMV-Script vector was used as a transfection control (Additional file 1: Table S2).

Endogenous ERα exon7 splicing pattern were detected in differently treated Ishikawa cells after single HNRNPG

### Table 1 Clinico-pathological features of patient cohort (Continued)

| Type I EC (n = 116) | Other related death | Further follow up |
|--------------------|---------------------|-------------------|
| Age (years)        |                     |                   |
| <65                |                     |                   |
| ≥65                |                     |                   |
| Histological type  |                     |                   |
| Endometrioid adenocarcinoma | 102 (87.9%)         |                   |
| Adenosquamous carcinoma | 14 (12.1%)         |                   |
| WHO Grade          |                     |                   |
| G1                 | 34 (29.3%)          |                   |
| G2                 | 59 (50.9%)          |                   |
| G3                 | 23 (19.8%)          |                   |
| Tumor size         |                     |                   |
| 1                  | 86 (74.1%)          |                   |
| 2                  | 14 (12.1%)          |                   |
| 3                  | 14 (12.1%)          |                   |
| 4                  | 2 (1.7%)            |                   |
| LN status          |                     |                   |
| Negative           | 95 (81.9%)          |                   |
| Positive           | 14 (12.1%)          |                   |
| Unknown            | 7 (6.0%)            |                   |
| Metastases         |                     |                   |
| Negative           | 79 (68.1%)          |                   |
| Positive           | 7 (6.0%)            |                   |
| Unknown            | 30 (25.9%)          |                   |
| Lymphagiosis       |                     |                   |
| Negative           | 24 (20.7%)          |                   |
| Positive           | 26 (22.4%)          |                   |
| Unknown            | 66 (56.9%)          |                   |
| FIGO stage         |                     |                   |
| I                  | 51 (44.0%)          |                   |
| II                 | 7 (6.0%)            |                   |
| III                | 24 (20.7%)          |                   |
| IV                 | 10 (8.6%)           |                   |
| Unknown            | 24 (20.7%)          |                   |
| Postoperative therapy |                 |                   |
| No therapy         | 35 (30.2%)          |                   |
| Brachytherapy      | 41 (35.3%)          |                   |
| Radiotherapy       | 28 (24.2%)          |                   |
| Chemotherapy       | 2 (1.7%)            |                   |
| Chemotherapy & radiotherapy | 6 (5.2%)   |                   |
| Unknown            | 4 (3.4%)            |                   |
| Recurrent EC and outcome |             |                   |
| EC recurrent       | 17 (14.7%)          |                   |
| EC related death   | 9 (7.8%)            |                   |
Figure 2 Functional implications of HNRNP G and HTRA2-BETA1 in EC in vitro. (A) HNRNP G and HTRA2-BETA1 protein expression in Ishikawa cells transiently transfected with expression and knock-down plasmids; (−) shRNA and (+) expression plasmid for HNRNP G and HTRA2-BETA1; (C) control: empty pCMV-plasmid. Western blot. (B) Influence of HTRA2-BETA1 and HNRNP G mRNA-levels on endogenous E7a-exon7 mRNA splicing. (C) cells transfected with: control: empty pCMV-plasmid; (HTRA2-BETA1+) HTRA2-BETA1-expression-plasmid; (HTRA2-BETA1−) HTRA2-BETA1-shRNA; (HNRNP G+) HNRNP G-expression-plasmid; (HNRNP G−) HNRNP G-shRNA. RT-PCR. (C) HNRNP G, HTRA2-BETA1, E7a-standard and E7a-exon6 mRNA expression in differently treated Ishikawa cells. (C) control:pCMV-plasmid; (HTRA2-BETA1+) HTRA2-BETA1-expression-plasmid; (HTRA2-BETA1−) HTRA2-BETA1-shRNA; (HNRNP G+) HNRNP G-expression-plasmid; (HNRNP G−) HNRNP G-shRNA. RT-PCR. (D) Exogenous level of E7a-exon7 splicing pattern. Influence of overexpression (+) and knock-down (−) HNRNP G and HTRA2-BETA1 on alternative E7a-exon7 minigene expression. In untransfected control cells, the reporter gene was alternatively spliced into 4 isoforms, two precisely spliced isoforms are exon7-skipping (137bp) and exon7-inclusion (321bp). Two lariat containing isoforms are: one containing a part of intron sequence between INS-exon2 and -3 (210bp), another containing an additional pseudo-exon from exon7 5’ intron sequence (544bp, all four isoforms were verified by sequencing). RT-PCR. (E,F) E7a exon7 alternative splicing regulation by HTRA2-BETA1 and HNRNP G in Ishikawa cells. (E) E7a-exon7 skipping/inclusion ratio; (F) HNRNP G/HTRA2-BETA1 ratio in differently treated Ishikawa cells. (G) E7a exon7 skipping/inclusion mRNA ratio difference between HTRA2-BETA1overexpression and HNRNP G overexpression group. **E7a exon7 skipping/inclusion ratio between the two groups was statistically significant p= 0.004. PCR-based tests originate on arithmetic mean of triplicate analyses. Student-T-test was applied for data shown in E-G, while statistical significance was assumed at p<0.05 at the two-sided test. Representative gel images in B-D demonstrate one out of three repeats.
or HTRA2-BETA1 plasmid transfection, while exogenous splicing pattern were analyzed in co-transfected HeLa cells with the ERα exon7 minigene as reporter.

RNA and protein extraction
Total cellular RNA and protein were extracted 48 hours after transfection applying TRIZol® reagent (Invitrogen, Karlsruhe, Germany) isolation protocol. Before RT-PCR, DNase I digestion was used to exclude gDNA contamination as well.

Western blot for hnRNPG and HTRA2-BETA1 detection
Western blot analyses were performed to evaluate the hNRNP and HTRA2-BETA1 expression plasmid as well as shRNA plasmid efficacy after transfection. The immune complexes were visualized by an ECL assay (Figure 2A). Rabbit polyclonal IgG HNRNP (RBMX) antibody (sc-48796, Santa Cruz Biotechnology, Inc.) and rabbit polyclonal IgG HTRA2-BETA1 antibody [18] (provided by Prof. Stefan Stamm, (Stamm’s Lab, Department of Molecular & Cellular Biochemistry, University of Kentucky, Lexington, U.S.A.)) were used.

ERα exon7 detection in transfected Ishikawa and HeLa cells
The primer design for ERα exon7 amplification (amplicon ranging from exon6 to exon8) allowed the detection of both exon7 inclusion and skipping isoforms. Since ERα exon6 is also subject to alternative splicing, ERα exon6 as well as ERα standard (primers located in exon1) PCR assays were used as ERα transcript level control for differently transfected cells (primer sequences: Additional file 1: Table S1). Conditions for ERα standard, exon 6 and exon7 PCR were as follows: 95°C 5 min, followed by 35 cycles 95°C 20 s, 60°C 20 s, 72°C 20 s.

The primers for the pCMV-INS based were complementary to INS exon 2 and exon 3, respectively and designed to detect different splicing pattern of ERα exon7 minigene after co-transfection. Post co-transfection PCR: 95°C 5 min, followed by 45 cycles 95°C 20 s, 60°C 15 s, 72°C 45 s. PCR products were separated by gel electrophoresis and quantitative analysis was conducted by application of imageJ software (http://rsbweb.nih.gov/ij/).

Sequence analysis
PCR products of endogenous and exogenous ERα exon7 alternatively spliced isoforms were subcloned into the CloneJET® PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany). PCR products of the HNRNP expression plasmid, ERα exon7 minigene and ERα exon7 isoform PCR products were sequenced by GATC BIOTECH (Konstanz, Germany). Furthermore, all PCR amplicons produced in different analyses were subject to verification via sequence analysis. Sequencing results were compared with NCBI reference sequences (http://www.ncbi.nlm.nih.gov/).

Statistical analyses
The expression levels of ERα were categorized as follows (normalization against RNA of HKG RPS18 for consecutive quantification:
- group 0: no ERα standard mRNA detectable; group 1: ERα standard mRNA amount ≤0.81; group 2: ERα standard mRNA amount >0.81, due to the mean mRNA level within ERα standard positive samples being 0.81. ERαD7 mRNA levels were defined as group 0: no ERαD7 mRNA detectable; group 1: ERαD7 mRNA amount ≤0.80; group 2: ERαD7 mRNA amount >0.80, due to the mean ERαD7 mRNA level within ERαD7 positive ones being 0.80.

The D7 real time PCR results were analyzed in regards to potential correlations with clinicopathological data by Spearman’s correlation test. Univariate and multivariate analyses were performed with Kruskal-Wallis H test and general linear model, respectively. When performing survival analyses, the records of patients who died of EC were considered to be uncensored; the records of patients who were alive during follow up or who died from other diseases were considered to be censored. Univariate analyses of disease-specific survival and progression-free survival were performed with Kaplan-Meier life-table curves and compared using the Log rank test. Multivariate prognostic analyses used multivariate Cox regression test in a forward step wise manner [19]. Student T test was used for RT-PCR results analyses. Statistical significance was assumed at p ≤ 0.05 at the two-sided test (SPSS 15.0 software, SPSS Inc.).

Results
HTRA2-BETA1 and HNRNP as antagonistic regulators of ERα exon7 splicing
Functional experiments in Ishikawa endometrial cancer cells with transient transfection of HTRA2-BETA1 and HNRNP expression plasmids revealed that endogenous exon7 inclusion was specifically induced by HTRA2-BETA1. In contrast, HNRNP acted as a splicing inhibitor with induced levels of exon7 skipping (Figure 2B). As a consequence, the exon7 skipping/inclusion ratio was significantly higher in HNRNP in comparison to HTRA2-BETA1 overexpression (Figure 2B, C, F, Additional file 1: Table S4). However, expression of endogenous ERα standard as well as ERα exon6 was not affected by the two splicing factors (Figure 2C).

Employing an ERα exon7 reporter gene the findings of a high specificity of the HTRA2-BETA1 and HNRNP effects were confirmed on the in vitro level (Figure 2D).

Induced ERαD7 expression is correlated to favorable clinico-pathological parameters
In type 1 EC, ERα mRNA expression could be detected in 87 samples (75%) of which 71 (61.2% of the complete cohort) expressed the ERαD7 isoform.
On the basis of categorization in groups 0–2 (see Methods, statistical analyses) both \( \text{ERa} \) standard and \( \text{ERaD7} \) mRNA levels were found to be inversely correlated to grading (−0.317, \( p = 0.001 \)) and FIGO stage (−0.222, \( p = 0.033 \)). Furthermore, increased \( \text{ERaD7} \) mRNA levels were detected in tumors without regional lymph node metastases (correlation coefficient = −0.206, \( p = 0.032 \), Table 2). The observed differences in \( \text{ERaD7} \) mRNA levels between well to moderately and poorly differentiated cancers, FIGO stage I/II and III/IV, as well as lymph negative and positive groups were all statistically significant (\( p = 0.030, p = 0.034, \) respectively, Kruskal-Wallis Test, Table 3).

The ratio of \( \text{ERaD7} \) to \( \text{ERa} \) standard is inversely related to \( \text{HTRA2-BETA1} \) expression

We chose real-time PCR quantification of \( \text{ERaD7} \) isoform since this methodical approach results in more accurate data on mRNA quantity. Real-time qPCR runs as a robust and reliable standard procedure in our lab and all randomly applied re-checks of qPCR products via classic gel electrophoresis accounted for the desired amplicons. Since our real-time PCR sense primer for \( \text{ERaD7} \) detection was designed to be complementary to the conjunction of exon6 and 8, the calculation of \( \text{ERaD7} \) mRNA level might be influenced by exon6 skipping, even though there were only 8 samples positive for \( \text{ERaD6} \). To overcome this problem, we also calculated the expression ratio of \( \text{ERaD7} \) in total \( \text{ERa} \) transcript amount (ratio = \( \text{ERaD7} \) mRNA level/\( \text{ERa} \) standard mRNA level) and performed additional analyses. Significant differences in the ratio between moderately and poorly differentiated, FIGO stage III/IV as well as lymph node positive tumors (\( p = 0.015, p = 0.016, p = 0.016, \) respectively, Kruskal-Wallis Test, Table 3) could be detected. Like the \( \text{ERaD7} \) mRNA level, its relative expression ratio in total \( \text{ERa} \) was also found to be associated with FIGO stage (\( R^2 = 2.311, p = 0.006, \) Additional file 1: Table S3). Furthermore, we were able to detect an inverse correlation of \( \text{ERaD7}/\text{ERa} \) standard ratio with \( \text{HTRA2-BETA1} \) mRNA levels (correlation coefficient = −0.198, \( p = 0.034 \), Table 2).

### Table 2

| Spearman’s correlation test | FIGO (I/II vs III/IV) | Grade (1/2 vs 3) | T (P vs N) | LN (P vs N) | M (P vs N) | L (P vs N) | hTra2β1 mRNA |
|-----------------------------|-----------------------|-----------------|------------|------------|------------|------------|--------------|
| \( \text{ERa} \) standard  | correlation            | −0.222*         | −0.317**   | N.S        | N.S        | N.S        | −0.214*      |
| coefficient                |                       | 0.033           | 0.001      | N.S        | N.S        | N.S        | 0.022        |
| p (2-tailed)               |                       | N                | 92         | 116        | 116        | 109        | 86           | 50           | 115          |
| \( \text{ERaD7} \)         | correlation            | −0.223*         | −0.203*    | N.S        | −0.206*    | N.S        | −0.332*      | −0.168       |
| coefficient                |                       | 0.033           | 0.029      | N.S        | 0.032      | N.S        | 0.019        | 0.073        |
| p (2-tailed)               |                       | N                | 92         | 116        | 116        | 109        | 86           | 50           | 116          |
| \( \text{ERaD7}/\text{standard} \) | correlation | −0.251*         | −0.227*    | N.S        | −0.232*    | N.S        | −0.407**     | −0.198**     |
| coefficient                |                       | 0.016           | 0.014      | N.S        | 0.015      | N.S        | 0.003        | 0.0034       |
| p (2-tailed)               |                       | N                | 92         | 116        | 116        | 109        | 86           | 50           | 116          |

T = Primary tumor; LN = lymph node metastasis; M = distant organ metastasis; L = lymphangiosis; P = positive; N = negative; p = p value; N.S = Not significant; * = significant at the 0.05 level (2-tailed); ** = significant at the 0.001 level.
Table 3  

|               | FIGO Grade | Grade | LN | L               | hTra2β1              |
|---------------|------------|-------|----|-----------------|----------------------|
|               | (I/II vs III/IV) | (1/2 vs 3) | (P vs N) | (P vs N) |                          |
| ERα standard  | mean rank  | 50.97 vs 38.87 | 63.74 vs 37.33 | 56.59 vs 44.21 | 29.25 vs 22.04 | 62.31 vs 53.61 |
|               | p (2-tailed) | 0.034 | 0.001 | 0.168 | 0.075 | 0.159 |
| ERαD7         | mean rank  | 50.90 vs 39.00 | 61.77 vs 45.26 | 57.42 vs 38.57 | 30.23 vs 21.13 | 62.62 vs 53.30 |
|               | p (2-tailed) | 0.034 | 0.030 | 0.032 | 0.020 | 0.123 |
| ERαD7/standard| mean rank  | 51.48 vs 38.00 | 62.17 vs 43.67 | 57.73 vs 36.46 | 31.31 vs 20.13 | 72.52 vs 49.00 |
|               | p (2-tailed) | 0.016 | 0.015 | 0.016 | 0.004 | 0.000 |

Figure 3  

Kaplan-Meier survival curves for disease-specific survival and progression-free survival in regard to ERα standard and ERαD7 expression (group 0: no ERα standard mRNA detectable; group 1: ERα standard mRNA amount ≤0.81; group 2: ERα standard mRNA amount >0.81, due to the mean mRNA level within ERα standard positive samples being 0.81. ERαD7 mRNA levels were defined as group 0: no ERαD7 mRNA detectable; group 1: ERαD7 mRNA amount ≤0.80; group 2: ERαD7 mRNA amount >0.80, due to the mean ERαD7 mRNA level within ERαD7 positive ones being 0.80.) Log rank test.
positive, and unknown status. As expected, differentiation grade was identified as an independent prognosticator for disease-specific survival, but ERα standard mRNA expression was also identified as an indicator for progression-free survival (Additional file 1: Table S3), respectively.

**Discussion**

ERα regulates gene expression either by binding to estrogen response elements (ERE) or through ERE-independent signaling (such as interactions with other transcription factors like AP-1, SP1, NF-KB) [20-24]. Those downstream effects from aberrant ERα regulation provoke changes in cellular function toward carcinogenesis. ERα as a prognosticator for EC has been studied for its potential influence on EC carcinogenesis. Horvath and colleagues reported a decrease of wild type ERα and an increase of ERαD7 isoform in estradiol treated EC tissue correlated with an estradiol-resistant growth phenotype with no growth rate change in comparison to controls [25]. These findings together with others suggested a competitive effect of ERαD7 on its wild type in determination of cellular hormone sensitivity [10,13]. Our study revealed an ERαD7 induction in type 1 EC and a correlation of its expression level to the FIGO stage. Higher ERαD7 mRNA levels were statistically significant correlated to an improved outcome with a better disease-specific survival as demonstrated by Kaplan-Meier survival curves (p = 0.034, Log-rank test, Table 3). The univariate survival analyses demonstrated a significant improved progression-free survival, defined as incidence of local or distant recurrence, for total ERα expression, which was already published in the literature. For ERαD7 a significant improved disease-specific survival, excluding all non-endometrial cancer related deaths, was also seen. In line with these observations the ratio of ERαD7/total ERα transformed into an improved progression-free survival (p = 0.037) in this EC subtype.

ERαD7 originates from an out-of-frame exon deletion that disrupts the ligand binding domain. Therefore supposedly functions as a dominant negative repressor of ERα transactivating properties [8]. Jazaeri et al. mention that ERα variants, e.g. ERαD7, may account for growth advantages in variant expressing cells under selective pressure caused by estrogens or anti-estrogens [8]. Furthermore they point out, that according to the heterodimer activity of ERα standard (wild-type) and variants, even small quantities of alternatively spliced isoforms can have a major effect on cell physiology [8]. We hypothesize, that the correlation of elevated ERαD7 expression and improved outcome in type 1 EC is based on the diminished cellular estrogen sensitivity. Malfunctioning estrogen receptor a-dependent transcription and associated tumor progression signaling pathways could account for the decrease of malignant behavior of ERαD7 expressing endometrial tumors. Furthermore, the pharmacological effect of anti-estrogens, e.g. tamoxifen, might be also reduced by ERαD7-mediated ERα resistance in regard to transcriptional activation of target genes. Functional studies demonstrated increased cellular levels of ERαD7 in response to both estrogen and tamoxifen exposure [25]. Interestingly, long-term exposure to either unopposed estrogen, e.g. hormone replacement therapies or tamoxifen treatment are major risk factors for EC [26].

So far the underlying mechanisms of regulation of ERα exon7 expression were not understood. Gotteland and colleagues described different ERα mRNA isoforms in physiological and malignant breast tissues, suggesting this phenomenon could be caused by alternative splicing, independent from cell transformation [27]. The analyses of the ERα exon7 sequence strongly supported the hypothesis of alternative splicing regulation (Figure 1A, B). Both, its 5´ polypyrimidine tract and 3´ intron sequence contain HNRNP I binding motifs [28-30]. It is known that HNRNP I represses exon splicing by looping out exons between its binding motifs, which has been found in various kinds of tissues (reviewed in [31-34]). This might explain why ERαD7 is the most frequently detectable isoform of ERα. It is well known that HTRA2-BETA1 preferentially promotes splicing of exons with GAA-rich domains in a concentration dependent manner [35,36]. The ERα exon7 sequence expresses two potential HTRA2-BETA1 binding motifs which could explain why HTRA2-BETA1 is promoting exon7 splicing on both, the endogenous as well as the exogenous level. HNRNPG is a known antagonizing factor of HTRA2-BETA1 activity in mRNA processing [37]. In our *in vitro* analyses, the expected antagonizing effects of HNRNPG on HTRA2-BETA1 became evident by the specific induction of ERα exon7 skipping (Figure 2B, D, G). Since exon7 contains the preferential HNRNP G binding sites AAGU and CC (A/C) [37,38] we hypothesize in accordance to other groups [37], that both splicing factors HTRA2-BETA1 and HNRNP G exhibit their antagonistic effects on ERα exon7 splicing by a concentration dependent competition (Figure 1C).

In previous studies, we analyzed ERα alternative splicing pattern in EC in regard to skipped exons or exon cassettes by use of combinatory primer pairs for PCR. Our prior analyses did not identify exon7/exon 8 skipping in EC samples, in detail: no EC cell line or EC tissue specimen (>20 specimen tested) exhibited this splicing possibility (data not shown).

Carcinogenesis is characterized by complex alterations in a magnitude of cellular mechanisms. Aberrant alternative splicing has a high impact on cellular processes that lead to cancer or promote cancer progression, including resistance to apoptosis and promotion of invasion, metastasis and angiogenesis, respectively [15]. Our previous study
demonstrated that HNRNP G and HTRA2-BETA1 trigger opposite effects on EC prognosis: a simultaneous higher level of HTRA2-BETA1 protein nuclear expression as well as mRNA is correlated to poor disease-specific as well as progression-free survival. On the contrary, high expression levels of nuclear HNRNP G protein and mRNA are associated with an improved clinical outcome in the same patient cohort. In our present study, we detected an inverse correlation between ERaD7 expression ratio and HTRA2-BETA1 mRNA level. Furthermore, our in vitro experiments demonstrated that HTRA2-BETA1 works as a splicing enhancer for ERa exon7, while HNRNP G acts as an opponent of HTRA2-BETA1 by antagonizing the HTRA2-BETA1 effect on ERa exon7 inclusion.

These functional data are in line with our observation regarding the correlation of ERaD7 expression and the clinicopathological features as well as outcome data of patients with type 1 EC.

Conclusions
The present study strongly supports our recently published hypothesis, that increased HNRNP G levels are associated with improved clinical outcome. This is due to the fact, that we were able to identify this nuclear protein as a specific regulator towards high levels of ERaD7 expression. However, the best proof for this theory is given by the fact that increased expression of ERaD7 was also characterized as a prognosticator towards an improved clinical outcome. The important biological role of ERαs in estrogen-dependent EC carcinogenesis is further supported by our study.

Taking all evidence into account, we hypothesize that expression pattern of splicing factors have profound effects on cancer cell biology. Our present study provides a new evidence for the pivotal impact of aberrations in alternative splicing pattern in carcinogenesis.

Additional file
Additional file 1: Table S1. Primers for real time and conventional PCR. Table S2. Plasmid transfection quantities. Table S3. Correlation of ERaD7 mRNA level with FIGO stage (Multivariate general linear regression test). Table S4. p value of ERα exon7 skipping/inclusion and HNRNP G/HTRA2-BETA1 mRNA ratio difference in differently treated cells.

Abbreviations
cDNA: Complementary deoxyribonucleic acid; EC: Endometrial cancer; ERα: Estrogen receptor alpha; ERαD3: Estrogen receptor alpha delta 3, splice variant; ERαD4: Estrogen receptor alpha delta 4, splice variant; ERαD5: Estrogen receptor alpha delta 5, splice variant; ERαD7: Estrogen receptor alpha delta 7, splice variant; ERΕ: Estrogen response elements; ESRI: Estrogen receptor 1, estrogen receptor alpha; FIGO: International Federation of Obstetrics and Gynecology; HKG: Housekeeping gene; HNRNP G: Heterogeneous ribonucleoprotein particle G; hNRNP I: Heterogeneous ribonucleoprotein particle I; HTRa2-beta1: Human Transform-2 sex-determining protein – beta 1; INS: Insulin; pCMV: Plasmid containing Cytomegalovirus sequence; real-time quantitative PCR: Real-time quantitative polymerase chain reaction; RT-PCR Reverse transcription - polymerase chain reaction.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MH and YQO substantially designed the experimental setup, were involved in practical realization and composed the manuscript. Statistical analysis was governed by YQO. MJ assisted in mRNA and protein identification procedures. The pathologists MOV and AH were responsible for tissue specimen provision and characterization. ES was significantly involved in experimental setup. ES and TE critically reviewed and approved the final manuscript. All authors read and approved the final manuscript.

Acknowledgements
We would like to thank Prof. Stefan Stamm for the provision of the HTRA2-BETA1 antibody.

The article processing charge was funded by the open access publication fund of the Albert-Ludwigs-University Freiburg.

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Received: 2 September 2014 Accepted: 19 February 2015
Published online: 27 February 2015

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