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Effects of SNP variants in the *17β-HSD2* and *17β-HSD7* genes and *17β-HSD7* copy number on gene transcript and estradiol levels in breast cancer tissue

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ABSTRACT

Breast cancers reveal elevated E_2 levels compared to plasma and normal breast tissue. Previously, we reported intra-tumour E_2 to be negatively correlated to transcription levels of 17β-HSD2 but positively correlated to 17β-HSD7. Here, we explored these mechanisms further by analysing the same breast tumours for *17β-HSD2* and *-7* SNPs, as well as *17β-HSD7* gene copy number.

Among the SNPs detected, we found the *17β-HSD2* rs4445895_T allele to be associated with lower intra-tumour mRNA ($p = 0.039$) and an elevated intra-tumour E_2 level ($p = 0.006$). In contrast, we found the *17β-HSD7* rs1704754_C allele to be associated with elevated mRNA ($p = 0.050$) but not to E_2 levels in breast tumour tissue.

Surprisingly, *17β-HSD7* – gene copy number was elevated in 19 out of 46 breast tumours examined. Elevated copy number was associated with an increased mRNA expression level ($p = 0.013$) and elevated tumour E_2 ($p = 0.025$). Interestingly, elevated *17β-HSD7* – gene copy number was associated with increased expression not only of *17β-HSD7*, but the *17β-HSD7_II* pseudogene as well ($p = 0.019$). Expression level of *17β-HSD7* and its pseudogene was significantly correlated both in tumour tissue ($r_s = 0.457$, $p = 0.001$) and in normal tissue ($r_s = 0.453$, $p = 0.002$). While *in vitro* transfection experiments revealed no direct impact of *17β-HSD7* expression on pseudogene level, the fact that *17β-HSD7* and *17β-HSD7_II* share a 95.6% sequence identity suggests the two transcripts may be subject to common regulatory mechanisms.

In conclusion, genetic variants of *17β-HSD2* and *17β-HSD7* may affect intra-tumour gene expression as well as breast cancer E_2 levels in postmenopausal women.

1. Introduction

While E_1 is the main unconjugated oestrogen produced in postmenopausal women [1], it must be reduced to E_2 to execute biological effects. The reversible inter-conversion between E_1 and E_2 is catalysed by a group of enzymes called 17β-hydroxysteroid dehydrogenases (17β-HSDs), named after their major redox activity at the 17β-position of the steroid backbone [2,3]. Multiple members of this enzyme family exist, and to date 14 different 17β-HSDs have been identified (reviewed in [4]). 17β-HSD type 1, 5, 7 and 12 catalyse the reduction of E_1 to E_2 [5–7], while 17β-HSD type 2, 10 and 14 inactivate E_2 by oxidising it to E_1 [8–10]. Although the 17β-HSDs reveal high structural similarity at the protein level, they are encoded by different genes, with a low degree of sequence identity.

One exception is *17β-HSD7* [6], located on chromosome 1q23, for which a pseudogene (referred to here as *17β-HSD7_II*) located on chromosome 10p11.2 exists [11]. These two genes (illustrated in Fig. S1) share 95.6% sequence identity, including strong similarity across the promoter region [12]. While the pseudogene is transcribed, nucleotide differences cause alternative splicing, and the *17β-HSD7_II* transcript lack the entire exon 6. In addition, insertions cause a shift in the open reading frame, resulting in a premature stop codon [11,12]. This shorter mRNA may encode

Abbreviations: E_2, estradiol; E_1, estrone; E_1S, estrone sulphate; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase; SNP, single nucleotide polymorphism; ER, estrogen receptor; TF, transcription factor.

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a hypothetical protein, but the C-terminal truncation seems to remove the membrane-associated helix, which may cause mislocalization of the protein to the cytosol and nucleus [13]. Still, the biological significance of *17β-HSD7_II* is uncertain, and conflicting results regarding enzyme activity *in vitro* has been reported [11,12].

Tumour tissue E_2 levels are often elevated compared to plasma [14] due to protein binding, but also local modulation. Recently, in a collaborative project [15], we demonstrated *17β-HSD7* and *17β-HSD2* expression to be up-regulated in breast tumour tissue. The fact that expression levels of *17β-HSD7* (favouring reduction of E_1 into E_2) showed a positive correlation and expression levels of *17β-HSD2* (favouring oxidation of E_2 into E_1) showed a negative correlation with E_2 level in breast cancers [15] indicated 17β-HSD enzymes to be involved in tumour tissue E_2 up-regulation.

The aim of this study was to explore the potential impact of genetic variants of *17β-HSD7* and *17β-HSD2* on intra-tumour gene expression as well as E_2-levels.

2. Materials and methods

2.1. Patients

The breast cancer patients included in this study have been described previously (see [16] for details). In brief, normal breast and breast cancer tissue specimens were collected from 46 breast cancer patients (13 pre- and 33 postmenopausal women) undergoing mastectomy at Haukeland University Hospital, Bergen, Norway. Women using oral hormone replacement therapy or contraceptives were excluded. The samples were snap-frozen in liquid nitrogen immediately upon removal in the operating theatre, and stored in liquid nitrogen until processing.

Some of the molecular analyses and statistical calculations presented were not performed in all 46 patients; premenopausal patients were excluded from all statistical calculations related to oestrogen levels, and complete oestrogen data were not available for the entire cohort. In addition, due to a limited amount of RNA we were not able to measure 17β-HSD2 mRNA-levels in the entire cohort of 46 patients. 17β-HSD2-mRNA levels for 34 of the 46 patients described in this study have been reported elsewhere [15]. Table S1 provides a detailed overview of the number of patients available for each parameter.

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2.2. Plasma and breast tissue oestrogen levels

Plasma and tissue oestrogen levels in these breast cancer patients have been reported previously [16]. The samples were analysed by highly sensitive and specific radioimmuno-assays involving sample pre-purification steps described in detail elsewhere [17–19].

2.3. DNA extraction

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from snap-frozen biopsies using Trizol reagent (Life technologies) according to the manufacturer's protocol, and dissolved in DEPC-treated deionised water as described by Knappskog et al. [20]. The RNA-concentrations were determined in all samples using a Nanodrop ND1000 spectrophotometer and adjusted to 25 ng/μL. Single strand cDNA was synthesised from 200 ng total RNA in a 20 μL reaction mix, using the Transcriptor reverse transcriptase system (Roche) according to the manufacturer's protocol. Both oligoT (16-mers) and random hexamers were used as primers in the cDNA-synthesis reaction mix.

2.5. Quantitative PCR (qPCR)

17β-HSD2 and 17β-HSD7 – mRNA levels for 34 of the 46 patients described in this study have been reported elsewhere [15]. To ensure a uniform protocol for samples to be compared with respect to mRNA expression levels, we re-synthesised cDNA from these 34 patients along with the cDNA synthesis for the remaining 12 patients. 17β-HSD7 and 17β-HSD7_II mRNA levels from the entire cohort ($n = 46$) were analysed using qPCR-primers specifically designed to distinguish between these two variants (Fig. S2). The quantification was performed using BlackBerry-quenched hydrolysis probes on a LightCycler 480 instrument (Roche). Expression level of the ribosomal protein P2 (RPLP2) was used as reference. The amplification primers and hydrolysis probes (TIB MOLBIOL) are listed in Table S2. Amplification was performed using the LC480 Probes Master (Roche) reaction mix, with 0.5 μM of each primer, 0.125 μM of each hydrolysis probe and 0.5 μL cDNA synthesised from 200 ng total RNA. The following thermo-cycling conditions were used: initial denaturation at 95 °C for 5 min, 50 cycles of denaturation at 95 °C for 10 s, annealing/elongation at 55 °C for 30 s, and a final cooling step at 40 °C for 10 s. Water were used as a negative control in each run. For each analysis, the results were converted into relative concentrations using an *in run* standard curve, and the observed relative concentrations for 17β-HSD7 and 17β-HSD7_II mRNA were normalised by the RPLP2 mRNA levels.

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2.6. Mutation screening

Screening for mutations and small insertions/deletions was performed by PCR-amplification and subsequent sequencing of the promoter regions and coding regions of *17β-HSD2* and *17β-HSD7*. The 5′-upstream region of *17β-HSD2* was covered from position -2274 to +429, and *17β-HSD7* from position -1452 to +154, relative to the transcription start sites. The *17β-HSD7* PCR-primers (Table S2) were designed specifically to avoid amplification of *17β-HSD7_II*. All amplifications were performed using either the KodXL (Novagen), or the DyNazyme EXT (Finnzymes) polymerase system. The KodXL amplifications were performed in a 50 μL reaction mix containing 1× PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 1.25 U Kod XL DNA polymerase and 1 μL gDNA/cDNA. The DyNazyme amplifications were performed in a 50 μL reaction mix containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate, 5% DMSO, 0.2 μM of each primer, 0.5 U DyNazyme polymerase and 1 μL gDNA or cDNA. Following amplification, the PCR product was treated with ExoSAP-IT® (USB® Products, Affymetrix, Inc.) at 37 °C for 30 min and 80 °C for 15 min according to the manufacturer's protocol. DNA sequencing was performed in a 10 μL reaction mix containing 1× sequencing buffer, 1 μM primer and 1× BigDye v.1.1. (Applied Biosystems). Capillary electrophoresis was performed on an automated DNA sequencer (ABI 3730), and the sequences were analysed using the Sequence Scanner v. 1.0 software (Applied Biosystems). When analysing *17β-HSD7* sequences, we carefully made sure that there was no contribution from *17β-HSD7_II*.

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2.7. Gene copy number analysis

We explored potential gene copy number changes in *17β-HSD7* by quantifying genomic DNA in duplex reactions with the reference gene *Beta-2-Microglobulin* (*B2M*), using the LightCycler 480 instrument (Roche). We used primers designed specifically for *17β-HSD7* (Fig. S3), and the qPCR-products were sequenced aiming at excluding any contribution from *17β-HSD7_II*. Primers and BlackBerry-quenched hydrolysis probes (TIB MOLBIOL) are listed in Table S2. Amplification was performed in a 20 μL reaction solution using the LC480 Probes Master (Roche) reaction mix, 0.5 μM of each primer, 0.125 μM of each hydrolysis probe and 2 μL gDNA as template. Negative controls (water) were included in each run. The data obtained through quantification were normalised by adjusting for *B2M* levels. These normalised values were divided by the corresponding values from a reference sample (pooled DNA from 6 healthy donors). As previously described for this type of analysis [21], the concentration of the reference was set to 1.0, and samples were considered to have reduced copy number if the sample/reference ratio was <0.65 (corresponding to 1.3 gene copies), and to have increased copy number if the ratio was >1.35 (corresponding to 2.7 gene copies).

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2.8. In silico predictions

Putative transcription factor (TF) binding sites and binding affinity in the promoter areas of *17β-HSD7* and *17β-HSD2* were predicted both for wild-type sequence and observed variant haplotypes. The predictions were performed using JASPAR (http://jaspar.genereg.net), an open-access database of annotated matrix-based eukaryotic TF binding site profiles [22]. The predictions were restricted to ±12 nucleotides relative to the observed SNPs, using an 80% profile score threshold (default settings).

2.9. Cell culture and transfection

For *in vitro* testing of the effects of *17β-HSD7* and *17β-HSD7_II* expression on each other, vectors containing the entire coding region as well as the 3′-UTR region of each variant were generated. Each vector (pCMV-cytoEGFP) expressed EGFP from an independent promoter. MCF-7 cells were cultured in RPMI 1640 medium (ATCC) supplemented with 10% FBS. Transfection was performed in 6-well plates using 1.85 μg plasmid and 4.4 μL Lipofectamin 2000 reagent (Invitrogen). The cells were harvested after 48 h, total RNA was extracted using Illustra triple prep kit (GE Healthcare), and cDNA was prepared from 1 μg total RNA using qScript cDNA SuperMix (Quanta Biosciences). Each experiment setup contained (1) cells transfected with pCMV_*17β-HSD7*, (2) cells transfected with pCMV_*17β-HSD7_II*, (3) cells transfected with pCMV-vectors containing no insert, (4) cells receiving only Lipofectamin 2000, and (5) untreated cells. A minimum of three parallels were used in each setup, and the experiment was repeated three times. When calculating fold change in mRNA-levels, the cells transfected with pCMV-vectors containing no insert was used as reference samples, and qPCR was otherwise performed as described in Section 2.5.

2.10. Statistical analysis

Statistical analyses (Kruskal–Wallis, Mann–Whitney and Spearman tests) were performed using the PASW Statistics 18.0 software package (IBM). Multivariate analysis was done using linear regression with both forward and backward selection methods. Factors predicting oestrogen levels with a p-value < 0.10 were considered as potential dependents in multivariate analysis. All p-values are given as two-sided.

3. Results

3.1. Screening for 17β-HSD2 and 17β-HSD7 variants

The promoters and coding regions of *17β-HSD2* and *17β-HSD7* were screened for mutations, single nucleotide polymorphisms (SNPs), insertions and/or deletions. The nucleotide changes are summarised in Table 1. We detected 5 SNPs previously described by others; rs4445895 [23] and rs117437228 [24] in *17β-HSD2*, and rs1704754, rs12563263 and rs2684875 in *17β-HSD7* [25]. In addition, we observed 4 novel sequence variants in *17β-HSD2*, and two 17β-HSD7 splice variants.

3.2. In silico predictions

The SNPs *17β-HSD2* rs4445895 (C → T) and *17β-HSD7* rs1704754 (T → C) were located 34 and 56 nucleotides downstream of the transcription start sites of *17β-HSD2* and *17β-HSD7*, respectively. Due to the proximity to the transcription start sites, these two variants were considered to be of particular interest with respect to a potential influence on gene expression levels. *In silico* predictions indicated these SNPs to create potential novel transcription factor (TF) binding sites and/or to influence the

Table 1
Mutation screening of *17β-HSD2* and *17β-HSD7*. A summary of the SNP identities, positions, nucleotide changes, amino acid changes and genotypes of the identified genetic alterations detected in promoter and coding regions of *17β-HSD2* and *17β-HSD7*.

Gene	dbSNP	Nucleotide position	Nucleotide change	Aa-change	Genotypes (n total = 46)		
17β-HSD2	N/A	−1960[a]	C > T	–	CC: n = 39	TC: n = 5	TT: n = 2
	N/A	−1540[a]	C > T	–	CC: n = 44	TC: n = 2	TT: n = 0
	N/A	−1120[a]	C > T	–	CC: n = 43	TC: n = 3	TT: n = 0
	rs4445895	+34[a]	C > T	–	CC: n = 19	TC: n = 20	TT: n = 7
	N/A	Exon 2 codon 106	G > T	Gly > Val	GG: n = 44	GT: n = 2	TT: n = 0
	rs117437228	Exon 4 codon 226	A > G	Met > Val	AA: n = 45	AG: n = 1	GG: n = 0
17β-HSD7	rs1704754	+56[a]	T > C	–	TT: n = 31	TC: n = 13	CC: n = 2
	rs12563263	Exon 8 codon 296	C > T	No	CC: n = 24	CT: n = 17	TT: n = 5
	rs2684875	Exon 9 codon 321	A > G	Lys > Glu	AA: n = 45	AG: n = 1	GG: n = 0
	17β-HSD7 splice variants				Total	Wild-type	Splice-variant
	Exon 3 missing		N/A	–	n = 46	n = 45	n = 1
	Exon 4 nucleotide 1–26 missing		N/A	Frameshift	n = 46	n = 39	n = 7

N/A: information not available.
[a] Nucleotide position relative to transcription start.

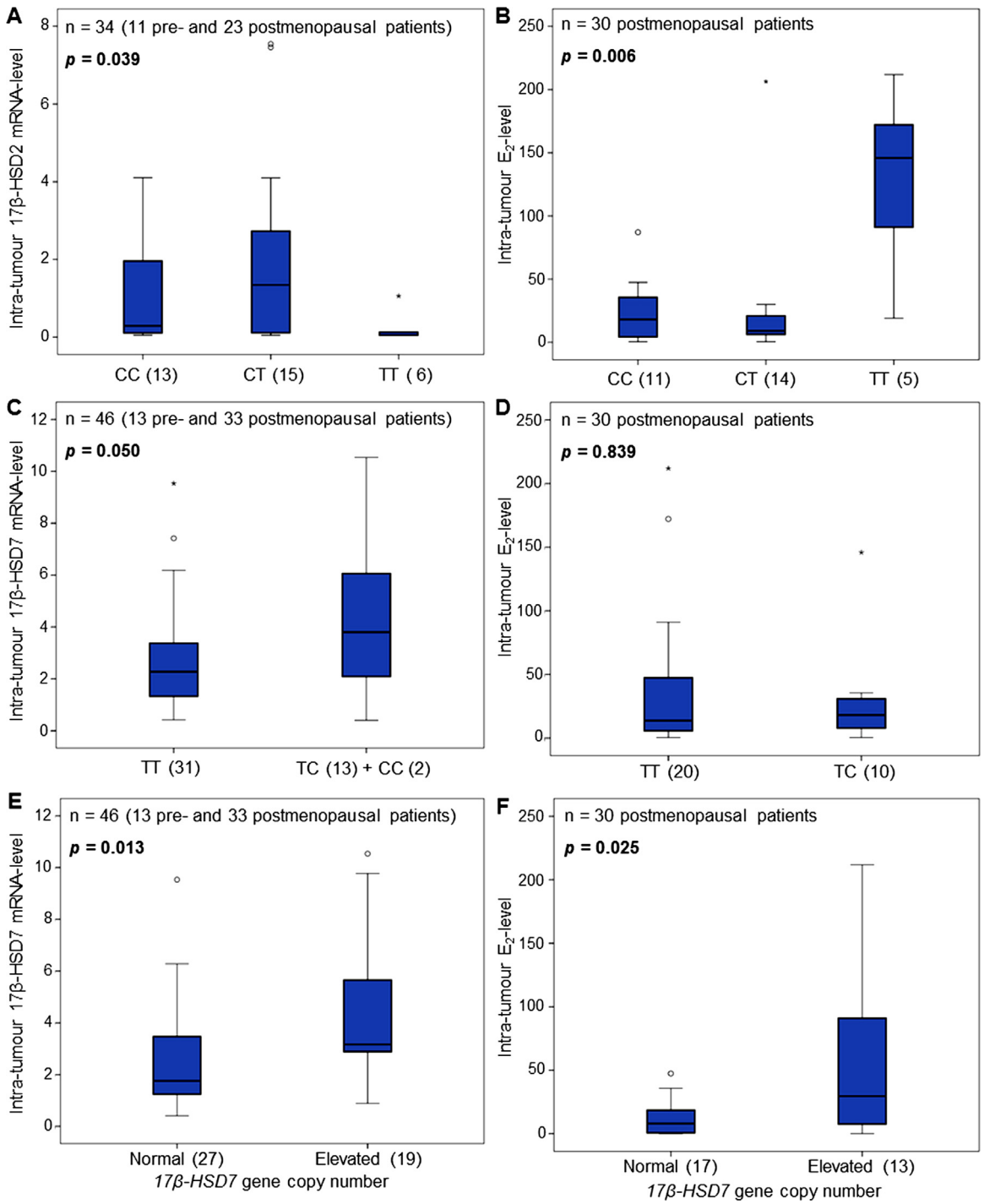

Fig. 1. Genetic alterations in *17β-HSD2* and *17β-HSD7* are associated with mRNA and E_2-levels. *17β-HSD2* SNP rs4445895 (C > T) is associated with a lower intra-tumour 17β-HSD2 mRNA level (Kruskal–Wallis $p = 0.039$) (A), and an elevated intra-tumour E_2-level (Kruskal–Wallis $p = 0.006$) (B). Analysing *17β-HSD7* SNP rs1704754 (T > C), we only detected two individuals harbouring the CC-genotype. When combining the individuals harbouring TC and CC-genotypes, we found this SNP to be associated with an elevated intra-tumour 17β-HSD7 mRNA level (Mann–Whitney $p = 0.050$) (C). Analysing the association between *17β-HSD7* SNP rs1704754 (T > C) and E_2-level, both individuals harbouring the CC-genotype had to be excluded, as one was premenopausal and E_2-data was not available for the other. Comparing the individuals harbouring TT- with CT-genotypes, no association between this variant and intra-tumour E_2-level was recorded (Mann–Whitney $p = 0.839$) (D). Elevated *17β-HSD7* gene copy number is associated with elevated intra-tumour 17β-HSD7 mRNA-level (Mann–Whitney $p = 0.013$) (E), and elevated intra-tumour E_2-level (Mann–Whitney $p = 0.025$) (F).

binding strength between TFs and already existing binding sites (Table S3).

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3.3. *17β-HSD2 rs4445895 and 17β-HSD7 rs1704754: associations to intra-tumour mRNA and oestrogen levels*

Assessing 17β-HSD2 mRNA levels in relation to *17β-HSD2* rs4445895 status, we performed a Kruskal–Wallis test comparing

all three genotypes (CC, CT and TT; $p = 0.039$, Fig. 1A). Paired comparison revealed homozygosity for the minor allele (genotype TT *vs.* CC + CT) to be associated with a significantly lower mRNA expression level ($p = 0.014$). Similarly, we performed a Kruskal–Wallis test to compare all three genotypes in relation to E_2-levels ($p = 0.006$, Fig. 1B). Again, paired comparison (TT *vs.* CT + CC) revealed significantly higher E_2 levels among individuals homozygous for the T variant allele ($p = 0.002$). These findings are in concordance with the known catalytic ability of the 17β-HSD2-enzyme favouring oxidation of E_2 into E_1.

Regarding *17β-HSD7* rs1704754 (T → C), we only observed homozygosity for the C-allele in two individuals (1 pre- and 1 postmenopausal) only. Thus, we combined homo- and heterozygous individuals (TC and CC), and compared this group with wild-type individuals (TT). We found elevated 17β-HSD7 mRNA level in tumour tissue from individuals carrying the TT genotype ($p = 0.050$, Fig. 1C). When analysing the association between this SNP and intra-tumour E_2, both individuals harbouring the CC-genotype had to be excluded, as one individual was premenopausal, while tumour E_2-data was not available for the other individual. Comparing individuals harbouring the TT- to those carrying the TC-genotype (Mann–Whitney test), no difference in E_2-levels was observed (Fig. 1D). Exploring different multivariate models including ER expression levels and either SNP status or expression levels for 17β-HSD2/17β-HSD7, we found 17β-HSD7 mRNA level to be the only significant determinant of tumour E_2 ($p < 0.001$) while a non-significant association for rs4445895 SNP-status ($p = 0.077$) was recorded.

In addition to *17β-HSD2 rs4445895* and *17β-HSD7* rs1704754, each of the nucleotide changes listed in Table 1 were analysed for potential associations to gene expression and E_2-levels. No associations were detected (data not shown).

3.4. *17β-HSD7* gene copy number in relation to mRNA- and oestrogen levels

19 out of 46 breast tumours revealed an elevated *17β-HSD7* gene copy number. The arithmetic mean gene copy number across this group was 3.12. The average of the gene copy numbers in the remaining patients was 2.36. Based on the gene copy number status (elevated or normal), we analysed the associations between gene copy number and intra-tumour 17β-HSD7-mRNA and E_2-level. We detected a higher level of 17β-HSD7-mRNA ($p = 0.013$, Fig. 1E), as well as E_2 level ($p = 0.025$, Fig. 1F) in breast tumours displaying an elevated *17β-HSD7* gene copy number (defined as >2.7 copies, as described in Section 2.7). To elucidate these results further, we performed a Spearman correlation test comparing 17β-HSD7 mRNA levels to the exact *17β-HSD7* gene copy number across the tumour samples ($n = 46$, $r = 0.377$, $p = 0.010$, Fig. 2A). Similarly, we detected a positive correlation between gene copy number and intra-tumour E_2-levels ($n = 30$, $r = 0.330$, $p = 0.075$, Fig. 2B), supporting the results from the Mann–Whitney test (Fig. 1E and F). Surprisingly, we also observed elevated 17β-HSD7_II mRNA levels in individuals harbouring an increased *17β-HSD7* tumour gene copy number ($p = 0.019$).

The promoter regions of *17β-HSD7* and *17β-HSD7_II* reveal high structural similarity. Notably, we observed a strong correlation between the expression levels of these two genes in tumour ($n = 46$; $r = 0.457$, $p = 0.001$, Fig. 3A) as well as in normal tissue ($n = 46$; $r = 0.453$, $p = 0.002$, Fig. 3B). Aiming to elucidate the unexpected finding of elevated 17β-HSD7_II mRNA levels in individuals harbouring an increased *17β-HSD7* gene copy number ($p = 0.019$), we calculated the Spearman correlation between 17β-HSD7 and 17β-HSD7_II mRNA levels in the tumours harbouring elevated and normal gene copy number separately. Contrary to our expectations, a significant correlation was recorded in the tumours harbouring an elevated copy number ($n = 19$, $r = 0.468$, $p = 0.043$, Fig. 3C), while this correlation was non-significant in tumours harbouring a normal gene copy number ($n = 27$, $r = 0.298$, $p = 0.132$; Fig. 3D).

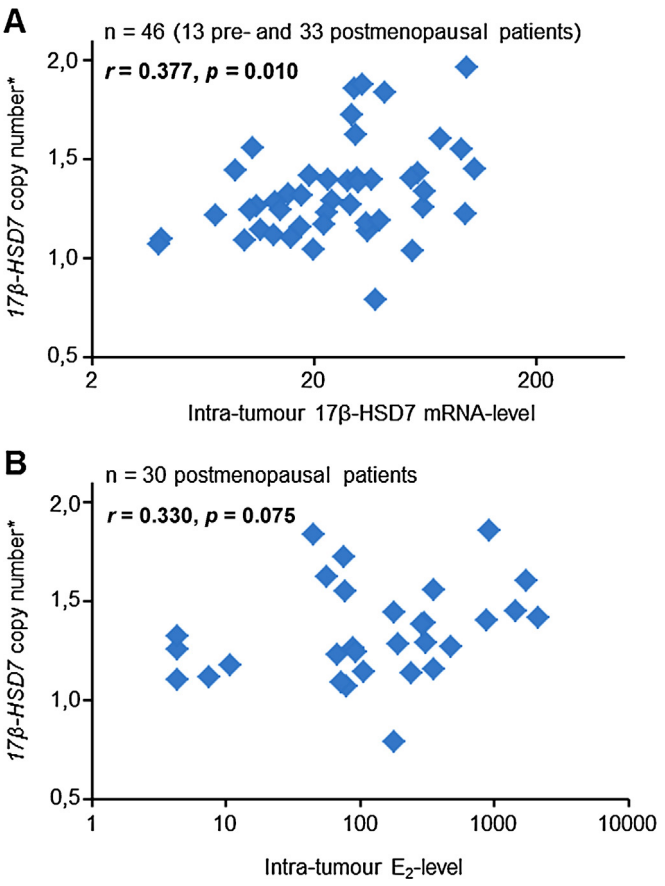

Fig. 2. *17β-HSD7* gene copy number is correlated with intra-tumour mRNA- and E_2. Spearman correlation between *17β-HSD7* gene copy number and intra-tumour 17β-HSD7-mRNA level ($n = 46$; $r = 0.377$, $p = 0.010$) (A). Spearman correlation between *17β-HSD7* gene copy number and intra-tumour E_2-level ($n = 30$, $r = 0.330$, $p = 0.075$) (B). *The data obtained through quantification were normalised by adjusting for *B2M* levels. These normalised values were divided by the corresponding values from a reference sample (pooled DNA from 6 healthy donors). As previously described [21], the concentration of the reference was set to 1.0, and samples were considered to have reduced copy number if the sample/reference ratio was <0.65, and to have increased copy number if the ratio was >1.35.

Recent evidence has suggested some pseudogenes may have biological functions at the RNA level. Taking the pseudogene for *PTEN* (*PTENP1*) as an example, Pandolfi's group suggested that this pseudogene may regulate PTEN expression levels by acting as a decoy for miRNAs targeting the *PTEN* transcript [26]. Based on these findings, we hypothesised that the correlations described above could be explained by a common miRNA targeting *17β-HSD7* and *17β-HSD7_II* in a competitive manner. To explore this hypothesis, we overexpressed each gene separately in MCF-7-cells with subsequent assessment of mRNA levels. The results (based on three individual experiments, with each experiment containing a minimum of three parallels) revealed that overexpression of one of the *17β-HSD7* variants did not significantly affect the mRNA levels of the other (Fig. 4).

4. Discussion

While contemporary evidence (reviewed in [27]) indicates plasma oestrogen levels to be the main determinator of local breast estrogens due to rapid equilibration between these two compartments, intra-tumour oestrogen levels are subject to local

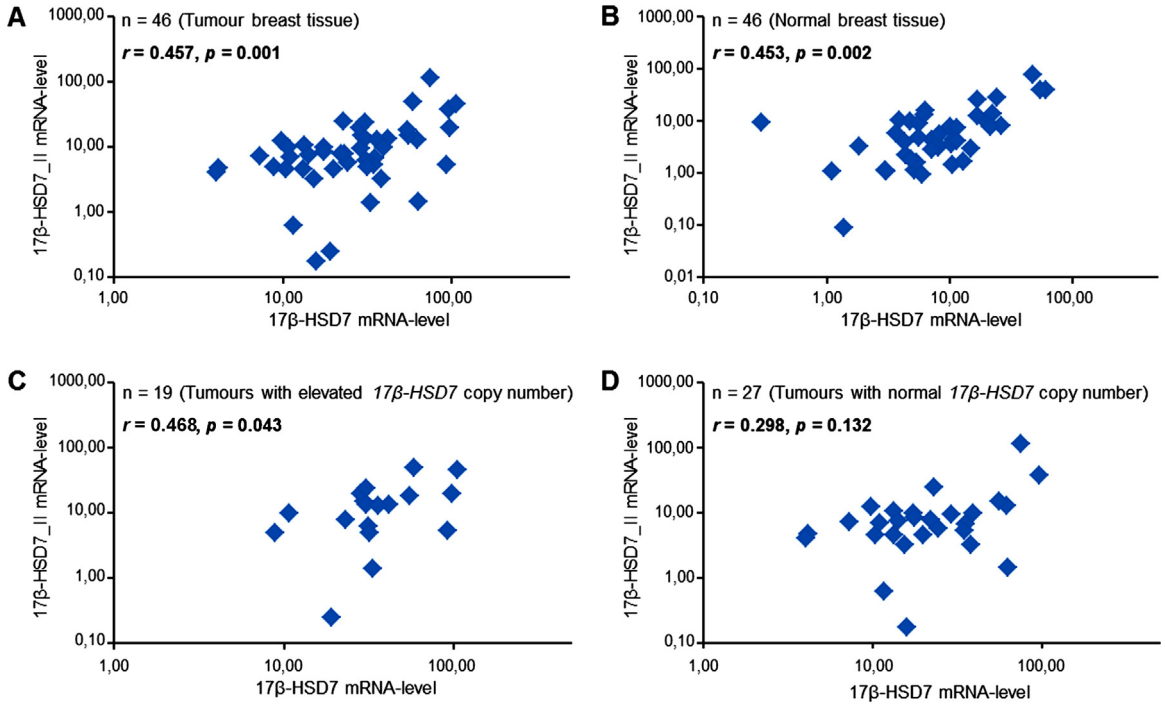

Fig. 3. The expression of *17β-HSD7* (wildtype) and *17β-HSD7_II* (pseudogene) is correlated. The top panel displays the Spearman correlation in all 46 individuals in tumour breast tissue ($r = 0.457$, $p = 0.001$) (A), and normal breast tissue ($r = 0.453$, $p = 0.002$) (B). The lower panel displays the Spearman correlation between *17β-HSD7* and *17β-HSD7_II* expression in breast tumour tissue among the individuals with an elevated *17β-HSD7* gene copy number ($n = 19$, $r = 0.468$, $p = 0.043$) (C), and among the individuals with a normal *17β-HSD7* gene copy number ($n = 27$, $r = 0.298$, $p = 0.132$) (D).

modulation through different dehydrogenases [15]. Here, we explored genetic variations in *17β-HSD7* and *17β-HSD2* aiming to elucidate the mechanisms by which these two dehydrogenases may be linked to elevated tumour E_2 levels [15].

Consistent with data from *in silico* predictions, we found homozygosity for the *17β-HSD2* rs4445895 T-allele to be associated with low 17β-HSD2 mRNA and elevated E_2 levels in breast tumour tissue. This finding fits well with the catalytic activity of 17β-HSD2 (conversion of E_2 to E_1); a lower transcript level would intuitively cause a lower enzyme level, leading to congestion of E_2.

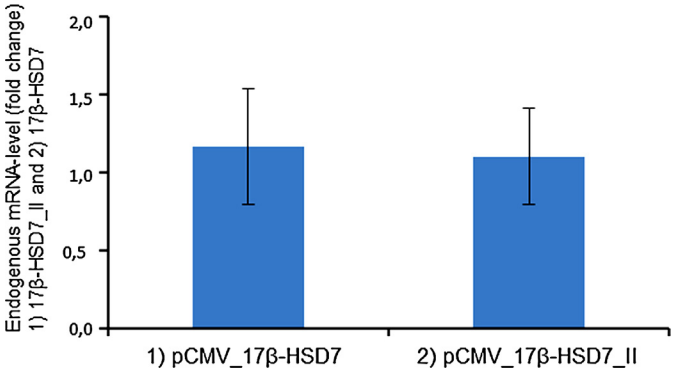

Fig. 4. *In vitro* testing of the effects of *17β-HSD7* (wildtype) and *17β-HSD7_II* (pseudogene) expression on each other. MCF7 cells were transfected with pCMV-vectors containing either *17β-HSD7* (wildtype) or *17β-HSD7_II* (pseudogene). Cells transfected with pCMV-vectors containing no insert was used as a reference when calculating fold change in mRNA-levels. Overexpression of the wildtype gene did not significantly affect the endogenous expression level of the pseudogene, illustrated in bar (1). Similarly, overexpression of the pseudogene did not significantly affect the endogenous expression level of wildtype gene, illustrated in bar (2). The data presented here is based on three individual experiments, where each experiment contained a minimum of three parallels.

The findings are less clear with respect to *17β-HSD7* rs1704754 (T → C). In support of the *in silico* predictions, we found individuals harbouring this SNP to display a higher 17β-HSD7 mRNA level in breast tumour tissue. The 17β-HSD7 enzyme is known to have a preference for reducing E_1 to E_2; thus, one would assume elevated E_2-levels in tumours expressing high 17β-HSD7 mRNA-levels. The lack of such an association may be due to the fact that only two individuals showed homozygosity for this SNP; one of these individuals was premenopausal, while tumour E_2-data was not available for the other individual.

In terms of gene copy number, elevated *17β-HSD7* copy number was associated with elevated mRNA as well as E_2 levels. These data indicate elevated gene copy number to potentially influence 17β-HSD7 enzyme activity and oestrogen metabolism.

An unexpected discovery was the association between *17β-HSD7* gene copy number and *17β-HSD7_II*-expression. These two genes have almost identical promoter regions, and may therefore be regulated by common trans-acting factors. However, the association between *17β-HSD7*-gene copy number and the expression level of *17β-HSD7_II* suggests other explanations may be involved as well. Recently it has been described that the levels of mRNAs with sequence similarities are balanced through their "competition" for the same miRNAs, elegantly shown for the *PTEN* gene and its pseudogene, *PTENP1* [26]. These findings have opened for new and interesting biological functions of transcribed pseudogenes, and made us hypothesise that the *17β-HSD7* and *17β-HSD7_II* transcripts may be targeted by common miRNAs. While the results from the *in vitro* experiment overexpressing *17β-HSD7* and *17β-HSD7_II* in MCF7-cells argues against this hypothesis, co-regulation by other trans-acting factors cannot be ruled out.

A weakness of this study relates to the limited number of samples available for analysis, and several of the associations may be considered preliminary findings that need validation in independent studies. Nevertheless, the observations indicate novel findings with respect to the biological activity of enzymes playing an important role to tissue oestrogen disposition.

In summary, we have identified genetic variants of *17β-HSD2* and *17β-HSD7* that may influence gene expression, as well as intra-tumour E_2 in postmenopausal breast cancer patients. Local oestrogen disposition may be a potential therapeutic target in endocrine manipulation of malignant disease, and the data presented here may add further information to our understanding of the mechanisms controlling breast cancer tissue E_2 levels.

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