

# Estrogen receptor splice variants as a potential source of false-positive estrogen receptor status in breast cancer diagnostics

Floris H. Groenendijk · Wilbert Zwart ·  
Arno Floore · Stephanie Akbari · Rene Bernards

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**Abstract** It is well established that only estrogen receptor (ER)-positive tumors benefit from hormonal therapies. We hypothesized that a subgroup of breast cancer patients expresses estrogen receptor  $\alpha$  (ER $\alpha$ ), but fails to respond to hormonal therapy due to the expression of a non-functional receptor. We analyzed a series of 2,658 ER $\alpha$ -positive HER2-negative breast tumors for ER $\alpha$  and progesterone receptor (PR) status as determined by mRNA expression and for their molecular subtypes (Luminal type vs Basal type, assessed by Blueprint<sup>TM</sup> molecular subtyping assay). In addition, we assessed the recurrence risk (low vs high) using the 70-gene MammaPrint<sup>TM</sup> signature. We found that 55 out of 2,658 (2.1 %) tumors that are ER $\alpha$  positive by mRNA analysis also demonstrate a Basal molecular subtype, indicating that they lack expression of estrogen-responsive genes. These ER $\alpha$ -positive Basal-type tumors

express significantly lower levels of both ER $\alpha$  and PR mRNA as compared to Luminal-type tumors ( $P < 0.0001$ ) and almost invariably (94.5 %) have a high-risk MammaPrint<sup>TM</sup> profile. Twelve of the MammaPrint<sup>TM</sup> genes are directly ER $\alpha$  responsive, indicating that MammaPrint<sup>TM</sup> assesses ER $\alpha$  function in breast cancer without considering ER $\alpha$  mRNA levels. We find a relatively high expression of the dominant negative ER $\alpha$  splice variant ER $\Delta$ 7 in ER $\alpha$ -positive Basal-type tumors as compared to ER $\alpha$ -positive Luminal-type tumors ( $P < 0.0001$ ). Expression of the dominant negative ER $\alpha$  variant ER $\Delta$ 7 provides a rationale as to why tumors are of the Basal molecular subtype while staining ER $\alpha$  positive by immunohistochemistry. These tumors may lack a functional response to estrogen and consequently may not respond to hormonal therapy. Our data indicate that such patients are of MammaPrint<sup>TM</sup> high recurrence risk and might benefit from adjuvant chemotherapy.

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F. H. Groenendijk · R. Bernards (✉)  
Division of Molecular Carcinogenesis, Cancer Genomics Centre,  
The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX,  
Amsterdam, The Netherlands  
e-mail: r.bernards@nki.nl

W. Zwart  
Division of Molecular Pathology, The Netherlands Cancer  
Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The  
Netherlands

A. Floore · R. Bernards  
Department of Research and Development, Agendia NV,  
Amsterdam, The Netherlands

S. Akbari  
Center for Breast Health, Virginia Hospital Center, Arlington,  
VA, USA

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## Introduction

The female hormone estradiol (E2) is a potent mitogen for estrogen receptor  $\alpha$  (ER $\alpha$ )-positive breast cancers. Hence, ER $\alpha$  protein levels, as determined by immunohistochemistry (IHC), are strongly predictive for response to endocrine therapies [1]. 75 % of all breast cancers express ER $\alpha$ , but not all tumors that express this steroid receptor respond to hormonal therapies. ER $\alpha$  is a member of the nuclear hormone receptor gene family that regulates transcription in a hormone-dependent fashion through sequence-specific

DNA binding [2]. Indeed, ER $\alpha$  binding sites are found proximal to many genes and consequently estrogen stimulation of breast cancer cells leads to significant changes in cellular gene expression [3, 4]. These responsive genes include the progesterone receptor (PR), one of the best-characterized ER $\alpha$  target genes. Hence, the PR is often co-expressed with ER $\alpha$  in breast cancers and PR testing is commonly performed in conjunction with ER $\alpha$  testing to assess hormone receptor status of a breast tumor. However, PR status is not a strong predictor of response to endocrine therapy, indicating that PR expression is not solely controlled by ER $\alpha$  activity [5].

Over a decade ago, the first large-scale gene expression profiling studies in breast cancer demonstrated that breast cancers consist of a number of “intrinsic” or “molecular” subtypes that are characterized by similarities in gene expression patterns [6]. Among these intrinsic subtypes are the “Luminal” and “Basal” tumors, which are thought to represent primarily ER-positive and -negative tumors, respectively. Consistent with this view, it was demonstrated that BluePrint<sup>TM</sup>, an 80-gene mRNA expression signature that identifies Luminal and Basal tumors, is significantly enriched in bona fide ER target genes [7]. These data suggest that this intrinsic subtype signature primarily measures the *functionality* of the ER, as judged by expression of its downstream target genes. As such, this signature also has the potential to identify a subgroup of breast cancer patients who are ER $\alpha$  positive by IHC and/or mRNA expression, but fail to elicit the hormone-induced transcriptional responses that normally result from ER stimulation (ER $\alpha$  target genes “off”; Basal type). Such a scenario would imply that breast cancers having this phenotype express a dysfunctional ER $\alpha$  protein that can nevertheless be detected by IHC.

Several different ER $\alpha$  variant mRNAs have been described in human breast cancer. Almost all of these naturally occurring variants are mRNA splicing variants, in which one or more exons are absent from the ER $\alpha$  mRNA. In most ER $\alpha$  splicing variants, except for variants lacking exon 3 or 4, translation runs out of frame after the site of the splicing variation, leading to a truncated protein [8–12]. Since the antibodies for ER $\alpha$  used in IHC often include those that recognize an epitope encoded by the first exon of the ER $\alpha$  gene [13], such splice variants are likely detected as IHC positive for ER $\alpha$ , even though their function may be different from the normal ER $\alpha$  protein. The functional activity of these variant ER $\alpha$  proteins can be negative, dominant negative, or dominant active on ER $\alpha$  target genes. Dominant negative variants are not only inactive themselves but also inactivate wild-type ER $\alpha$  through heterodimerization. Two variants, the ER $\Delta$ 3 and the ER $\Delta$ 7 variants, have been described as dominant negative receptor forms in the presence of wild-type ER $\alpha$  [8–12]. The ER $\Delta$ 7 mRNA has been reported to be the major alternatively spliced form

in most human breast tumors and cancer cell lines [14]. The ER $\Delta$ 7 is especially interesting because the hormone-binding domain, the transcription activation function-2 domain, and the dimerization domain are all partially located in exon 7 (Fig. 1). It has been shown that the ER $\Delta$ 7 variant has the ability to suppress the E2-dependent transcriptional activation by both wild-type ER $\alpha$  and ER $\beta$  [14].

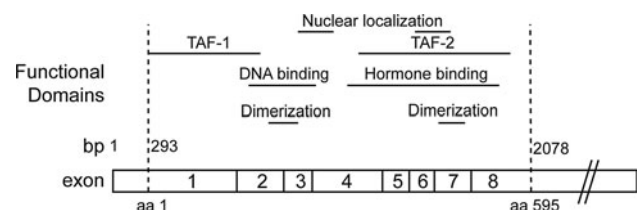
According to the guideline recommendations from the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) for IHC testing of ER $\alpha$  and PR in breast cancer, it is recommended that ER $\alpha$  assays should be considered positive if there are at least 1 % (weakly) positive tumor nuclei in the sample [13]. This threshold is based on a cut-point analysis correlating IHC scores with outcome in patients treated with adjuvant endocrine therapy alone, where patients with a score correlating to 1–10 % weakly positive cells had a statistically significant better prognosis than patients with scores correlating with <1 % positive cells [15]. However, Iwamoto et al. have shown recently that only a minority of the borderline (1–9 % positive nuclei) IHC ER $\alpha$ -positive tumors are of the Luminal subtype (as identified by the PAM50 classifier [16]) and that most of these borderline ER $\alpha$ -positive samples are of the Basal molecular subtype [17].

Here, we identify in a large cohort of molecular profiled breast cancers a subgroup of around 2 % of breast tumors that are ER $\alpha$  positive by mRNA expression analysis, but are of the Basal molecular subtype. These tumors express significantly lower levels of both ER $\alpha$  and PR mRNA than the Luminal-type tumors and have almost invariably (94.5 %) a high-risk MammaPrint<sup>TM</sup> profile. Furthermore, we show that these tumors have relatively high levels of the dominant negative ER $\Delta$ 7 splice variant, in agreement with the notion that they may lack a functional response to estrogen and consequently may not respond to hormonal therapy.

## Patients and methods

### Patient samples and molecular profiling

A total of 3,527 breast cancer patient specimens were retrospectively analyzed. This selection was based on the



**Fig. 1** Organization of the ER $\alpha$  mRNA and functional domains. TAF-1 transcription activation function 1, TAF-2 transcription activation function 2, aa amino acid, bp base pair

availability of MammaPrint™, TargetPrint™, and Blueprint™ molecular profiling results as performed in the Agendia testing laboratories. The ER $\alpha$  status on mRNA levels was determined by TargetPrint, a microarray-based gene expression test, which offers a quantitative assessment of the patient's level of ER $\alpha$ , PR, and HER2 expression [18]. The TargetPrint probe for ER $\alpha$  mRNA detection is located in the 3' UTR region. The ER $\alpha$ , PR, and HER2 TargetPrint score is a value between  $-1$  and  $1$ , where the null cutoff value is calibrated to 1 % IHC ER $\alpha$ -positive cells, as identified in a reference laboratory according to ASCO/CAP guidelines. Tumors are reported ER $\alpha$  or PR positive when the TargetPrint score is above 0, corresponding to  $>1$  % IHC-positive cells [18]. Molecular subtyping was performed using the 80-gene Blueprint™ molecular subtyping profile for the classification of breast cancer into Basal type, Luminal type, and ERBB2 type (HER2 positive) molecular subclasses [7]. In addition, the tumors were classified as low risk or high risk for distant recurrence using the 70-gene MammaPrint™ signature, a FDA-cleared breast cancer recurrence assay, performed by Agendia Inc. [19].

#### ER $\Delta$ 7 variant analysis

We obtained RNA from 15 ER $\alpha$ -positive Luminal-type tumors and from 12 ER $\alpha$ -positive Basal-type tumors to analyze the relative ER $\Delta$ 7 mRNA expression. cDNA was synthesized from 500 ng RNA using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer primers. The total ER $\alpha$  and ER $\Delta$ 7 mRNA expression was determined by qRT-PCR. For total ER $\alpha$  expression, the forward primer was located in exon 1 and the reverse primer in exon 2. For ER $\Delta$ 7 expression, the forward primer was located in exon 6 and the reverse primer was designed to specifically detect ER $\Delta$ 7 and located partially in exon 6 (12 nucleotides) and partially in exon 8 (14 nucleotides) (Primer sequences in Supplementary Materials). All qRT-PCR reactions were performed in duplicates using SYBR Green reaction mix containing 5  $\mu$ l cDNA. The expression levels were quantified using a reference standard dilution curve. The relative expression of the ER $\Delta$ 7 variant was calculated by dividing the ER $\Delta$ 7 mRNA expression by the total ER $\alpha$  mRNA expression.

#### Identification of ER $\alpha$ target genes in the 70-gene MammaPrint™ breast cancer signature

The 70 MammaPrint genes were analyzed for ER $\alpha$  binding events within 20 kb from the transcription start site (TSS), representing the most commonly detected window for ER-mediated gene regulation [20]. ER $\alpha$ -binding sites were identified by ChIP-seq analyses [21], using available

datasets for the Luminal breast cancer cell line MCF-7 [22] and 2 ER-positive Luminal breast tumor samples (paper in submission; GSE40867). Publically available data on E2-stimulated gene expression were used from [3], where Global Run-On sequencing was applied to assess gene transcription after 0-, 10-, 40-, and 160-min E2 treatment. Only genes with a differential expression as compared to control conditions with a false discovery rate of  $\sim 0.1$  % were considered as E2 regulated.

## Results

### ER $\Delta$ 7 splice variant expressed in an ER $\alpha$ -positive basal-type breast cancer

We have recently developed an 80-gene signature (Blueprint™) that identifies the three major intrinsic subtypes (Basal, Luminal, and HER2) of breast cancer [7]. Of these 80 genes, 58 are used to identify the Luminal subtype. Importantly, 32 out of these 58 Luminal subtype reporter genes have ER $\alpha$ -binding sites adjacent to the TSS [7]. This indicates that the genes that identify Luminal-type breast cancer are significantly enriched for bona fide ER $\alpha$  target genes and suggests that the Luminal subtype is characterized by tumors that have a functional ER $\alpha$  pathway. Conversely, Blueprint Basal-type tumors would be expected to have either no significant ER $\alpha$  expression or a non-functional ER $\alpha$  pathway; these same bona fide ER $\alpha$  target genes show an inverse expression pattern in Basal-type tumors [7].

Following argumentation as outlined above, one would expect that breast tumors that are ER $\alpha$  positive, but Basal type by Blueprint analysis, would either have a very low level of ER $\alpha$  protein or harbor a defective ER $\alpha$  protein. To test this hypothesis directly, we mined the Agendia database for patients who are ER $\alpha$  positive by TargetPrint, but Basal type by Blueprint molecular subtype analysis. We initially identified a patient (Table 1, patient 1; 60-year-old woman with 9 mm, moderately differentiated, HER2 negative, ER/PR  $> 90$  % by IHC, invasive ductal carcinoma), who had undergone MammaPrint, TargetPrint, and Blueprint tests. She had MammaPrint high-risk result, was ER/PR positive by TargetPrint, but Basal subtype by Blueprint, suggesting that the ER $\alpha$  was present both at the protein (IHC  $> 90$  %) and mRNA levels, but that ER $\alpha$  target genes were not expressed in this tumor (hence Basal type). The tumor was also analyzed using the OncotypeDX™ breast cancer assay (Genomic Health Inc.), classifying the tumor as low risk for distant recurrence (Recurrence Score 8, Table 1).

We used the same tumor mRNA sample as was used to perform the MammaPrint, TargetPrint, and Blueprint assays for detailed analysis of the ER $\alpha$  mRNA transcript in this patient. We first PCR amplified the coding sequence of

**Table 1** Characteristics of ER $\alpha$ -positive Basal-type tumors for which the ER $\Delta$ 7 expression was determined ( $N = 12$ )

Patient	Age	Stage	IHC ER $\alpha$	IHC PR	FISH HER2	TargetPrint ER $\alpha$ index <sup>c</sup>	TargetPrint PR index <sup>d</sup>	TargetPrint HER2 index <sup>a</sup>	BluePrint classification	MammaPrint classification	Oncotype recurrence score
1 <sup>b</sup>	60	T1bN0M0	>90 %	>90 %	NA <sup>c</sup>	0.33	0.25	-0.77	Basal type	High risk	8 (low-risk)
2	56	pT1bN0Mx	2+	Negative	Negative	0.18	-0.16	-0.53	Basal type	High risk	NA
3	47	NA	NA	NA	NA	0.26	0.22	-0.52	Basal type	High risk	NA
4	64	pT1cN0Mx	60–70 %	40–50 %	Negative	0.41	0.16	-0.73	Basal type	High risk	NA
5	87	NA	NA	NA	NA	0.25	-0.19	-0.39	Basal type	High risk	NA
6	58	pT1cN0Mx	80 %	<5 %	Negative	0.03	-0.35	-0.78	Basal type	High risk	NA
7	60	NA	NA	NA	NA	0.04	-0.28	-0.51	Basal type	High risk	NA
8	67	T1N0Mx	Negative	Negative	Negative	0.15	-0.28	-0.62	Basal type	High risk	NA
9	40	NA	NA	NA	NA	0.03	-0.32	-0.57	Basal type	High risk	NA
10	71	pT2N0Mx	Negative	<5 %	Negative	0.01	-0.24	-0.64	Basal type	High risk	NA
11	74	T1cN0M0	Positive	Positive	Negative	0.28	0.01	-0.59	Basal type	High risk	31 (intermediate risk)
12	67	pT2N0M0	3+	2–3+	Negative	0.55	0.21	-0.56	Basal type	High risk	NA

IHC immunohistochemistry, ER $\alpha$  estrogen receptor  $\alpha$ , PR progesterone receptor, FISH fluorescence in situ hybridization, NA not available

<sup>a</sup> A TargetPrint index >0.00 is considered as positive, a index  $\leq 0.00$  is considered as negative (described in “Patients and methods” section)

<sup>b</sup> Patient in whom we initially identified the ER $\Delta$ 7 variant by cDNA sequencing as is described in the “Results” section

<sup>c</sup> FISH for HER2 not available, but tumor scored negative for HER2 by IHC

ER $\alpha$  with specific oligonucleotides that span the start codon of ER $\alpha$  at the 5' end and the stop codon at the 3' end (Primer sequences in Supplementary Materials). Agarose gel electrophoresis of the PCR product revealed a smaller DNA fragment next to the expected DNA fragment coding for the open reading frame of ER $\alpha$ . Inspection of the DNA sequence of the smaller product revealed an ER $\alpha$  sequence-lacking exon 7 of the coding sequence (data not shown). This transcript corresponds to the previously reported ER $\Delta$ 7, an ER $\alpha$  splice variant that inhibits the function of the normal (wild-type) ER $\alpha$  in a dominant fashion [14].

#### Frequency of ER $\alpha$ -positive basal-type tumors

To determine the frequency at which ER $\alpha$ -positive Basal-type breast tumors occur, we searched the Agendia database for additional cases. Out of 3,527 cases, we identified 2,658 ER $\alpha$ -positive, HER2-negative breast tumors, as judged by TargetPrint mRNA expression, for which Blueprint intrinsic subtyping data were available. From these 2,658 tumors, 2,603 (97.9 %) were classified as Luminal type and 55 (2.1 %) were classified as Basal type (Table 2). The mean ER $\alpha$  and PR TargetPrint indices for the ER $\alpha$ -positive Basal-type tumors were significantly lower than for the ER $\alpha$ -positive Luminal-type tumors ( $P < 0.0001$ ).

#### ER $\Delta$ 7 splice variant expression in ER $\alpha$ -positive basal-type breast cancers

We further analyzed an additional 11 of these 55 ER $\alpha$ -positive Basal-type tumors for expression of total ER $\alpha$  as well as the ER $\Delta$ 7 variant by qRT-PCR. The specificity of the primer pairs was tested with cDNA from MCF7 breast

cancer cells overexpressing either wild-type ER $\alpha$  or ER $\Delta$ 7 and the calculated ER $\Delta$ 7/total ER $\alpha$  ratio was correlated with ER $\alpha$  protein expression in these cells. The ER $\alpha$  antibody clone 1D5 (Dako) was used for western blot analysis, for which the epitope is located in the N-terminal domain of ER $\alpha$  and therefore recognizes both wild-type ER $\alpha$  and ER $\Delta$ 7. We show in these cells that the relative ER $\Delta$ 7 levels as measured by qRT-PCR are highly concordant with protein expression (Supplementary Fig. 1).

The average total ER $\alpha$  mRNA expression by qRT-PCR was significantly lower for the 12 analyzed ER $\alpha$ -positive Basal-type tumors compared to 15 randomly chosen ER $\alpha$ -positive Luminal-type tumors (Fig. 2a;  $P = 0.0019$ ), consistent with the TargetPrint results (Table 2). There was no significant difference in average ER $\Delta$ 7 mRNA expression between the ER $\alpha$ -positive Basal-type and Luminal-type samples (Fig. 2b;  $P = 0.4088$ ). However, the relative ER $\Delta$ 7 mRNA expression was significantly higher for the ER $\alpha$ -positive Basal-type group compared to the ER $\alpha$ -positive Luminal-type group (Fig. 2c;  $P < 0.0001$ ), due to the lower overall ER $\alpha$  mRNA expression in the Basal-type tumors.

The characteristics of the 12 ER $\alpha$ -positive Basal-type tumors, for which ER $\Delta$ 7 splice variant expression was determined, are shown in Table 1. For 8 of the 12 patients, we were able to retrieve the ER $\alpha$  and PR IHC scoring. Based on the ER $\alpha$  IHC, six out of eight (75 %) patients were classified as ER $\alpha$  positive. In two patients, we found a discrepancy between TargetPrint and ER $\alpha$  IHC classification; in one of these patients, the TargetPrint ER $\alpha$  index was just above the ER $\alpha$ -positive threshold (patient 10). The PR IHC was in concordance with the PR classification based on TargetPrint in six of eight patients, and for two patients (patient 6 and 8), a small percentage of

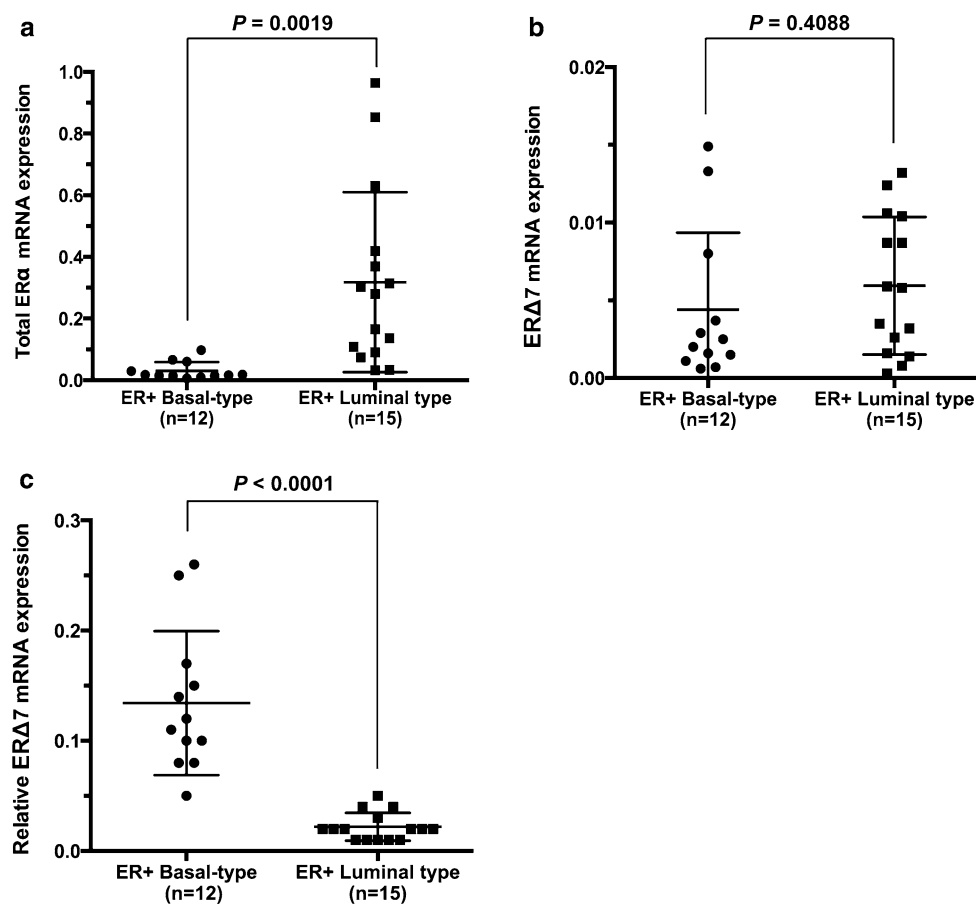
**Table 2** TargetPrint ER $\alpha$ /PR index, PR classification, and MammaPrint classification of 2,658 ER $\alpha$ -positive, HER2-negative tumors according to their Blueprint molecular subtype (Basal type vs Luminal type)

	Blueprint classification		<i>P</i> value
	Basal type ( <i>n</i> = 55, 2.1 %)	Luminal type ( <i>n</i> = 2,603, 97.9 %)	
ER $\alpha$ index (mean $\pm$ SD)	0.20 ( $\pm$ 0.15)	0.57 ( $\pm$ 0.17)	<0.0001 <sup>a</sup>
PR index (mean $\pm$ SD)	-0.04 ( $\pm$ 0.27)	0.28 ( $\pm$ 0.31)	<0.0001 <sup>a</sup>
PR classification			<0.0001 <sup>b</sup>
PR positive	24 (43.6 %)	2047 (78.6 %)	
PR negative	31 (56.4 %)	556 (21.4 %)	
MammaPrint classification			<0.0001 <sup>b</sup>
Low risk	3 (5.5 %)	1434 (55.1 %)	
High risk	52 (94.5 %)	1169 (44.9 %)	

ER $\alpha$  estrogen receptor  $\alpha$ , PR progesterone receptor, SD standard deviation

<sup>a</sup> Unpaired *t* test, two-tailed

<sup>b</sup> Fisher's exact test, two-tailed



**Fig. 2** ER $\alpha$ -positive Basal-type tumors have a relatively high ER $\Delta$ 7 expression compared to ER $\alpha$ -positive Luminal-type tumors. **a** Scatter plot of total ER $\alpha$  mRNA expression analysis by qRT-PCR in ER-positive Basal-type ( $n = 12$ ) and ER-positive Luminal-type ( $n = 15$ ) tumors. The qRT-PCR primers are located in exon 1 and exon 2. *Points* indicate individual tumors; *lines* indicate mean with SD. **b** Scatter plot of specific ER $\Delta$ 7 mRNA expression analysis by qRT-PCR in ER-positive Basal-type ( $n = 12$ ) and ER-positive Luminal-type ( $n = 15$ ) tumors. The qRT-PCR Primers are located in exon 6

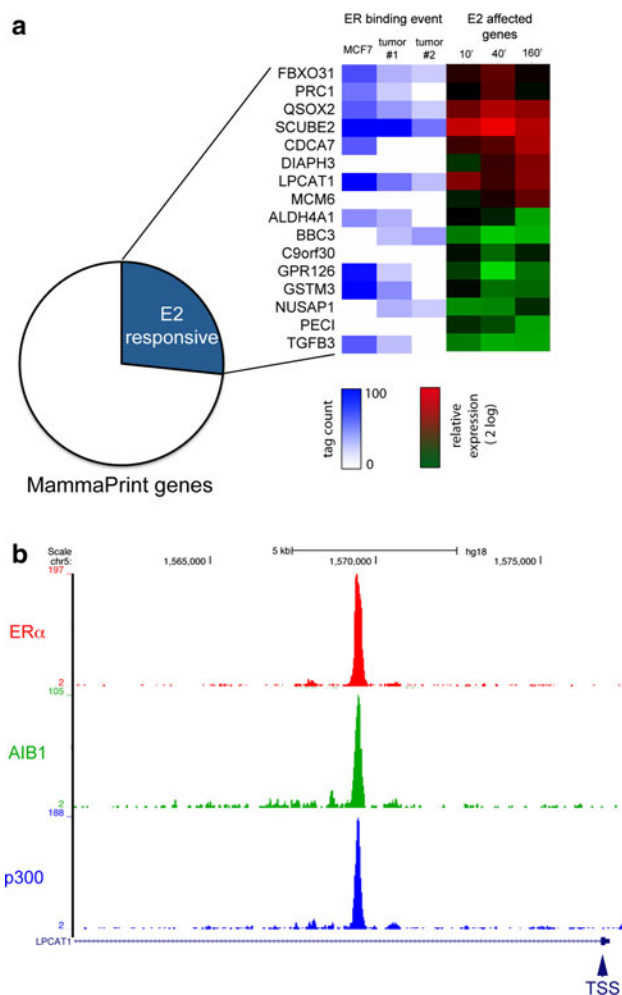
(forward) and over the exon 7 splice site (reverse). *Points* indicate individual tumors; *lines* indicate mean with SD. **c** Scatter plot of relative ER $\Delta$ 7 expression calculated by dividing the ER $\Delta$ 7 mRNA expression with the total ER $\alpha$  mRNA expression in ER-positive Basal-type ( $n = 12$ ) and ER-positive Luminal-type ( $n = 15$ ) tumors. *Points* indicate individual tumors; *lines* indicate mean with SD. *P*-values are calculated by unpaired *t* tests with Welch's correction and are two-tailed

PR-positive cells was detected by IHC where the Target-Print PR index was negative. The HER2 negative status was confirmed by fluorescence in situ hybridization (FISH) in all available cases. All patients (12/12) were stratified as high risk of distant recurrence by the MammaPrint prognostic gene signature.

#### MammaPrint measures ER $\alpha$ function independent of ER $\alpha$ expression

MammaPrint measures 70 genes that were selected from the entire complement of human genes, but ER $\alpha$  is not among the MammaPrint genes [23]. Nevertheless, we observed that 52 of the 55 (94.5 %) ER $\alpha$ -positive Basal-type tumors were MammaPrint high risk, while only 44.9 % of the ER $\alpha$ -positive Luminal-type tumors were classified as high risk of recurrence (Table 2;  $P < 0.0001$ ).

Since the MammaPrint assay identifies nearly all these ER $\alpha$ -positive Basal-type tumors as high risk, it suggests that the test measures ER $\alpha$  activity independent of the ER $\alpha$  mRNA expression level itself. To investigate this further, we determined how many of the 70 MammaPrint prognosis genes are directly responsive to E2 treatment. For this, a publicly available dataset was used that assessed gene expression changes after 10, 40, and 180 min of E2 treatment [3]. We found that 16 MammaPrint reporter genes annotated in the most recent build of the human reference genome sequence are E2 regulated (Fig. 3a). Next, we tested whether these E2-responsive MammaPrint genes can be classified as direct ER $\alpha$  target genes. Using a publicly available ChIP-seq dataset [22], the genome-wide chromatin-binding landscape of ER $\alpha$  in MCF7 cells was analyzed for the occurrence of an ER $\alpha$  binding event within 20,000 bp from the TSS of any of the MammaPrint genes.



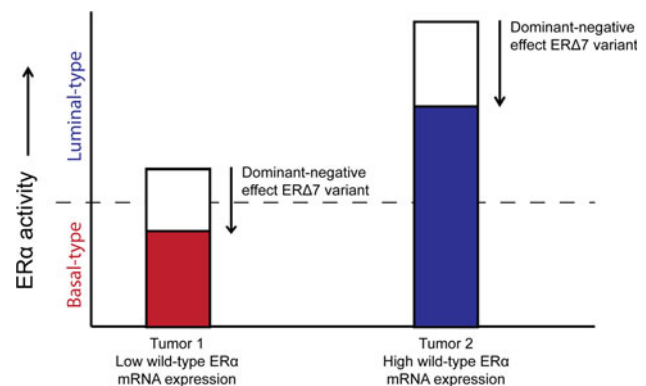
**Fig. 3** Functional ER $\alpha$  target genes in MammaPrint 70-gene set. **a** Pie chart, depicting the proportion of MammaPrint genes, which are affected by E2 treatment. Heat map (*right panel*) depicts proximal ER $\alpha$  ChIP-seq signal by tag count (*blue*) as well as relative gene expression values as measured by GRO-seq, after 10, 40, and 160 min of E2 treatment (*green–black–red* heat map). **b** Genome browser snapshot, depicting a shared ER $\alpha$  (*red*), AIB1 (*green*), and p300 (*blue*) proximal to the *LPCAT1* transcription start site. Chromosome number, genomic coordinates, and tag count are indicated

This window was chosen since most ER $\alpha$ -mediated gene regulation is found within this distance from a TSS [20]. Ten out of 16 genes had an ER $\alpha$  binding event within 20,000 bp from the TSS (Fig. 3a), as exemplified for the *LPCAT1* locus (Fig. 3a). Importantly, the essential ER $\alpha$  coactivators AIB1 (also known as SRC3) and p300 were also present at this specific binding site, indicating that ER $\alpha$  is likely to be functional here [24]. Furthermore, we confirmed that ER $\alpha$  binding events in E2-regulated MammaPrint genes are also found in 2 ER-positive Luminal human breast tumor samples, for which ER $\alpha$  ChIP-seq data are available (Fig. 3a). In total, 12 out of 16 E2-regulated genes had an ER $\alpha$ -binding site in either MCF7 cells or in the two studied tumors (Fig 3a). Cumulatively, these data

indicate that bona fide ER $\alpha$  target genes are enriched in the MammaPrint gene signature, providing a plausible explanation for why the MammaPrint can measure ER $\alpha$  functionality rather than its mere presence, in contrast to other available assays.

## Discussion

The present study identifies approximately 1 in 50 ER-positive breast cancer patients as Basal molecular subtype. Basal-type breast tumors are characterized by an absence of expression of ER $\alpha$  target genes, which is generally thought to result from the absence of ER $\alpha$  expression [25]. However, the group of tumors identified here is ER $\alpha$  positive on the mRNA level, suggesting that their Basal phenotype is the result of a lack of ER $\alpha$  protein expression or a lack of functionality of the ER $\alpha$  protein present in these tumors. Indeed, we find that these tumors not only express relatively low levels of ER $\alpha$  mRNA but also express a splice variant of ER $\alpha$ -missing exon 7 (ER $\Delta$ 7, Fig. 2a, b). This ER $\alpha$  variant has been shown previously to act in a dominant negative fashion, meaning that this variant can inhibit the function of the wild-type ER $\alpha$  protein when co-expressed in the same cell [14]. We note that the *absolute* levels of ER $\Delta$ 7 are comparable in ER $\alpha$ -positive Basal-type versus ER $\alpha$ -positive Luminal-type tumors, but that the *relative* abundance of ER $\Delta$ 7 is higher in the ER $\alpha$ -positive Basal-type tumors (Fig. 2c). We interpret these data as follows: When the levels of wild-type ER $\alpha$  in a breast tumor are high, the inhibitory effects of dominant negative ER $\Delta$ 7 are by comparison minor, leaving the cell with considerable ER $\alpha$  activity and thus with a luminal phenotype (Fig. 4, right). In contrast, lower levels of wild-type ER $\alpha$  in the weakly ER $\alpha$ -positive breast tumors are inhibited to a greater extent by the presence of ER $\Delta$ 7, leaving the tumor cells with insufficient ER $\alpha$  activity to



**Fig. 4** Proposed model by which ER $\Delta$ 7 mRNA expression can affect ER $\alpha$  activity in low ER $\alpha$  wild-type expressing tumors (*left*) and in high ER $\alpha$  wild-type expressing tumors (*right*)

regulate ER $\alpha$  target gene expression and thus with a Basal phenotype (Fig. 4, left). It remains to be explained why lower levels of ER $\alpha$  result in a relative increase in abundance of the ER $\Delta$ 7 splice variant. It is possible that ER $\alpha$  also controls the expression of certain components of the splicing machinery and that low ER $\alpha$  activity therefore results in a different processing of the ER $\alpha$  (and potentially also other) precursor mRNAs.

A clinically relevant question is whether this identified group of ER $\alpha$ -positive Basal-type tumors is likely to respond to hormonal therapy. The finding that ER $\alpha$  target genes are not expressed suggests that the mitogenic responses in such tumors are not driven by E2 and that such tumors would be unlikely to derive significant benefit from hormonal therapy. It was reported by Ellis et al. [26] in a cohort of postmenopausal women with clinical stage II to III ER-positive breast cancer that the single patient in their study with a basal-like intrinsic subtype was resistant to endocrine therapy. While it remains to be formally proven, there are other suggestions in the literature that the presence of ER $\Delta$ 7 is associated with a lack of response to tamoxifen. Van Dijk [27] analyzed the relative ER $\Delta$ 7 mRNA expression in a group of 21 primary breast tumors from postmenopausal early breast cancer patients treated with adjuvant tamoxifen. It was found that out of eleven ER $\alpha$  mRNA variants tested, only the ER $\Delta$ 7 mRNA was significantly differentially expressed between primary breast tumors of patients who developed a tumor recurrence (13/21) and tumors of patients without recurrence (8/21). Tumors from patients with a recurrence expressed on average 24 % ER $\Delta$ 7 mRNA (relative to wild-type ER $\alpha$  mRNA expression), while tumors from patients without recurrence expressed on average 9 % ER $\Delta$ 7 mRNA [27]. While it may be premature to withhold hormonal therapy from this group of ER $\alpha$ -positive breast cancer patients, as this would require a large randomized outcome study, there are reasons to consider adding chemotherapy to the treatment regimen for these patients. We find that 94.5 % of the ER $\alpha$ -positive Basal-type breast cancer patients are high risk by the MammaPrint assay, making them potential candidates to benefit from chemotherapy based on their high recurrence risk. Moreover, Basal-type breast cancers have been shown to be significantly more responsive to neoadjuvant chemotherapy as compared to luminal breast cancers, again indicating that addition of chemotherapy could be effective in this patient group [7]. The St. Gallen consensus guidelines state that patients with an (borderline) ER $\alpha$ -positive Basal-type tumor are classified as incompletely endocrine responsive [28]. This relative lack of endocrine responsiveness together with a designation of “high risk” of relapse by MammaPrint will contribute to a clinician’s recommendation of whether endocrine therapy alone may be sufficient or supplementary chemotherapy may be beneficial for these patients.

Our finding that ER $\alpha$ -positive Basal-type tumors are in general borderline ER $\alpha$  positive on mRNA level is in agreement with the conclusions of Iwamoto et al. who found that most of the 1–9 % IHC ER $\alpha$ -positive tumors show molecular features similar to ER $\alpha$ -negative basal-like tumors [17]. The strength of our study is the high number of cases and therefore the better estimate we can make of the frequency of ER $\alpha$ -positive Basal-type tumors. In addition, we show that a majority of these tumors have a high-risk prognostic profile. One limitation of our study is that we do not have all the clinical information for the entire group of patients which was studied here. For instance, we did not have access to the IHC data for all the patients in this study and had to rely on TargetPrint to assess ER $\alpha$  levels. However, IHC data were available for 8 of the 12 ER $\alpha$ -positive Basal type for which ER $\Delta$ 7 expression was determined (Table 1) and showed that 6 of 8 tumors scored clearly positive for ER $\alpha$  protein by IHC.

ER $\alpha$ -positive breast tumors have in general a better prognosis than ER $\alpha$ -negative tumors [29]. In spite of this, the group of ER $\alpha$ -positive Basal-type breast tumors consists nearly exclusively of high-risk patients as judged by the MammaPrint assay (Table 2). Our present data also provide a possible explanation for this finding. In contrast to the OncotypeDX<sup>TM</sup> prognostic signature, the 70-gene MammaPrint<sup>TM</sup> signature does not include ER $\alpha$  [23, 30]. We find that 16 MammaPrint genes are responsive to E2 treatment and that 12 of these are classified as direct ER $\alpha$  targets based on ER $\alpha$ /DNA associations in close proximity to the TSS, indicating that MammaPrint determines ER $\alpha$  activity rather than merely its expression. We believe that this likely explains why the first patient (Table 1, patient 1) having the ER $\alpha$ -positive basal phenotype was characterized by the OncotypeDX assay as “low risk”, but “high risk” by MammaPrint and patient 11 also had a discordant risk assessment in these two assays (Table 1). The ER $\alpha$  mRNA is expressed at a relatively high level in these patients, which is a “good prognosis” factor in the OncotypeDX assay. However, MammaPrint identified this tumor as lacking a functional ER $\alpha$  and came to a “high risk” reading.

In conclusion, by combining TargetPrint and Blueprint molecular subtyping analysis, we have identified a subgroup of some 2 % of breast cancer patients who lack ER $\alpha$  function while expressing ER $\alpha$  at the mRNA and protein level. Our data indicate that such patients are frequently at high recurrence risk and might benefit from adjuvant chemotherapy.

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**Conflict of interest** Arno Floore and Rene Bernards are employees of Agendia NV.

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