Recurrent hormone-binding domain truncated \( ESR1 \) amplifications in primary endometrial cancers suggest their implication in hormone independent growth

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The estrogen receptor alpha (ER\textsubscript{\( \alpha \)}) is highly expressed in both endometrial and breast cancers, and represents the most prevalent therapeutic target in breast cancer. However, anti-estrogen therapy has not been shown to be effective in endometrial cancer. Recently it has been shown that hormone-binding domain alterations of ER\textsubscript{\( \alpha \)} in breast cancer contribute to acquired resistance to anti-estrogen therapy. In analyses of genomic data from The Cancer Genome Atlas (TCGA), we observe that endometrial carcinomas manifest recurrent \( ESR1 \) gene amplifications that truncate the hormone-binding domain encoding region of \( ESR1 \) and are associated with reduced mRNA expression of exons encoding the hormone-binding domain. These findings support a role for hormone-binding alterations of ER\textsubscript{\( \alpha \)} in primary endometrial cancer, with potentially important therapeutic implications.

Endometrial cancer (EC) is the fourth most common malignancy of women and the most common pelvic gynecological malignancy in countries with advanced industrialization\textsuperscript{12}. But approved targeted therapies are still not in use today\textsuperscript{13,14}. ER\textsubscript{\( \alpha \)}, encoded by the gene \( ESR1 \), is known to be an important driver of cell proliferation\textsuperscript{9} and has been identified as a risk locus in breast cancer\textsuperscript{16,17}. Both breast as well as endometrial cancer are estrogen dependent and express the estrogen receptor alpha (ER\textsubscript{\( \alpha \)}) to a similar extent\textsuperscript{18-21}.

While ER\textsubscript{\( \alpha \)} constitutes the most frequently inhibited therapeutic target in breast cancer\textsuperscript{9}, anti-estrogen therapy has shown inconsistent results and mostly a very limited effect in endometrial cancers\textsuperscript{12-18}. The estrogen antagonist Tamoxifen can even increase the risk of carcinogenesis\textsuperscript{19-21}. Consequently anti-estrogen therapy does not constitute a component of standard therapy of EC\textsuperscript{3,4}. Since mutations and alternative splicing of \( ESR1 \)
that alter the hormone-binding domain have been shown to generate hormone independence or resistance to anti-estrogen therapy in breast and endometrial cancers, related genetic alterations could play a role for therapy outcome in primary endometrial carcinoma. Recent studies identified mutations of ESR1 in breast cancer that alter their hormone binding domain coding sequence, to be linked to endocrine therapy resistance in a metastatic setting. One study by Li et al. even demonstrates an ESR1 fusion in endocrine treatment resistant breast cancer, truncating the hormone-binding domain coding exons, while a later study by Veeraraghavan et al. identified evidence for another type of recurrent ERα-altering gene fusions in this tumor type. However, structural genetic alterations of ESR1 have not been suggested to play a role in endometrial cancer carcinogenesis. Due to the potential importance of such ESR1 alterations in endometrial cancer, we analyzed an tumor test subset of 29 primary endometrial cancers for somatic gene copy-number alterations (SCNA) and explored The Cancer Genome Atlas (TCGA) for concerning SCNA and mRNA expression data of endometrial carcinoma.

**Results**

Across a cancer study subset of 29 primary endometrial carcinomas that had gone on to metastasize, we characterized the copy-number changes by GeneChips and validated amplifications of ESR1 in these cancers by fluorescence in-situ hybridization (FISH). The Pearson correlation of ESR1 GeneChip copy numbers with FISH determined absolute average ESR1 copy numbers per nucleus and average ESR1 to centromere 6 (CEN6) ratios were $r = 0.743$ ($p < 0.001$) and $r = 0.774$ ($p < 0.001$) respectively (Appendix A, Fig. 1, Supplementary Figures S1 and S2, Supplementary Optical Dataset S1).

Four of these tumors exhibited focal ESR1 amplification determined by GeneChips, of which two amplifications showed 3' truncations of ESR1 ($\Delta$ exon 6–8 or 7–8) that would remove the hormone-binding domain...
We therefore explored the prevalence of ESR1-truncating amplifications across uterine corpus endometrial carcinoma within The Cancer Genome Atlas (TCGA). In the TCGA data subset of 539 endometrial carcinomas analyzed, we identified 88 (16.3%) cases with amplifications encompassing or overlapping ESR1. 46.6% of these were histologically defined serous and 75.0% of the tumors with ESR1 amplification were clustered within the serous-like copy-number high molecular subtype according to TCGA. The ESR1 amplifications were focal (less than half a chromosome arm in length) in 36 cases (6.7%) of tumors, and had a significantly higher rate of amplification than the genome-wide average (q = 5.75 × 10^-4). Mapping of the overlap between amplifications across tumors identified ESR1 only as the most likely gene target (see methods).

These amplifications appeared to truncate the hormone-binding domain encoding region in seven cases (1.3% of the entire dataset; and 19.4% of cases with focal ESR1 amplification) and to retain exons 1–4 or 1–3, encoding the N-terminal ESR1 transactivation domain (AF1) and DNA-binding domains. Another case without ESR1 amplification exhibited a heterozygous deletion of exons encoding the hormone-binding domain (Fig. 2), for a total apparent ESR1 truncation rate of 1.5% over all tumors. In one additional TCGA case, we detected a hormone-binding domain (exons 4–8) truncating ESR1–SYNE1 mRNA fusion (Appendix B). Eight of these
nine tumors were molecularly classified as being in the serous like copy-number high subgroup (4.3% of this subgroup)\(^{35}\).

**Association of ESR1 exon copy numbers with mRNA expression.** The ESR1 truncation events are associated with decreased mRNA expression of the truncated exons encoding the hormone-binding domain (exons 5–8) compared to the transactivation and DNA-binding domains (exons 1–4) (p < 0.001) (Fig. 2 and Appendix C). We compared the normalized ESR1 expression values estimated from RNA-Seq data for the eight tumors exhibiting amplified, truncated ESR1 to those from eight tumors selected on the basis of exhibiting similarly focal ESR1 amplifications that lack intragenic breakpoints. The average ratio between expression levels of exons 1–4 and 5–8 is 2.1-fold higher among truncated tumors relative to these controls (p = 0.003). We also confirmed this relation after replacing the eight ESR1-amplified controls with all 545 tumors profiled by TCGA. In this comparison, the ratio of expression levels between exons 1–4 and 5–8 is 2.2-fold higher in ESR1-truncated tumors (p < 0.001).

In contrast, TCGA breast cancers exhibit ESR1 truncations on DNA-level less than half as often (7 of 1080; 0.65%) as observed in endometrial cancer and had increased expression of exons 1–2, but not of the full DNA-binding domain (Appendix D). These data suggest that the amplified truncations and associated mRNA profiles we describe in endometrial cancer are not frequent in breast cancer.

**Discussion**

The gene truncations we report in endometrial carcinoma disrupt the hormone-binding domain encoding sequence of ESR1. Similarly, mRNA splice variants lacking one or more of exons 5–8, encoding the hormone-binding domain, have been described in normal\(^{35–38}\) and malignant\(^{22,23,35–37,39}\) breast as well as in normal\(^{24,40–45}\) and malignant\(^{42–46}\) endometrial tissue. Point mutations of the ligand binding domain encoding sequence of ESR1 have also been described to occur in both breast and endometrial cancers\(^{25–27,30,47,48}\).

Both splice variants and point mutations involving the ESR1 hormone-binding domain have been associated with hormone-independent E\(\alpha\) activity. The point mutations found in both breast and endometrial cancers have been shown to enable ligand-binding independent transcriptional activity\(^{26,30,48–50}\) and have been related to acquired resistance to anti-estrogen therapy in breast cancer\(^{26–28}\). Excisions of exons 5 and 7 by alternative splicing have also been shown to constitutively activate E\(\alpha\)\(^{22,23,30}\) and have been associated with hormone independent growth in both breast and endometrial cancers\(^{22–24,31}\). These findings raise the hypothesis that the ESR1 truncations we report may also generate hormone-independent E\(\alpha\) activity.

In breast cancer, point mutations in the ligand-binding domain occur in 20–50% of tumors that have acquired resistance to anti-estrogen therapy\(^{26–27}\) but only in 0.2% of primary cancers\(^{51}\). In endometrial cancer, however, point mutations and in-frame deletions altering the ligand binding domain occur in 2.8% of primary endometrial cancers\(^{26,31}\). Similarly the recurrent ESR1 truncations we report appear to be much more frequent in primary endometrial carcinoma than in primary breast cancers.

Anti-estrogen therapy with estrogen antagonists or aromatase inhibitors is standard first-line treatment for E\(\alpha\)-positive breast cancers, but has been associated with only a low rate (~10%) of overall response among endometrial cancers\(^{16–18}\) and is not a standard treatment for endometrial cancer\(^{34}\). In some cases, anti-estrogens such as Tamoxifen can even induce proliferation effect on endometrial cancer cells\(^{52,53}\) and normal endometrial tissue\(^{54}\) and increase the risk of endometrial carcinogenesis\(^{19–21}\). Splice variants of ESR1 that alter the hormone-binding domain have been associated with E\(\alpha\) activation by Tamoxifen in endometrial cancer cells\(^{24}\). The effect of estrogen antagonists on E\(\alpha\) encoded by the truncated forms of ESR1 that we have detected should also be tested, and all alterations of the ESR1 ligand-binding domain should be evaluated as potential biomarkers of anti-estrogen therapy resistance. Conversely, the absence of such alterations should be evaluated as a biomarker of anti-estrogen sensitivity, potentially opening up a new therapeutic option for a subset of patients with endometrial cancer.

**Methods**

**GeneChip analysis.** For our study subset of 29 primary endometrial tumors, gene copy-number data were determined by Affymetrix SNP 6.0 microarray analysis as described earlier\(^{55}\). GeneChip probe intensities are normalized across samples and circular binary segmentation is performed. Areas harboring germline CNVs are removed from the final segmented copy-number output. The range of birdseed call rates is 92.6–99.3% with an average call rate of 97.1%. For TCGA copy-number data, log2 copy-number values are calculated as ratios relative to the genome wide average according to standard procedures\(^{56–59}\). Genes with log2 copy-number data were used in analysis. For both datasets, log2 copy-number values are calculated as ratios relative to the genome wide average according to standard procedures\(^{56–59}\). These gene copy-number data were visualized using the IGV viewer software\(^{60}\). Linear gene level copy-number data were derived by GISTIC\(^{35,59}\). All TCGA DNA copy-number data (2015-06-01 stddata 2015-04-02 regular peel-off) can be accessed through the TCGA Copy Number Portal\(^{57}\).

**RNA-Seq analysis.** Reads per kilobase per million (RPKM)\(^{61}\) RNA exon expression quantification values were normalized and RPKM 0 was assigned 0.1 (Appendices C+D). Exons were compared using inverted log2 of normalized values. A two tailed Mann-Whitney-U-Test was applied to test for statistical significance of differences. P-values < 0.05 were considered statistically significant. Paired-end RNA-seq fusion transcript analysis of TCGA RNA-sequencing data from 295 tumors to detect mRNA fusions was performed using SnowShoes-FTD as described earlier\(^{62–64}\). Parameters used to define a fusion transcript of high confidence were at least two unique fusion junction spanning split reads within the dataset and at least five encompassing reads\(^{65}\). RNA-Seq data were taken from the TCGA database http://cancergenome.nih.gov.
**FISH analysis.** FISH was performed without RNase treatment as described earlier⁶⁴. Pearson correlation coefficients and regarding p-values (two sided t-test) were generated using SPSS (Statistical Package of Social Science) version 20.0.0 applying standard bootstrapping. P-values < 0.05 were considered statistically significant.

**Tumor samples and DNA extraction.** This study has been approved by the Norwegian Data Inspectorate (961478-2), the Norwegian Social Science Data Services (15501) and the local Institutional Review Board (REKIII nr. 052.01) and the BROAD institute, MA, USA and methods were carried out in accordance with these approved guidelines. The 29 metastatic high grade primary tumor samples were obtained with documented informed consent in a patient based setting (Sept 2002-Sept 2012) from the Department of Obstetrics and Gynaecology, Section of Gynaecological Cancer, Haukeland University Hospital, Bergen, Norway. Biopsies were snap frozen in nitrogen and stored at minus 80 °C until DNA extraction. Tumor purity was assessed based on histology sections obtained by microtome prior to DNA extraction. DNA extraction was performed using samples with estimated tumor purity ≥ 50% as previously described⁷.

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Author Contributions
F.H. and A.D.C. designed and conducted the analyses, and prepared the manuscript, together with support from E.A.H., W.J.G., A.T., S.E.S., Y.W.A., P.G., J.T., B.M.N., E.A.T., M.M., R.B. and H.B.S.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: Frederik Holst has royalty interest associated with intellectual property of ZytoVision GmbH concerning patent US8101352B2 “Detection of ESR1 Amplification in Breast Cancer” and according EU patent application. Mathew Meyerson and Andrew Cherniack receive research support from Bayer AG.

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Corrigendum: Recurrent hormone-binding domain truncated ESR1 amplifications in primary endometrial cancers suggest their implication in hormone independent growth

Frederik Holst, Erling A. Hoivik, William J. Gibson, Amaro Taylor-Weiner, Steven E. Schumacher, Yan W. Asmann, Patrick Grossmann, Jone Trovik, Brian M. Necela, E. Aubrey Thompson, Matthew Meyerson, Rameen Beroukhim, Helga B. Salvesen & Andrew D. Cherniack

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In the original version of this Article, there were errors in Affiliation 2 which was incorrectly given as 'KG Jebsen Center for Precision Medicine in Gynecologic Cancer, Department of Gynecology and Obstetrics, Haukeland University Hospital Bergen, Norway'. The correct affiliation is listed below:

'Department of Gynecology and Obstetrics, Haukeland University Hospital Bergen, Norway'.

This error has now been corrected in the PDF and HTML versions of the Article.

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This Article contains errors in the frequencies of ESR1 amplifications concerning uterine corpus endometrioid carcinoma (UCEC) in TCGA, which were incorrectly given as 88 cases (16.3%) and 36 cases (6.7%) for overall and focal amplifications respectively. These numbers represent the regarding amplification frequencies of partial ESR1 sequences that manifest the GISTIC peak.

The correct frequencies of ESR1 amplification in UCEC of TCGA are 90 cases (16.7%) and 39 cases (7.2%) for overall and focal amplification respectively. Accordingly, the seven cases with ESR1 amplifications that appear to truncate the hormone-binding domain encoding region manifest 17.9% instead of 19.4% of cases with focal amplification.

In addition, the GISTIC q-value for ESR1 amplification is incorrectly given as "$q = 5.75 \times 10^{-4}$". The correct term and number for this value is "residual $q = 2.29 \times 10^{-4}$ after ‘GISTIC peel-off’".

As a result, in the Results section under subheading 'Hormone-binding domain truncated ESR1 amplifications in primary endometrial cancers'.

“In the TCGA data subset of 539 endometrial carcinomas analyzed, we identified 88 (16.3%) cases with amplifications encompassing or overlapping ESR1. 46.6% of these were histologically defined serous and 75.0% of the tumors with ESR1 amplification were clustered within the serous like copy-number high molecular subtype according to TCGA. The ESR1 amplifications were focal (less than half a chromosome arm in length) in 36 cases (6.7%) of tumors, and had a significantly higher rate of amplification than the genome-wide average ($q = 5.75 \times 10^{-4}$). Mapping of the overlap between amplifications across tumors identified ESR1 only as the most likely gene target (see methods).

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should read:

“In the TCGA data subset of 539 endometrial carcinomas analyzed, we identified 90 (16.7%) cases with amplifications encompassing or overlapping ESR1. 45.6% of these were histologically defined serous and 75.0% of the tumors with ESR1 amplification were clustered within the serous like copy-number high molecular subtype according to TCGA. The ESR1 amplifications were focal (less than half a chromosome arm in length) in 39 cases (7.2%), and had a significantly higher rate of amplification than the genome-wide average (residual $q = 2.29 \times 10^{-4}$ after “GISTIC peel-off”). Mapping of the overlap between amplifications across tumors identified ESR1 only as the most likely gene target (see methods).

These amplifications appeared to truncate the hormone-binding domain encoding region in seven cases (1.3% of the entire dataset; and 17.9% of cases with focal ESR1 amplification) and to retain exons 1–4 or 1–3, encoding the n-terminal ESR1 transactivation domain (AF1) and DNA-binding domains. Another case without ESR1 amplification exhibited a heterozygous deletion of exons encoding the hormone-binding domain (Fig. 2), for a total apparent ESR1 truncation rate of 1.5% over all tumors. In one additional TCGA case, we detected a hormone-binding domain (exons 4–8) truncating ESR1-SYNE1 mRNA fusion (Appendix B). Eight of these nine tumors were molecularly classified as being in the serous like copy-number high subgroup (4.3% of this subgroup).”

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