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

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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 α (ERα), but fails to respond to hormonal therapy due to the expression of a non-functional receptor. We analyzed a series of 2,658 ERα-positive HER2-negative breast tumors for ERα and progesterone receptor (PR) status as determined by mRNA expression and for their molecular subtypes (Luminal type vs Basal type, assessed by BluePrint™ molecular subtyping assay). In addition, we assessed the recurrence risk (low vs high) using the 70-gene MammaPrint™ signature. We found that 55 out of 2,658 (2.1 %) tumors that are ERα positive by mRNA analysis also demonstrate a Basal molecular subtype, indicating that they lack expression of estrogen-responsive genes. These ERα-positive Basal-type tumors express significantly lower levels of both ERα and PR mRNA as compared to Luminal-type tumors ($P < 0.0001$) and almost invariably (94.5 %) have a high-risk MammaPrint™ profile. Twelve of the MammaPrint™ genes are directly ERα responsive, indicating that MammaPrint™ assesses ERα function in breast cancer without considering ERα mRNA levels. We find a relatively high expression of the dominant negative ERα splice variant ERΔ7 in ERα-positive Basal-type tumors as compared to ERα-positive Luminal-type tumors ($P < 0.0001$). Expression of the dominant negative ERα variant ERΔ7 provides a rationale as to why tumors are of the Basal molecular subtype while staining ERα 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™ high recurrence risk and might benefit from adjuvant chemotherapy.

Keywords Breast cancer · Estrogen receptor variants · Intrinsic subtypes · Molecular subtypes · Tamoxifen

Introduction

The female hormone estradiol (E2) is a potent mitogen for estrogen receptor α (ERα)-positive breast cancers. Hence, ERα protein levels, as determined by immunohistochemistry (IHC), are strongly predictive for response to endocrine therapies [1]. 75 % of all breast cancers express ERα, but not all tumors that express this steroid receptor respond to hormonal therapies. ERα 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, ERα 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 ERα target genes. Hence, the PR is often co-expressed with ERα in breast cancers and PR testing is commonly performed in conjunction with ERα 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 ERα 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™, 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 ERα positive by IHC and/or mRNA expression, but fail to elicit the hormone-induced transcriptional responses that normally result from ER stimulation (ERα target genes “off”; Basal type). Such a scenario would imply that breast cancers having this phenotype express a dysfunctional ERα protein that can nevertheless be detected by IHC.

Several different ERα 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 ERα mRNA. In most ERα 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 ERα used in IHC often include those that recognize an epitope encoded by the first exon of the ERα gene [13], such splice variants are likely detected as IHC positive for ERα, even though their function may be different from the normal ERα protein. The functional activity of these variant ERα proteins can be negative, dominant negative, or dominant active on ERα target genes. Dominant negative variants are not only inactive themselves but also inactivate wild-type ERα through heterodimerization. Two variants, the ERΔ3 and the ERΔ7 variants, have been described as dominant negative receptor forms in the presence of wild-type ERα [8–12]. The ERΔ7 mRNA has been reported to be the major alternatively spliced form in most human breast tumors and cancer cell lines [14]. The ERΔ7 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 ERΔ7 variant has the ability to suppress the E2-dependent transcriptional activation by both wild-type ERα and ERβ [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 ERα and PR in breast cancer, it is recommended that ERα 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 ERα-positive tumors are of the Luminal subtype (as identified by the PAM50 classifier [16]) and that most of these borderline ERα-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 ERα positive by mRNA expression analysis, but are of the Basal molecular subtype. These tumors express significantly lower levels of both ERα and PR mRNA than the Luminal-type tumors and have almost invariably (94.5 %) a high-risk MammaPrint™ profile. Furthermore, we show that these tumors have relatively high levels of the dominant negative ERΔ7 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 ERα mRNA and functional domains. TAF-1 transcription activation function 1, TAF-2 transcription activation function 2, aa amino acid, bp base pair](image-url)
availability of MammaPrint™, TargetPrint™, and BluePrint™ molecular profiling results as performed in the Agenda testing laboratories. The ERα 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 ERα, PR, and HER2 expression [18]. The TargetPrint probe for ERα mRNA detection is located in the 3’ UTR region. The ERα, PR, and HER2 TargetPrint score is a value between −1 and 1, where the null cutoff value is calibrated to 1 % IHC ERα-positive cells, as identified in a reference laboratory according to ASCO/CAP guidelines. Tumors are reported ERα 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].

**ERΔ7 variant analysis**

We obtained RNA from 15 ERα-positive Luminal-type tumors and from 12 ERα-positive Basal-type tumors to analyze the relative ERΔ7 mRNA expression. cDNA was synthesized from 500 ng RNA using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer primers. The total ERα and ERΔ7 mRNA expression was determined by qRT-PCR. For total ERα expression, the forward primer was located in exon 1 and the reverse primer in exon 2. For ERΔ7 expression, the forward primer was located in exon 6 and the reverse primer was designed to specifically detect ERΔ7 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 μl cDNA. The expression levels were quantified using a reference standard dilution curve. The relative expression of the ERΔ7 variant was calculated by dividing the ERΔ7 mRNA expression by the total ERα mRNA expression.

**Identification of ERα target genes in the 70-gene MammaPrint™ breast cancer signature**

The 70 MammaPrint genes were analyzed for ERα binding events within 20 kb from the transcription start site (TSS), representing the most commonly detected window for ER-mediated gene regulation [20]. ERα-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 cancer cell line (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 ~0.1 % were considered as E2 regulated.

**Results**

**ERΔ7 splice variant expressed in an ERα-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 ERα-binding sites adjacent to the TSS [7]. This indicates that the genes that identify Luminal-type breast cancer are significantly enriched for bona fide ERα target genes and suggests that the Luminal subtype is characterized by tumors that have a functional ERα pathway. Conversely, BluePrint Basal-type tumors would be expected to have either no significant ERα expression or a non-functional ERα pathway; these same bona fide ERα 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 ERα positive, but Basal type by BluePrint analysis, would either have a very low level of ERα protein or harbor a defective ERα protein. To test this hypothesis directly, we mined the Agenda database for patients who are ERα 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 positive 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 ERα was present both at the protein (IHC > 90 %) and mRNA levels, but that ERα 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 ERα mRNA transcript in this patient. We first PCR amplified the coding sequence of
Table 1 Characteristics of ERα-positive Basal-type tumors for which the ERA7 expression was determined (N = 12)

| Patient | Age | Stage | IHC ERα | IHC PR | FISH HER2 | TargetPrint ERα index | TargetPrint PR index | TargetPrint HER2 index | BluePrint classification | MammaPrint classification | Oncotype recurrence score |
|---------|-----|-------|---------|--------|-----------|------------------------|----------------------|------------------------|------------------------|--------------------------|--------------------------|
| 1⁷      | 60  | T1bN0M0 | >90 %  | >90 %  | NA⁶       | 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, ERα estrogen receptor α, PR progesterone receptor, FISH fluorescence in situ hybridization, NA not available

⁷ Patient in whom we initially identified the ERA7 variant by cDNA sequencing as is described in the “Results” section
⁵ A TargetPrint index >0.00 is considered as positive, a index ≤0.00 is considered as negative (described in “Patients and methods” section)
⁶ FISH for HER2 not available, but tumor scored negative for HER2 by IHC
ERα with specific oligonucleotides that span the start codon of ERα 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 ERα. Inspection of the DNA sequence of the smaller product revealed an ERα sequence-lacking exon 7 of the coding sequence (data not shown). This transcript corresponds to the previously reported domain of ERα and therefore recognizes both wild-type ERα and ERΔ7. We show in these cells that the relative ERΔ7 levels as measured by qRT-PCR are highly concordant with protein expression (Supplementary Fig. 1).

The average total ERα mRNA expression by qRT-PCR was significantly lower for the 12 analyzed ERα-positive Basal-type tumors compared to 15 randomly chosen ERα-positive Luminal-type tumors (Fig. 2a; \( P = 0.0019 \)), consistent with the TargetPrint results (Table 2). There was no significant difference in average ERΔ7 mRNA expression between the ERα-positive Basal-type and Luminal-type samples (Fig. 2b; \( P = 0.4088 \)). However, the relative ERΔ7 mRNA expression was significantly higher for the ERα-positive Basal-type group compared to the ERα-positive Luminal-type group (Fig. 2c; \( P < 0.0001 \)), due to the lower overall ERα mRNA expression in the Basal-type tumors.

The characteristics of the 12 ERα-positive Basal-type tumors, for which ERΔ7 splice variant expression was determined, are shown in Table 1. For 8 of the 12 patients, we were able to retrieve the ERα and PR IHC scoring. Based on the ERα IHC, six out of eight (75 %) patients were classified as ERα positive. In two patients, we found a discrepancy between TargetPrint and ERα IHC classification; in one of these patients, the TargetPrint ERα index was just above the ERα-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 ERα/PR index, PR classification, and MammaPrint classification of 2,658 ERα-positive, HER2-negative tumors according to their Blueprint molecular subtype (Basal type vs Luminal type) |
|-----------------|-----------------|----------------|
| Blueprint classification | Luminal type (\( n = 2,603, 97.9 \% \)) | \( P \) value |
| ERα index (mean ± SD) | 0.20 (±0.15) | 0.57 (±0.17) | <0.0001a |
| PR index (mean ± SD) | −0.04 (±0.27) | 0.28 (±0.31) | <0.0001a |
| PR classification | | | |
| PR positive | 24 (43.6 %) | 2047 (78.6 %) | <0.0001b |
| PR negative | 31 (56.4 %) | 556 (21.4 %) | |
| MammaPrint classification | | | <0.0001b |
| Low risk | 3 (5.5 %) | 1434 (55.1 %) | |
| High risk | 52 (94.5 %) | 1169 (44.9 %) | |

ERα estrogen receptor α, PR progesterone receptor, SD standard deviation

a Unpaired \( t \) test, two-tailed

b Fisher’s exact test, 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 ERα function independent of ERα expression

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

Since the MammaPrint assay identifies nearly all these ERα-positive Basal-type tumors as high risk, it suggests that the test measures ERα activity independent of the ERα 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 publically 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 ERα target genes. Using a publically available ChIP-seq dataset [22], the genome-wide chromatin-binding landscape of ERα in MCF7 cells was analyzed for the occurrence of an ERα binding event within 20,000 bp from the TSS of any of the MammaPrint genes.
This window was chosen since most ERα-mediated gene regulation is found within this distance from a TSS [20]. Ten out of 16 genes had an ERα binding event within 20,000 bp from the TSS (Fig. 3a), as exemplified for the LPCAT1 locus (Fig. 3a). Importantly, the essential ERα coactivators AIB1 (also known as SRC3) and p300 were also present at this specific binding site, indicating that ERα is likely to be functional here [24]. Furthermore, we confirmed that ERα binding events in E2-regulated MammaPrint genes are also found in 2 ER-positive Luminal human breast tumor samples, for which ERα ChIP-seq data are available (Fig. 3a). In total, 12 out of 16 E2-regulated genes had an ERα-binding site in either MCF7 cells or in the two studied tumors (Fig 3a). Cumulatively, these data indicate that bona fide ERα target genes are enriched in the MammaPrint gene signature, providing a plausible explanation for why the MammaPrint can measure ERα 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 ERα target genes, which is generally thought to result from the absence of ERα expression [25]. However, the group of tumors identified here is ERα positive on the mRNA level, suggesting that their Basal phenotype is the result of a lack of ERα protein expression or a lack of functionality of the ERα protein present in these tumors. Indeed, we find that these tumors not only express relatively low levels of ERα mRNA but also express a splice variant of ERα-missing exon 7 (ERΔ7, Fig. 2a, b). This ERα variant has been shown previously to act in a dominant negative fashion, meaning that this variant can inhibit the function of the wild-type ERα protein when co-expressed in the same cell [14]. We note that the absolute levels of ERΔ7 are comparable in ERα-positive Basal-type versus ERα-positive Luminal-type tumors, but that the relative abundance of ERΔ7 is higher in the ERα-positive Basal-type tumors (Fig. 2c). We interpret these data as follows: When the levels of wild-type ERα in a breast tumor are high, the inhibitory effects of dominant negative ERΔ7 are by comparison minor, leaving the cell with considerable ERα activity and thus with a luminal phenotype (Fig. 4, right). In contrast, lower levels of wild-type ERα in the weakly ERα-positive breast tumors are inhibited to a greater extent by the presence of ERΔ7, leaving the tumor cells with insufficient ERα activity to...
regulate ERα target gene expression and thus with a Basal phenotype (Fig. 4, left). It remains to be explained why lower levels of ERα result in a relative increase in abundance of the ERα7 splice variant. It is possible that ERα also controls the expression of certain components of the splicing machinery and that low ERα activity therefore results in a different processing of the ERα (and potentially also other) precursor mRNAs.

A clinically relevant question is whether this identified group of ERα-positive Basal-type tumors is likely to respond to hormonal therapy. The finding that ERα 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 may be premature to withhold hormonal therapy from patients treated with adjuvant tamoxifen. It was found that 94.5% of the ERα-positive breast tumors have in general a better prognosis than ERα-negative tumors [29]. In spite of this, the group of ERα-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™ prognostic signature, the 70-gene MammaPrint™ signature does not include ERα [23, 30]. We find that 16 MammaPrint genes are responsive to E2 treatment and that 12 of these are classified as direct ERα targets based on ERα/DNA associations in close proximity to the TSS, indicating that MammaPrint determines ERα activity rather than merely its expression. We believe that this likely explains why the first patient (Table 1, patient 1) having the ERα-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 ERα 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 ERα 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 ERα function while expressing ERα 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 Bernard are employees of Agendia NV.

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