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Gene expression of ERβ isoforms in laser microdissected human breast cancers: Implications for gene expression analyses

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Abstract. *Background*: Despite many published studies on ERβ, progress towards understanding its role in breast cancer remains slow. This is largely due to discordant data between mRNA and protein studies as well as failure to take into account the biologically distinct ERβ isoforms and their heterogeneous expression profile.

Methods: We compared expression of ERβ1, -2 and -5 genes in HB2 and MCF-7 breast cell lines, primary breast fibroblasts ($n = 5$) and whole tissue and laser microdissected epithelial and stromal cells obtained from 25 human breast tumours.

Results: Our study shows that the level of gene expression of ERβ isoforms depends on the cell population within a given tumour and varies dramatically in different cellular compartments. This has implications for gene expression analyses and could explain some of the contradictory data published to date, rendering "grind and bind" analyses of ERβ uninformative.

Conclusion: With the technology now available, we suggest a more refined approach be adopted to help resolve some of the controversy surrounding ERβ.

Keywords: Estrogen receptor β, laser microdissection, breast cancer

1. Introduction

Estrogen receptor (ER) β was first described in 1997 [1]. Initial studies on its putative role in breast cancer were conducted using RT-PCR on whole tumour extracts [2–8]. Whilst this was the only method available at the time to study ERβ, there have since been significant advances. Development of robust antibodies have permitted immunohistochemical studies which have demonstrated that unlike its counterpart ERα, which is generally only seen in epithelial cells, ERβ expression is widespread having been found in epithelial, myoepithelial, endothelial and stromal cells as well as lymphocytes and adipocytes [9,10]. We also know that there are five separate ERβ isoforms (ERβ1–5) formed by alternative splicing of exon 8, the last coding exon [11,12]. Recent immunohistochemical studies have demonstrated that expression of these isoforms can identify breast cancer patients with different prognoses or responses to endocrine therapy, underlining their separate functions [13,14].

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Expression analyses using qRT-PCR is a useful screen for candidate genes in cells and tissues. Studies have been performed to account for different ERβ isoforms [4–6,8,15] however the heterogeneous cellular expression profile of ERβ is mostly overlooked; over ten years since the discovery of ERβ recent literature shows that many researchers still continue to study whole tissue [16–18]. This could have implications for data interpretation and may partly explain discordant data between mRNA and protein studies [19–21]. The aim of this study was to use laser microdissection (LMD) to selectively capture epithelial and stromal cells from breast tumour sections and compare gene expression levels of ERβ1, -2 and -5, the 3 main isoforms expressed in breast tumours [13] to that of whole tissue and breast cell lines.

2. Methods

2.1. Samples

Clinical material

Ethical approval was obtained from the Leeds (East) Regional Ethical Committee at St. James's Univer-

Table 1
Clinicopathological characteristics of the tumours studied

Characteristic	Number
Histological type	
DNST	21
Lobular	3
Other	1
Grade	
I	3
II	10
III	10
n/a	2
Lymph node	
+	13
−	12
ER	
+	15
−	8
n/a	2
PR	
+	13
−	7
n/a	3

sity Hospital, Leeds, UK. Fresh tissue was transported to the laboratory, trimmed of adipose tissue and either embedded in Lambs OCT then snap frozen in liquid nitrogen-cooled isopentane for cryosectioning ($n = 25$) or enzymatically processed to yield fibroblasts ($n = 5$) as previously described [22]. Tumour characteristics are shown in Table 1. For cryosectioning (Leica CM3050S), the chamber was cooled to -28°C for at least 45 min prior to use. Four 7 μm sections were cut onto PALM membrane slides (Zeiss). Cryo-sections were fixed in 100% alcohol for 1 min and dipped in RNase free water for 30 s. Sections were either used immediately or stored at -80°C. Prior to laser microdissection (LMD) sections were stained with a mix of 2 μl of 1% eosin/10 μl 1% toluidine blue made up in RNase-free deionised water for 45 s. LMD and pressure catapulting was performed at ×20 magnification on a PALM microbeam microdissector. Areas of 5 mm^2 were catapulted into sterile opaque adhesive caps (PALM).

Cell lines

MCF-7 and HB2 cell lines were maintained according to Masanat et al., 2008 [23].

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted according to the manufacturer's instructions (Stratagene Nanoprep) except that the elution volume was increased to 15 μl to provide sufficient RNA for cDNA synthesis and appropriate controls. First strand cDNA synthesis was performed on 11.5 μl of RNA extract using Affinity script multiple temperature reverse transcriptase (Stratagene) according to the manufacturer's instructions, using random hexamers (Invitrogen). For cell lines and fibroblasts total RNA was extracted and cDNA synthesised as previously described [24]. For qRT-PCR relative quantification of the test mRNA in relation to the housekeeping gene RPLP0 was carried out using the ΔCt method [25]. MCF-7 cDNA prepared from the same RNA batch was included as a standard in all PCR runs. Samples were batched to ensure each of the 3 ERβ isoforms were represented on each PCR plate, thus allowing comparative expression of each gene. Primer sequences and optimised concentrations are shown in Table 2, and were designed to span exon–intron boundaries, with the exception of ERβ5. qRT-PCR was carried out using Brilliant II SYBR green qPCR master mix (Stratagene) using a Stratagene Mx3000P thermal cycler. Cycle threshold (Ct) values were determined using MxPro software using the adaptive baseline correction algorithm. Primer concentrations were optimised and the amplification efficiencies were 90–110%, calculated from the slope of log [template] vs. Ct graphs, which had RSq-values $\geqslant 0.985$. Dissociation curves were performed after each run to ensure amplification of a single product. Negative controls (minus RT and minus template) were also included.

3. Results

3.1. Cell lines

Expression of ERβ1, -2 and -5 genes in MCF-7 and HB2 cells and in 5 different breast fibroblast cultures derived from primary breast tumours is shown in Fig. 1. In terms of relative expression, ERβ5 > ERβ2 > ERβ1, with approximately 10-fold difference in the level of expression between each of the 3 isoforms. When levels of expression of individual genes were compared, ERβ1 was expressed at similar levels in MCF-7 and HB2 cells however considerable variation was seen in fibroblasts, most likely due to biological differences between donors. ERβ5 was generally more abundant in epithelial cells, while ERβ2 expression was variable.

Table 2
Sequences and optimised primer concentrations of ERβ isoform-specific and RPLP0 reference gene qRT-PCR primers

Primer	Sequence (5′–3′)	Concentration (nM)
ERβ1 Forward	dCGCCTGGCTAACCTCCTGATG	300
ERβ1 Reverse	dGAGCAGATGTTCCATGCCCTTG	300
ERβ2 Forward	dCGCCGTGACCGATGCTTTG	400
ERβ2 Reverse	dCCTTTTCTGCCCTCGCA	300
ERβ5 Forward	dGCATCTCCTCCCAGCAGCAATC	500
ERβ5 Reverse	dGCACATAATCCCATCCCAAGCC	500
RPLP0 Forward	dGAAACTCTGCATTCTCGCTTCC	100
RPLP0 Reverse	dGATGCAACAGTTGGGTAGCCA	100

3.2. Clinical samples

We then compared expression of ERβ1, -2 and -5 in whole sections and laser microdissected epithelial and stromal cells microdissected from breast tumours. As anticipated, greater gene expression was observed in whole tissue sections compared to microdissected material (Fig. 2a). Scatter plots of laser miscrodissected epithelial and stromal cells showed that ERβ1 was significantly more abundant in the stromal component while ERβ5 was significantly expressed in the epithelium. ERβ2 expression was low in both cell types when compared to its expression in tissue (Fig. 2b). We then tested the hypothesis that the wide variation in expression of ERβ1, -2 and -5 in whole tissue sections (Fig. 2a) was due to tissue heterogeneity. This was confirmed in Fig. 3, where considerable heterogeneity in ERβ isoform expression in whole sections and microdissected epithelial and stromal samples across individual tumours was observed. This probably reflects phenotypic differences in tissue composition (Fig. 3c, f, i).

4. Discussion

Progress towards understanding the potential clinical role of ERβ has been slow. While there are now good antibodies to measure the protein immunohistochemically, many studies still continue to use "grind and bind" techniques to determine its expression at a genetic level in tissues such as breast, prostate, lung and colon where ERβ has been shown to have a putative clinical role [26,27]. While the contribution of isoforms is often taken into consideration by designing isoform-specific oligonucleotide primers [4–6,8, 15] the contribution of different cellular components to

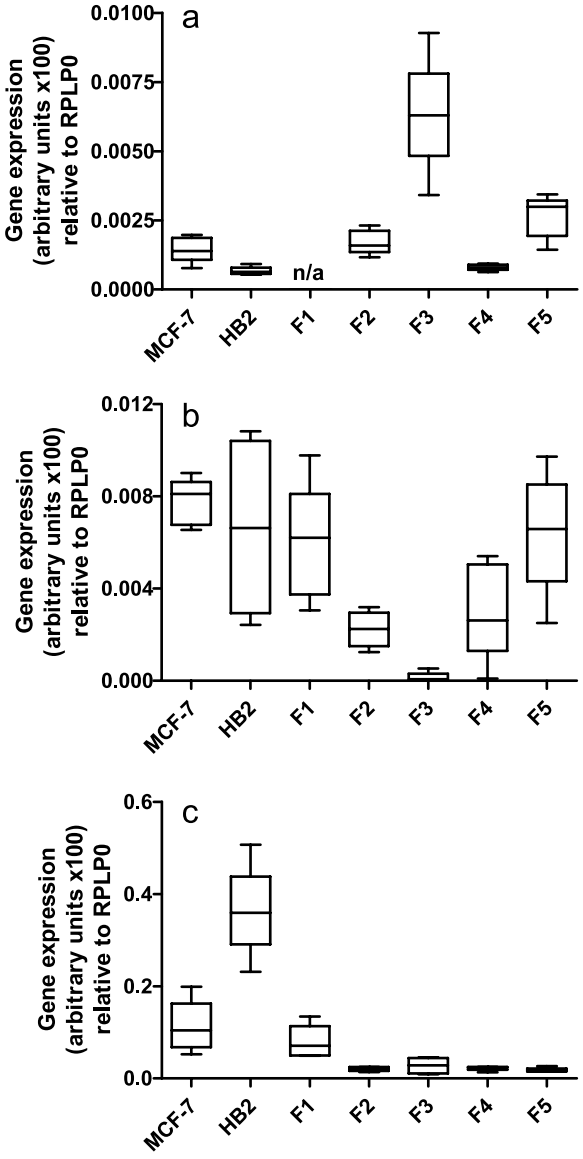

Fig. 1. qRT-PCR expression of ERβ1 (a), -2 (b) and -5 (c) in benign (HB2) and malignant (MCF-7) breast cell lines and fibroblasts (F). Expression levels were calculated relative to the housekeeping gene, *RPLP0* and are presented as boxplots. n/a – not analysed (insufficient cDNA).

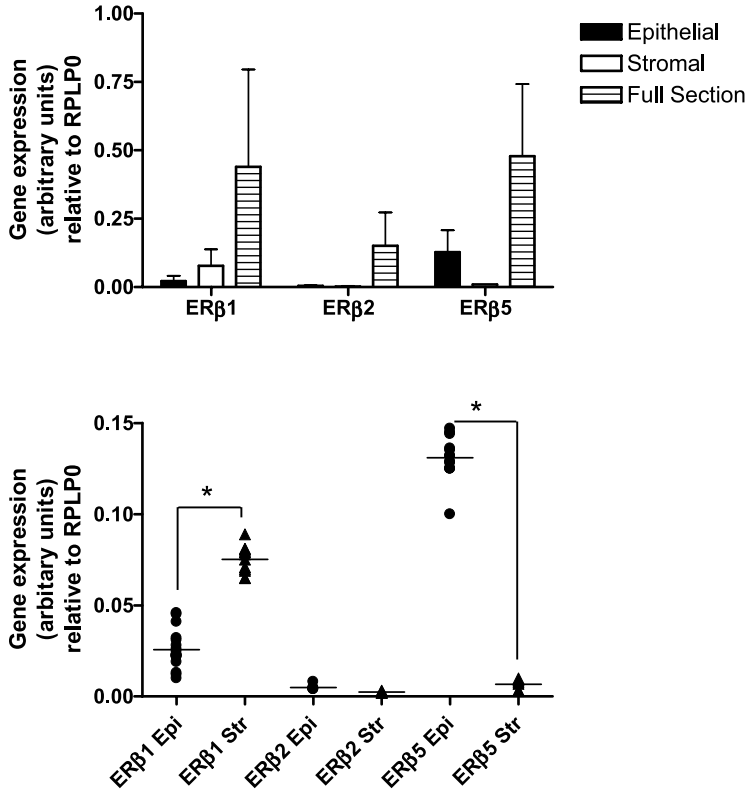

Fig. 2. qRT-PCR expression of ERβ1, -2 and -5 in whole sections and epithelial and stromal cells microdissected from breast cancer cell lines and fibroblasts (a). Mean data for epithelial and stromal components is re-plotted in (b) and shows significantly more ERβ1 and ERβ5 in the stromal and epithelial compartments, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$. Data is presented as mean values $\pm$ SD from 25 individual breast tumours.

the gene expression signature tends to be overlooked. This study has shown that gene expression of ERβ isoforms varies considerably in different cellular compartments following LMD of human breast tumours.

In mammary cell lines and fibroblasts expression of ERβ isoforms was ranked: ERβ5 $>$ ERβ2 $>$ ERβ1. The high expression of ERβ5 mirrors what Wong and colleagues [28] observed in colon cancer however a similar study in breast cancer revealed mixed expression of ERβ5 in a panel of breast cancer cell lines and 30 primary breast tumours [5]. Similar varied expression of ERβ1 and ERβ2 was reported in the latter study. In fibroblasts ERβ isoform expression seemed to be tissue specific. In mammary fibroblasts derived from both normal and malignant adult mammary glands and in endometrium ERβ2 was the principal isoform [29,30], while in Sertoli cells of the testis ERβ2 but not ERβ1 was expressed [31]. We showed ERβ1 was significantly more abundant in the stromal component, however there was variability in expression in stromal cultures derived from different donors, with one sample in particular (F3) expressing considerably more ERβ1 than the others. Interestingly F3 had very low expression of ERβ2. Work by Hurtado and colleagues have shown that in a prostate cell line, ERβ1 and ERβ2 mRNA and protein expression are cell cycle-associated [32]. It is possible that the proportion of cells within a given phase of the cell cycle may influence ERβ isoform expression.

In general pooled gene expression data showed that levels of specific ERβ isoforms in breast tumours mirrored that observed in benign and malignant breast cell lines and in cultured human mammary fibroblasts. However care must be taken when considering individual tumours as we have shown that, unsurprisingly, their gene expression profiles change with respect to tissue composition. This is reinforced in gene expression profiling of fine needle aspirates from breast carcinoma patients which showed tumour heterogeneity could impact on gene expression profiles [33]. Inspecting H&E sections under the supervision of a pathologist prior to proceeding to extraction should be

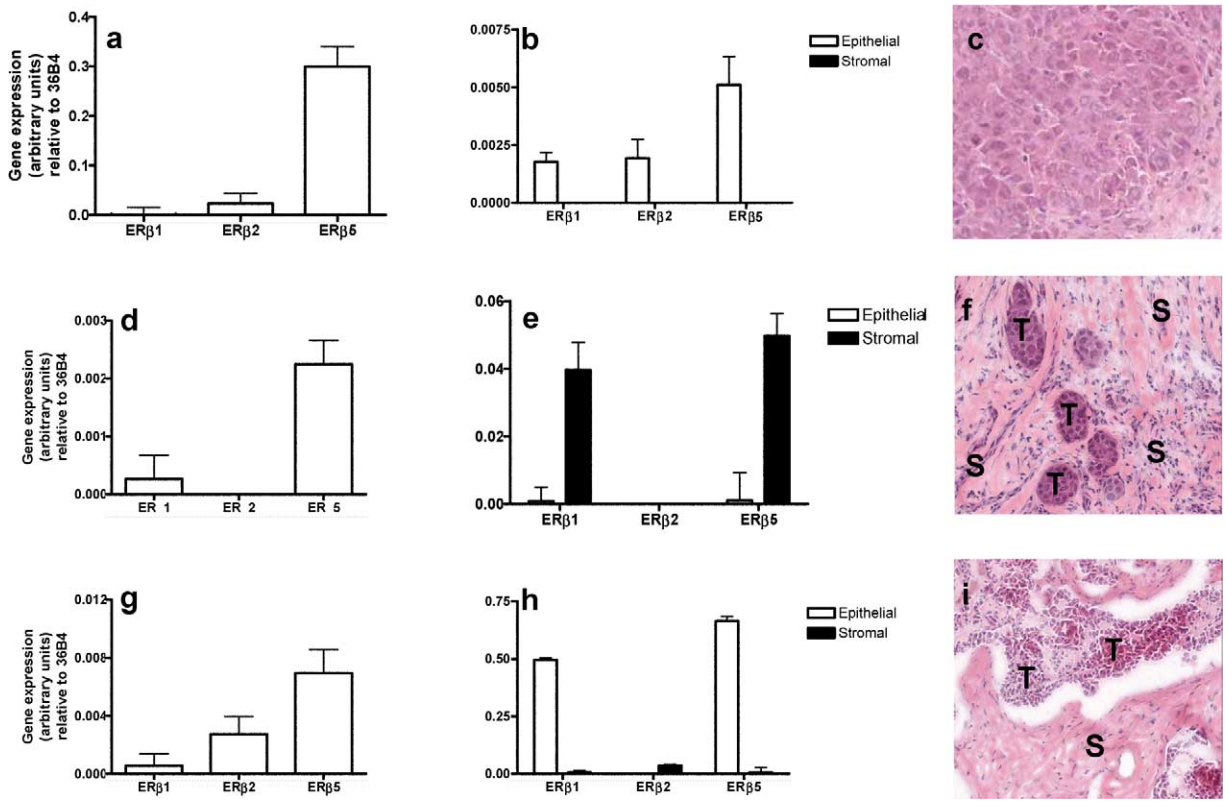

Fig. 3. Representative examples of qRT-PCR expression of ERβ1, -2 and -5 in 3 whole tissue (a, d, g) and epithelial and stromal cells microdissected from 3 different breast tumours (b, e, h). Expression varies according to tissue cellularity (c, f, i). Data is presented as mean values ± SD. T – tumour cells, S – stromal cells.

considered. An additional concern is that studies on whole tissue may give an overestimate of the level of expression of ERβ as a result of failure to account for the multiple cell types which often exist within a given tumour (as exemplified in Fig. 3f which has a considerable stromal element) and which can express ERβ e.g. endothelial cells, lymphocytes and adipocytes [34]. Stromal density of different tumours could also influence this; the stroma in Fig. 3f is dense compared to that of Fig. 3i and is reflected in the different stromal gene expression signatures in each of these cases. This has been considered recently for the pre-invasive breast lesions LCIS [35], but to our knowledge this is the first time it has been accounted for in invasive breast cancer.

Studies in prostate cancer examined ERβ gene expression in LMD epithelium and stroma, but did not account for specific isoforms [36,37]. In lung LMD was used to microdissect normal lung and NSCLC but subsequent downstream gene expression analysis did into account for isoforms [38]. This was also true for microdissected breast stroma [39]. Our study indicates that both should be taken into account in order to generate the most informative data. We suggest that other reports analysing ERβ gene expression in pulverised frozen tumours must be treated with caution as this is not the most appropriate way of conducting gene expression analysis studies. We already know that ERβ is subject to complex regulation [40] with on-going work from our group showing this involves 5′-UTRs [41] and also miRNAs (Al-Nakhle, Speirs unpublished observations). Such complex regulation may also help explain the non-concordance of ERβ at the mRNA and protein level which has been consistently reported [19–21] and could render these comparisons uninformative.

In summary, the level of expression of ERβ isoforms at the genetic level depends on the cellularity of a given tumour. As breast cancer is an heterogeneous disease a more refined approach for gene expression analysis of ERβ is clearly more rationale in order to avoid producing potentially misleading or uninformative data.

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