Gene Expression Profiles in Breast Cancer to Identify Estrogen Receptor Target Genes

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Abstract: The estrogens play important role in the homeostatic maintenance of several target tissues including those in the mammary gland, uterus, bone, cardiovascular system, and brain. Most of estrogen's action is thought to be mediated through its nuclear estrogen receptors, ERα and ERβ, which are members of the nuclear receptor superfamily that act as ligand-induced transcription factors. Acting via its receptors, estrogen also plays an essential role in the development and progression of human breast cancer. The ER and progesterone receptor (PR), which are regulated by estrogen via ER, have been used as prognostic markers in the clinical management of breast cancer patients. However, the prognosis of a patient with ER+/PR+ breast cancer can be highly variable and a significant proportion of hormone receptor positive breast cancers does not respond to endocrine therapy. The identification of estrogen receptor target genes may improve our understanding of the role played by estrogens in breast cancer making it possible to better tailor hormone treatments and improve a patient's response to hormonal therapy. In this review, we explore the literature for data regarding the identification of estrogen receptor-regulated genes in breast cancer cell lines and breast tumor biopsies using high throughput technologies such as serial analysis of gene expression (SAGE) and cDNA microarrays.

Key Words: Breast cancer, estrogen receptor, prognostic marker, gene expression profiling.

INTRODUCTION

Estrogens are small lipophilic molecules produced mainly by the ovary and carried out through the blood stream to specific target tissues. The major form and most potent natural estrogen is 17β-estradiol (E_2) followed by estrone and estriol which are less effective than estradiol (Fig. **1**). Besides the critical role played by estrogens in the development and maintenance of the reproductive system, there are compelling evidences that they also play important roles in regulating the physiological functions of various organs, such as bone, brain, and heart [1, 2].

In the mammary gland, the estrogens mediate key physiological processes that are essential for the normal growth and differentiation. However, there is a large body of evidence showing that estrogens, especially E_2, also play a critical role in the development and progression of breast cancer [3]. Most of the complex biological functions of the estrogens are mediated through the estrogen receptors, ERα and ERβ, *via* the transcriptional regulation of ER target genes. Estrogen's actions can be partially blocked by selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, or by selective estrogen receptor down-regulators (SERDs), such as fulvestrant ("faslodex", ICI 182,780), that is a pure antagonist, which binds to ER leading to its destabilization and degradation (Fig. **1**) [4]. In addition, the third-generation non-steroidal aromatase inhibitors (AIs), anastrozole and letrozole, which inhibit estrogen production can be combined with fulvestrant to overcome resistance and improve patient's response to hormone therapy [5].

In fact, the ER is a useful prognostic and predictive marker for breast cancer. Approximately two-thirds of breast tumors express ER and are considered hormone-dependent, with hormone therapy widely accepted as the most important treatment for these patients. However, a proportion of the ER-positive breast cancer patients either does not respond to hormonal therapy, or become resistant to it [6]. In the last years, various molecular technologies that allow high throughput analysis of gene expression profiling have been used to identify the gene expression signature associated with the hormone-dependence of breast cancer that might improve our understanding of the ER-positive breast cancers and help select therapy for individual patients.

ESTROGEN RECEPTORS

The estrogen receptors belong to a family of structurally related and highly conserved proteins, the nuclear hormone receptor super-family, which includes receptors for progesterone, androgen, glucocorticoid, thyroid hormones, retinoic acids, and vitamin D. These steroid hormone receptors are ligand-activated transcriptional factors composed of a modular structure that includes several functional domains. These domains were designated A to F and carry out specific functions (Fig. **2**). The A/B domain is located in the N-terminal region and comprises the hormone-independent transcriptional activation function (AF1). The highly conserved C domain is located in the central region of the receptor molecule and corresponds to the DNA-binding domain, which contains two zinc-fingers motifs that directly interacts with the hormone response elements (HREs) in the promoter regions of the target genes. The D domain refers to a hinge

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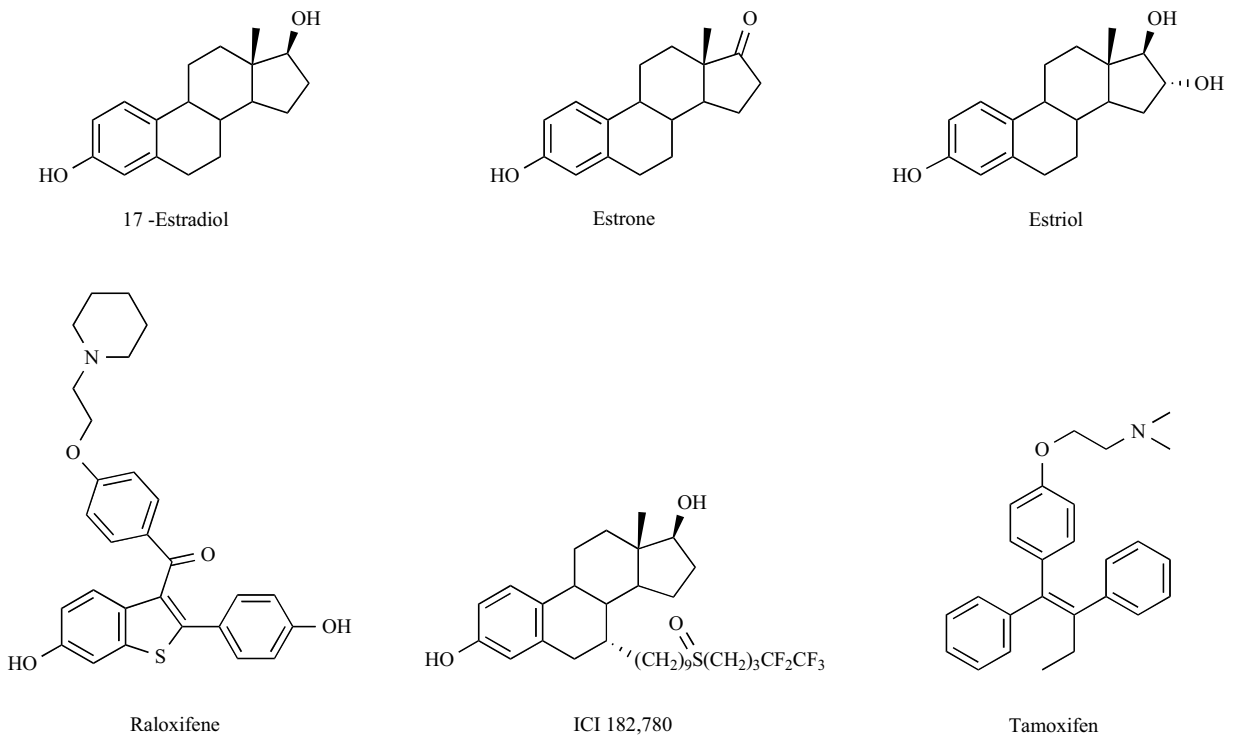

Fig. (1). Chemical structure of natural estrogens (17β-estradiol, estrone and estriol) (A); Chemical structures of selective estrogen receptor modulators (SERMs), tamoxifen, and raloxifene, and the pure antagonist ICI 182,780 ("faslodex"; fulvestrant) (B).

region associated with receptor dimerization and interaction with co-regulatory proteins. The E/F domain is located at the C-terminal region and contains the ligand-binding domain [7].

The two functional isoforms of the ERs, ERα and ERβ, are encoded by two distinct genes. The *ESR1* gene is located on chromosome 6q25.1 and encodes the 66 kDa ERα protein [8], and the *ESR2* gene is located on chromosome 14q23.2 and encodes the 54 kDa ERβ protein [9]. The expression pattern of ERα and ERβ in adults is tissue specific. ERα is ubiquitously expressed, and found predominantly in breast, uterus, cervix, and vagina, while ERβ expression is usually observed in the ovary, prostate, testis, spleen, and hypothalamus. ERβ is often co-expressed with ERα in breast carcinoma [10, 11]. These receptors share high similarity in their DNA-binding domain (97%) (Fig. **2**).

MECHANISMS OF ER ACTION

Due to their lipophilic properties, the estrogens can cross the cell membrane to enter into the cell cytoplasm and nucleus and bind to ERs, which dissociates from Hsp90 and acquire an activated state. Once activated, the hormone-receptors form either homo- or heterodimers, and bind to estrogen-response elements (EREs) on DNA to activate or repress gene transcription. The EREs are specific DNA sequences located in the promoter region of ER target genes to which the ERs directly interact with high affinity inducing the recruitment of co-regulatory factors and the basal core of transcriptional proteins leading to transcriptional regulation. ER transcriptional activity is enhanced by co-activators, such as the members of the SRC family (SRC1, like SRC2 and SRC3) and is repressed by the interaction of co-repressors such as NCOR1, NCOR2, and RIP140 [12]. In addition, cis-regulatory domains such as the Forkhead motif coupled with

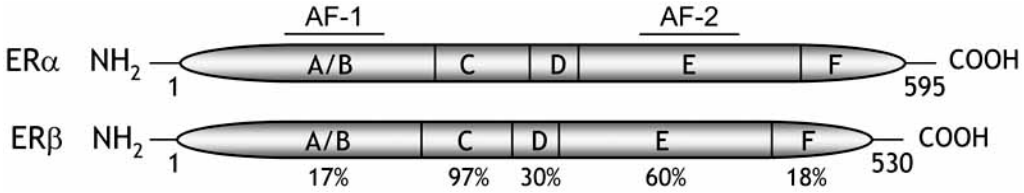

Fig. (2). Diagrammatic representation of the domain structure of the human estrogen receptors, ERα and ERβ. The ERs molecules are composed of six structural and functional domains (A-F). The variable A/B domain contains the ligand-independent transcriptional-activating function (AF-1). The highly conserved DNA-binding domain (C) contains two zing finger structures that recognizes and bind to specific DNA sequences. The D domain is associated with receptor dimerization. The E/F domain contains the ligand-binding domain (LBD) and the ligand-dependent activation function (AF-2). Percentages of amino acid identity between ERα and ERβ in the corresponding functional regions are represented.

the Foxa1 transcription factor may facilitate and enhance ER-mediated transcription [13, 14]. This classical model of ER action involves direct interaction of the hormone-receptor complex with specific EREs.

The minimum consensus ERE consists of a 13 base pair perfect palindromic inverted repeat sequence separated by 3 non-conserved bases, GGTCANNNTGACC, derived from the sequence of the promoter regions of highly estrogen-induced genes [15]. These DNA binding domains are essential for the specificity of target-gene activation, and sequence deviations of the consensus ERE sequence reduces the affinity and specificity of the receptor interaction and the effectiveness of the transactivation activity [16]. Both ERα and ERβ form homo- or heterodimers and bind to specific EREs with similar specificity, but usually ERβ displays weaker transactivation activity than ERα [17, 18]. Most of the estrogen-target genes identified so far do not contain a perfect palindromic consensus sequence, which is expected to occur once every 4 million base pairs in random DNA sequences [15]. However, the ERs are able to induce transcriptional activation by binding to perfect or imperfect palindromic sequences separated by more than 3 base pairs, and also to direct repeats of half EREs, but with lower affinity [19]. A combination of experimentally defined and computationally predicted data can provide useful information about human ER target genes with putative EREs [20-22].

The classical ligand-dependent mechanism of ER action was for decades accepted as the only way through which the ER could induce transcriptional transactivation of target genes. During the last decade, it became clear that the mechanism through which the ER mediates the transcriptional regulation of gene expression is more complex. Besides the classical ligand-dependent mechanism of ER action, in which the hormone-receptor complex regulates gene transcription through its interaction with ERE consensus DNA sequences, the ER can also regulate gene transcription *via* protein-protein interaction with other transcriptional factors that bind to other promoter elements such as AP1, SP1, and CREs [18, 23, 24]. In fact, approximately one third of the ER-responsive genes have no ERE-like sequences in their promoter regions [19]. Moreover, extensive analysis of structural and functional properties of ERα and ERβ have led to additional complexity in this area, showing that these ER isoforms can transduce different hormonal signals depending on the ligand and the nature of the ERE [25, 26]. Thus, ERα and ERβ can transduce different hormonal signals depending on the ligand, and the nature of the hormone responsive element (HREs).

The transactivation elicited by receptors complexes with E_2 may result in opposite signal transduction, leading to opposite biological responses in the presence of AP1 and/or CRE sites [27, 28]. In addition, several E_2-responsive genes are regulated by DNA-independent or –dependent interactions of the ERα and SP1 proteins. Otherwise, the signal transduction by growth factors and their tyrosine kinase receptors, such as EGFR, IGFR, erbB-2, and other molecules such as cAMP and dopamine, may lead to a ligand-independent ER activation, resulting from the phosphorylation of serine and tyrosine amino acid residues in the AF1 and AF2 domains in the estrogen receptor molecule [29]. ER phosphorylation promotes receptor dimerization, association with co-regulatory proteins, and transcription transactivation.

On the other hand, estrogens are also responsible for rapid biological effects, called non-genomic actions that are independent of mRNA and protein synthesis [30]. Although controversial, these non-genomic effects are thought to be displayed by E2 through a subpopulation of ER associated with the cell membrane, which induces the activation of intracellular second messengers such as calcium, nitric oxide formation, and protein kinase cascades such as ras-raf-MAPK (mitogen-activated protein kinase), ERK1 and ERK2 (extracellular-signal related kinases) or PI3K-AKT (phosphoinositide 3 kinase-protein kinase B) [31, 32]. In summary, there are at least four models of ER action: A, ligand-dependent (classical); B, ERE-independent; C, ligand-independent (cross-talk with growth factors), and D, ER cell membrane signaling (non-genomic) (Fig. **3**).

GLOBAL GENE EXPRESSION PROFILING OF ER ACTION

Although estrogens have been implicated as a major etiological factor in the tumorigenic process of breast cancer, the details of the effects of E_2 on downstream gene targets are far from fully understood. With the improvement of high-throughput experimental technologies such as SAGE and microarrays, information about estrogen signaling has been accumulating rapidly, showing that estrogen affects hundreds of genes. Gene expression profiles in response to E_2 have been carried out, particularly in single breast tumor cell lines, and most of the data have come from experiments with MCF-7, T47D, BT-474, and ZR-75 cells [33-46]. These cell lines are estrogen dependent breast cancer cells whose growth can be blocked by antiestrogens [47]. Although many investigators used similar platforms, there are differences in breast cancer cell lines expression profiles performed over a limited time course of hormone treatment (Table **1**). In particular, reports carried out using several distinct lengths of estrogen exposure revealed a diversity of temporal patterns of gene regulation by E2, with genes showing rapid changes in mRNA levels that could be sustained or not at later times, and transcripts induced or repressed beginning at later time points [36, 46, 48].

Several previous genes identified by a variety of methodological approaches and focused on one gene or a few genes at a time such as trefoil factor-1, PR, cyclin D1, GATA-3, catepsin D, pS2/TFF-1 and c-myc were confirmed in some studies and several novel genes were described. Many of these ER up-regulated genes are important for cell proliferation and survival. However, E_2 down regulation of multiple transcriptional repressors could also contribute to increased cell proliferation. Suggested mechanisms by which E2 has been shown to repress gene expression involve the sequestering of co-activators or inhibition of NF-κß [49]. In all those studies, the term ER was referring to ERα.

The definition of ER direct target genes was further refined and a group of genes that are responsive to E_2, sensitive to ICI 182,780, and insensitive to the protein synthesis inhibitor cycloheximide (CHX) was identified in ER+ breast cancer cell lines (T47D, ZR-75, and MCF-7). This group of genes was considerably small as compared with the total

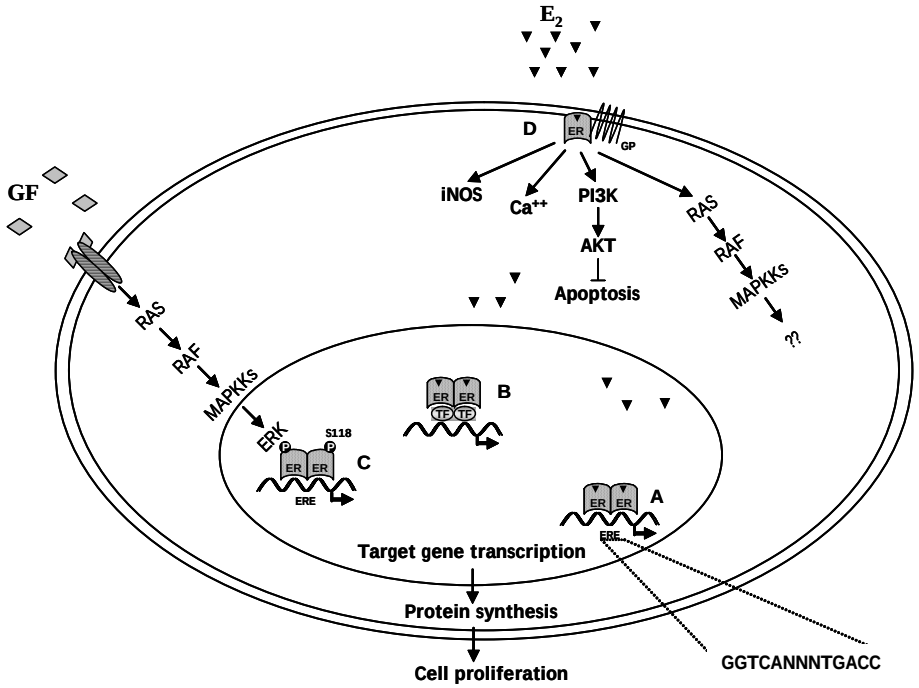

Fig. (3). Schematic illustration of ER mechanisms of action. A, Classical ligand-dependent: the E_2-ER complexes as homo- or heterodimers interact with EREs in the promoter region of target genes; B, ERE-independent: the E_2-ER complexes interact with other transcription factors, such as AP1 and SP1 that bind to their cognate DNA binding sites; C, ligand-independent: growth factors, such as EGF and IGF-I, activate protein kinase cascades leading to ER phosphorylation (P) and activation; and D, non-genomic: estrogens binding to membrane–associated ERs lead to the activation of intracellular protein kinases cascades, such as MAPK and PI3K signaling pathways. E_2, estradiol (triangles); ER, estrogen receptor; GF, growth factors; TF, transcriptional factors; ERE, estrogen response element; iNOS, inducible nitric oxide synthase; PI3K, phosphoinositide-3-kinase; AKT, v-akt murine thymoma viral oncogene homologue; RAS, v-ras oncogene homologue; RAF, v-raf murine leukemia viral oncogene homologue; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases.

number of genes induced by E_2, suggesting the possibility of ER independent mechanisms in those cells [34, 39, 42]. Using these stringent criteria, a core set of ER direct target genes showed similar behavior in T47D and MCF-7 cells [42].

Therefore, using the initial inventory of responsive genes, one of the next questions addressed was to confirm the interaction between ER and ERE-like sites in the promoters of putative direct target genes. The Chip-on-chip technique that combines chromatin immunopreciptation with microarrays has important application for genome-wide identification of DNA binding sites for transcription factors, such as EREs in the promoter regions of ER target genes (Table **1**). Chip-on-chip experiments were performed to monitor recruitment of ERα to the EREs in several genes known to be regulated directly by E2 in MCF-7 cells and binding was observed for practically all tested genes [21]. Similar results in T47D and MCF-7 cells were observed by Lin *et al.* 2004 [42], suggesting that the ERE is the major response element mediating the specific regulation of ER direct target genes. In the report of Carrol *et al.*, 2006 [14], it was also verified that E_2 up-regulated genes which have adjacent estrogen receptor binding sites are more likely to contain EREs, and that genes down regulated generally contain AP1 sites. In summary, in comparison to the overall number of E_2 regulated genes in ERα positive breast cancer cell lines, the E_2 direct response pathway accounts for only a portion of the molecular signature and a significant enrichment of EREs in the regulatory regions of these direct target genes was observed [42, 50, 51].

Many studies have shown distinct patterns of gene expression related to ER status in breast cancer biopsies and identified genes related to ER signaling [51-62]. The results provided evidence that ER+ and ER- or ER+PR+, ER-PR-tumors display remarkably different gene-expression phenotypes, but the association between ER discriminator genes and genes regulated by E2 are unclear.

A key question to be answered is whether the *in vitro* observations in cell lines reflect biological significance *in vivo*. Lin *et al.* [42] compared the E_2-induced expression profiles of MCF-7 cells and the behavior of these genes in ER-positive tumor samples and observed that the number of direct estrogen responsive genes was small in comparison to the overall number of genes that define the ER+ breast tumors. According to the work of Harvell *et al.*, 2006 [45], a comparison of *in vivo* estrogen-regulated genes in a model of human breast tumor xenografts compared with the identical cell grown *in vitro* revealed only an 11% of overlap. In spite of differences in individual genes, similar functions were maintained in general. On the other hand, another report found a good agreement between the estrogen-regulated pattern in MCF-7 cells *in vitro* and that obtained in the same

Table 1. Expression Profiling of ER-Regulated Genes in Breast Cancer Cell Lines

Cell Line	Experimental Conditions	Plataform	Differentially Expressed Genes	Reference
ZR-75	5 day with 10% CSS + E_2 10^{-8}M +CHX for 6 0r 24 h;or + 4-OHT 10^{-7}M, Ral 10^{-7} M or ICI 10-7M for 24 h	Affymetrix HuGeneFL	53	Soulez and Parker, 2001 [34]
MCF-7	48 h with 5% CSS and treated with E_2 0 to 100 pM for 48 h	Affimetrix U133A	792 E_2 sensitive genes (190 up- and 602 down-regulated)	Coser *et al.*, 2003 [38]
MCF-7	4 days with 5% CSS + E_2 10 nM for 4, 8 and 48 h (+ CHX) alone or in the presence of 4-OHT, ICI or Ral 1μM	Affimetrix Hu95A GeneChips	438 (30% up- and 70% down-regulated)	Frasor *et al.*, 2004 [40]
T47D	4 days with CSS + E_2 10 nM for 6 and 24 h and mice tumor xenografts E2 for 7 wk	Affimetrix HG-U133A	1592 E_2 up 1277 E_2 down (cells vs xenografts, 11% overlap	Harvell *et al.*, 2006 [45]
MCF-7	4 days with CSS + E_2 10 nM for 4, 8 and 24 h or + Tam 1 or 6 μM for 48 h and mice tumor xenografts E_2 for 6 wk	Affimetrix HG-U133A	1989 E_2 up 1512 E_2 down (cells vs xenografts, >40% over-lap)	Creighton *et al.*, 2006 [46]
MCF-7, T47D and BT-474	3 days with CSS + E_2 10^{-8}M for 24 h or 4-OHT or ICI 1 μM for 24 h	Affymetrix U133A Genechip	E_2 sensitive gene in MCF-7 – 674 T47D – 140 BT0474 - 33	Rae *et al.*, 2005 [43]
MCF-7	4 days with CSS + E_2 100 nM for 0, 3, 6 and 12 h	Affimetrix U133Plus 2.0 and ChIP on-chip	3 h – 275 6 h – 723 12 h – 1,023	Carroll *et al.*, 2006 [14]
MCF-7	4 days with CSS + E_2 10 nM for 24 h	ChIP-on-chip (9000 GC-rich genomic sequence)	236	Bourdeau *et al.*, 2004 [21]
MCF-7	3 days with 3% CSS and treated with E_2 10 nM for 24 h	ChIP-on-chip (8124 promotor sequences)	70	Jin *et al.*, 2004 [41]
MCF-7	3 days with CSS + E2 100 nM for 45 min	ChIP-on-chip (18,668 promoter regions)	153	Langanière *et al.*, 2005 [13]

CSS, charcoal stripped serum; CHX, cyclohexamide; Ral, reloxifene; ICI, ICI 182,780; Tam, tamoxifen

cells grown as xenografts (over 40%). Interestingly, a significant number of genes induced by E_2 *in vitro* were correlated with tumor profiles in ERα breast cancers from patients within a narrow age range of 41-44 years [46].

Abba and co-workers [51] used SAGE to analyze the gene profile of breast carcinomas based on ERα status coupled with the identification of putative high affinity EREs in the promoter regions of the SAGE-identified up modulated genes. Approximately 31% of ERα associated transcripts were involved in biological process related to cell growth. The authors suggested that many of these genes were transcriptionally regulated by non-ERE mediated mechanisms such as those involving ER-binding to the AP1 or SP1 transcription factors. Comparison of the *in vitro* transcripts (MCF-7) and *in vivo* profiles revealed that only few transcripts behaved similarly in both studies, confirming the observation of Meltzer and co-workers that the majority of genes regulated in cell culture do not predict ER status in breast cancer [39, 52].

As most of the data were based on expression of mRNAs isolated from tumor masses which includes fibroblasts and lymphocytes and the proportion of tumor cells in clinical samples varies significantly, the multiple cell population may compromise the gene expression data associated with ER that is expressed on epithelial cells. In the report on Yang *et al.*, [61], epithelial tumor cells obtained by laser capture micro dissection that allows one to isolated nearly pure cell populations from a heterogeneous environment retained only 43% of the genes, unique to this category. Several genes classified in this category have been demonstrated in ERα tumors such as trefoil factors 1 and 3, GATA 3, GREB1, XBP1 and keratin 18. Other cause of discrepancy might be the presence of ERß, which seems to have a significant impact on the pattern of gene expression in breast cancer cells

that contain ERα [11]. However, it was demonstrated that a complex pattern of genes (not including ER) could also identify most ER-negative from ER-positive breast tumors and could be used to predict clinical ER status, suggesting that the differences between ER- and ER+ cancers may not simply be attributable to the absence or presence of ER function but rather reflect different molecular phenotypes [56].

In accordance, a novel molecular taxonomy has been proposed which stratified breast cancer into several clinical relevant subtypes. Among them, the luminal A type tumors are characterized by high expression of the ER and for being responsive to adjuvant hormonal treatment and associated with improved survival [62]. Part of the genes that co-clustered with ER was previously identified in E2-responsive carcinoma cell lines [63]. However it is possible that these expression cassettes segregating with ER status are reflecting other tumor aspects such as slower growth, enhanced differentiation that are only partly related to the presence of ER, reflecting different molecular phenotypes perhaps arisen from different precursors in the breast. All the studies outlined above emphasize the molecular complexicity of the mechanisms by which estrogen receptor dictates tumor status and showed that other molecular events could influence sensitivity to hormonal therapy and clinical outcome.

Since its introduction more than 30 years ago tamoxifen has been the most widely used drug in endocrine therapy for the treatment of women with advanced breast cancer. However, almost all patients with metastatic disease and as many as 40% receiving adjuvant tamoxifen eventually relapse due to intrinsic (de novo) or acquired resistance and need further treatment options [64]. Some multigene prognostic predictors of tamoxifen response are already being proposed [65-68] and interestingly although experimental and computational studies revealed that a large number of genes is potentially regulated by ER signaling pathways, the discriminatory signatures consist of a relatively small number of genes.

The treatment with aromatase inhibitors (AIs) and fulvestrant has been demonstrated to be active in a proportion of tamoxifen-resistant breast cancer patients and fulvestrant can also be an appropriate option after failure of AIs therapy [5].

The mechanistic bases of the different antiestrogens described are not yet fully understood. Microarray analysis used to identify transcriptional programs regulated by tamoxifen, raloxifen and ICI in ER positive cells, showed a very low degree of overlapping indicating that each individual compound exhibited a very specific gene expression profile [40, 68, 69]. The identification of antiestrogenic effects on gene regulation *in vivo* may be expected to provide a better understanding of the mechanisms that lead to a poor antiproliferative sequential response. Two recent reports used a neodjuvante protocol with aromatase inhibitors and analyzed gene expression in individual biopsies taken before and after treatments highlighting that decreased expression of proliferation-related genes were particularly prominent [70, 71].

As illustrated in this review, several studies have been published reporting interesting results of estrogen responsive gene profiles in breast cancer cell lines and tumor biopsies and they clearly pointed that only the analysis of multiple genetic elements could classify tumors in terms of response to therapy more accurately than conventional biomarkers. The main challenge in the area is to establish a group of limited number of genes that are suitable for determining sensitivity to anti-hormone therapy.

FUTURE CONSIDERATIONS

Although the gene expression profile technology is promising, several problems elicited by the poor association between bioinformatics and biology has to be solved. One of the most important is due to the confounding effect of coordinated expression of thousands of genes that are associated with clinical phenotype and because of technical noise in the microarray data. Further optimization and standardization of methodology including mathematical and statistical analyses, similar criteria of patients and protocols selection and properly designed clinical trials [72, 73] will be required to improve our understanding of breast cancer molecular-phenotype and its relationship with anti-cancer agents response.

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