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Antiestrogen Resistance and the Application of Systems Biology

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Abstract

Understanding the molecular changes that drive an acquired antiestrogen resistance phenotype is of major clinical relevance. Previous methodologies for addressing this question have taken a single gene/pathway approach and the resulting gains have been limited in terms of their clinical impact. Recent systems biology approaches allow for the integration of data from high throughput "-omics" technologies. We highlight recent advances in the field of antiestrogen resistance with a focus on transcriptomics, proteomics and methylomics.

Keywords

Systems biology; breast cancer; estrogens; antiestrogens

Introduction

Estrogen receptor-α (ER) positive breast cancers account for over two-thirds of the nearly quarter of a million cases of invasive breast cancers diagnosed each year in the U.S. Antiestrogens, such as Tamoxifen (TAM) and Faslodex (FAS; Fulvestrant; ICI 182,780), are among the least toxic and most effective means to treat these cancers. Unfortunately, more than half of all ER+ breast tumors will display *de novo* resistance to these therapies [1] and many initially responsive tumors will eventually recur. Thus, resistance to endocrine therapies remains a significant clinical problem. Many potential mechanisms of antiestrogen resistance have been proposed and reviewed elsewhere [2–5]. However, mechanisms of resistance to these therapies remain unclear, reflecting an incomplete understanding of the signaling affecting cell fate decisions and their hormonal regulation. Recent advances in systems biology have provided an integrated approach to understanding the complex interplay among DNA/RNA/protein/metabolites within the human interactome, allowing for a richer understanding of the molecular interactions driving the endocrine resistant phenotype [5].

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Conflict of Interest

The authors have no conflict of interest to declare.

Systems Biology

Previous approaches to ascertaining the cause(s) of endocrine resistance focused primarily on single genes/signaling pathways. Given the complexity and redundancy inherent in biological systems, coupled with increasing evidence that the resistance phenotype is driven by both genomic and non-genomic mechanisms, it is clear that following a single gene/pathway approach is an ineffective means for investigating this multifaceted problem [6]. Thus, a systems biology approach is required to integrate the knowledge gained from clinical and laboratory research with computational and mathematical modeling. This approach allows for the assimilation and analysis of complex data from multiple sources using interdisciplinary tools, leading to the creation of models that have the power to predict both quantitatively and qualitatively how a given biological system functions. Furthermore, they allow for the building of *in silico* models predictive of system (network) functions; model predictions can then be validated using basic laboratory models [5]. Many of the primary data in modern systems biology come from relatively recent advances in the high throughput "-omics" revolution including transcriptomics, proteomics, and methylomics. New insights from the emerging field of high throughput metabolomics are anticipated but have yet to make a major impact in the study of antiestrogen resistance. This review will highlight some of the key advances in the field of antiestrogen resistance that recently have arisen from a systems biology approach, with a focus on observations from transcriptomics, proteomics, and methylomics/epigenomics.

We have chosen not to include a detailed discussion of genomics in this review because this is unlikely to contribute substantially to acquired resistance. Patients with tumors that respond and then recur within 0–3 years likely exhibit *de novo* resistance conferred by the biology of a pre-existing population(s) well represented within their disease at the time of diagnosis. The resistance phenotype of this population may or may not be driven by genetic modifications; only those driven by genetic alterations would be detected by genomics. For a truly acquired resistant phenotype, it seems unlikely that a single cell could acquire a genetic event (mutation, deletion, amplification, rearrangement) and expand to become the bulk of the tumor mass in 0-36 months. In marked contrast, adaptation of a common signaling network within a population of cells exposed to the same stressor could occur within minutes (or faster) if the adaptation(s) occurs in the transcriptome, proteome, methylome, or metabolome.

Taking high dimensional –omics data and building predictive models of system function is an area of active interest but much work has yet to be done to understand how best to approach modeling of endocrine resistance. We recently provided the first roadmap for building mathematical models [7]; other roadmaps seem likely to emerge to further guide research in this field. Success in this area also will require a greater understanding of the properties of high dimensional data spaces, and how these can be addressed to extract the most appropriate information to support modeling [7–9].

Transcriptome

Microarray technologies allow for the investigation of global gene expression changes within specific cellular and/or drug treatment contexts. Time course microarray analyses in ER+ breast cancer cells have been used to establish estrogen (E2)-induced transcriptional changes that likely drive the proliferative and antiapoptotic effects of E2 in ER+ breast cancer cells. Data from these studies implicate an upregulation of growth factors, proliferation, and cell-cycle related genes, with a concomitant downregulation of transcriptional repressors, and both proapoptotic and antiproliferative genes [10–12].

Teasing apart the E2-regulated pathways that, when dysregulated, allow for the development of endocrine resistance has been the focus of several investigations. Studies in ZR-75-1 breast cancer cells (ER+) treated either with E2 or the antiestrogens TAM, FAS, or raloxifene (RAL) identified a subset of genes that were regulated either by E2 only (and not by antiestrogens) and *vice versa. In silico* promoter analysis revealed that only 13% of the hormonally regulated genes contained an estrogen response element (ERE) in their promoter region, reflecting a non-genomic (or indirect genomic) regulation of gene expression by ER. Furthermore, they found that 25% of antiestrogen responsive genes responded to only one antiestrogen illustrating specific and discernible effects of the different antiestrogens. [13]. This observation was substantiated in MCF-7 cells where a subset of genes preferentially regulated by TAM, but not by FAS or RAL, was identified. The experimental design of this study did not allow for the identification of genes unique to FAS or RAL. The overexpression of some TAM regulated genes correlated with recurrence in patients (*e.g.,* the YWHAZ gene that encodes the 14-3-3 zeta/delta protein) [10].

We have identified new topological features of stable hormone resistant signaling using data derived from SAGE, 2D-gel, and gene expression microarray experiments in antiestrogen sensitive (LCC1) *vs.* resistant (LCC9) MCF-7 cells. Our ER-based network is comprised of a series of interconnected modules that both drive cell fate decisions and perform specific functions such as cell cycle, apoptosis, unfolded protein response (UPR), and autophagy that contribute to effective execution of the cell fate decision [5,14,15]. We apply both computational and mathematical modeling using a novel seed-gene based approach where individual seeds (single genes or very small networks of a few genes) are used to "grow" larger models that can better represent signaling. Among these seeds are genes that we have confirmed experimentally to be relevant to hormone resistance including BCL2, BCLW, RELA, NPM1, IRF1, and XBP1 [16–21].

In another MCF-7 based model of antiestrogen resistance, microarray analysis confirmed that TAM resistant cells, although no longer growth stimulated by E2, retain ERα expression and a transcriptional response to E2 [22]. Genes upregulated in TAM resistant (MCF-7-T) cells included caveolins, annexins, MAPK phosphatases, and those involved in PKA signaling, calcium binding, and inhibiting differentiation. Only 25% of E2 regulated genes in TAM sensitive MCF-7 cells maintained E2 regulation in resistant cells. These data support our hypothesis that the survival/growth of TAM resistant breast cancer cells relies upon an altered network of ER signaling [6]. Cells resistant to FAS (MCF-7-F) upregulated their expression of growth promoting genes, *e.g.,* cytokines and cytokine receptors, and genes involved in EGFR/ErbB2, Wnt/β-catenin, Notch, and interferon signaling. In marked contrast, MCF-7-F cells showed a 90% reduction in ER expression and almost complete loss of E2-activated gene transcription, suggesting that the acquisition of FAS resistance is associated with a loss of E2- induced gene expression and the adoption of an ER-negative phenotype, at least in this cell model [22].

As hormonally-regulated signaling networks are being delineated, new mechanisms controlling gene expression, and the complexity of this regulation, have very recently been discovered [23]. E2-regulated microRNAs have been shown to alter estrogen regulated gene expression [24,25]. Using microRNA specific microarrays, Manavalan *et al.* showed the differential expression of 97 TAM regulated microRNAs in antiestrogen resistant LY2 (cross-resistant to TAM, FAS, and RAL) vs. antiestrogen sensitive MCF-7 parental cells. Putative target genes of these microRNAs (e.g., ERα, BCL2, CYP1B1 and ERBB3) were identified from a publicly available dataset [26]. Further studies are required to assess the contribution of microRNAs to the acquisition of TAM resistance.

Hicks *et al.* identified small nucleotide polymorphisms (SNP) associated with breast cancer risk by combining genome-wide association studies (GWAS) with gene expression data [27]. Recently, they extended these studies to analyze the contribution of SNPs to TAM responsiveness using the same GWAS based method. In an analysis of 43 GWAS studies and gene expression data from 423 ER+ breast cancer patients, they identified 54 signature genes containing SNPs whose differential expression was able to significantly distinguish between TAM treated and untreated patients. These genes were clustered into functionally related and co-regulated clusters and included pathways involved in E2 action, apoptosis, extracellular matrix and immune response. Further, they showed that signaling pathways/networks containing these genetic variants were dysregulated in response to TAM treatment [28]. How these SNPs may contribute functionally to TAM resistance is currently unknown and warrants further study but they may be more closely associated with *de novo* rather than acquired resistance.

In addition to aiding our understanding of E2/antiestrogen regulated gene expression and how these relate to resistant phenotypes, microarray-based technologies have allowed for the identification of potential prognostic biomarkers in TAM treated patients [1]. Loi *et al.* developed an outcome-based predictor representing 13 gene clusters that predict risk of distant metastasis in TAM treated ER+ tumors. This method differs from others in that they utilized clusters of highly correlated genes to develop their predictor. This classification scheme was validated in a multi-institution independent sample of early and metastatic ER+ tumors treated with TAM. Genes involved in cell cycle, proliferation, and inflammation were significantly predictive of response [29].

Symmans *et al.* also have developed a predictor for endocrine responsiveness [30]. Using microarray data from a discovery cohort of patients, they developed a genomic index for sensitivity to endocrine therapy (SET) comprised of 165 genes. SET is derived from genes whose expression correlated either positively or negatively with ER expression. The SET index was validated in an independent cohort of 245 patients on adjuvant TAM therapy. SET, and not ER mRNA expression, was significantly predictive of distant relapse and death in these patients. SET was further tested in several cohorts of patients with differing treatment histories (TAM only, chemotherapy followed by TAM/aromatase, no systemic therapy). SET was found to be predictive of a survival benefit from adjuvant endocrine therapy but not predictive of prognosis, as there was no association in the untreated patients [30].

The challenge with developing molecular classifiers to learn about biology is evident in the recent observation that most gene expression profiles associated with breast cancer outcomes are no better as clinical predictors that random gene sets [31]. Indeed, the classification scheme that defined the various luminal, basal, Her2, and “normal-like” breast cancer subgroups also has been shown not to be adequately reproducible [32]. The problem here is multifaceted and beyond the scope of this review. However, some of the statistical approaches used can be defeated by the properties of the data spaces explored [8]. Biological interpretation is confounded by the fact that the classifiers select the signals best able to separate groups statistically; an approach that does not specifically link statistical properties of genes with their biological function. Some classification schemes are platform dependent and work only with a specific tool (*e.g.,* the Affymetrix GeneChip or other platform), further highlighting the challenges of linking differential expression with biological function [7].

Other approaches take a different perspective to identify biologically meaningful associations from within high dimensional transcriptome data. For example, Chen *et al.* created a network-constrained support vector machine (netSVM) designed to identify breast cancer biomarkers. This method allows for the integration of protein-protein interaction

network data with gene expression data and is focused on generating hubs which, when validated, may be clinically useful biomarkers [33]. This method is an improvement upon traditional analytical tools for microarray data in that it has higher reproducibility across datasets. The study successfully identified gene hubs involved in signaling to breast cancer metastasis from publicly available datasets, implicating those involved in MAPK, TGF-β, and JAK/STAT signaling pathways. This method, although used to identify biomarkers of metastasis, likely can be used to predict signaling pathways involved in hormone resistance [33].

The challenges noted here apply more broadly than the transcriptome studies described above; many also are represented in high dimensional proteome, methylome, metabolome, and genome data sets. Many new analytical tools continue to emerge and the optimal approach for data analysis has yet to be described definitively. Nonetheless, the development of better tools will improve our ability to find the most important genes and their interactions as needed to support the goals of *in silico* modeling in systems biology.

Proteome

Previously, 2-D gel approaches, combined with either HPLC-MS/MS or MALDI (matrix-assisted laser desorption/ionization), were the primary methods to explore the proteome. More recent shot-gun proteomics have allowed for the identification of a larger pool of potential proteins involved in various biological activities. Using an accurate mass and time (AMT) tag proteomics approach, Umar *et al.* [34] compared protein expression in TAM responsive vs. resistant tumors from patients with recurrent disease. A total of 2556 unique proteins were identified (>100 times the number of proteins than with conventional 2-D gel methods). Of these, 100 were found to correlate with responsiveness to TAM including structural, metabolic, apoptotic, and signaling proteins. Many of these proteins were previously associated with aggressiveness or TAM resistance. The top discriminating protein, EMMPRIN, was expressed at higher levels in TAM resistant tumors and associated with early tumor progression in an independent patient cohort [34]. Shotgun approaches have been developed that do not fractionate proteins prior to HPLC-MS analysis and thus reduce the undersampling inherent in techniques that utilize fractionation. MCF-7 breast cancer cells ± TAM were compared using a precursor acquisition independent from ion count (PAcIFIC) method. More than 2,000 proteins were identified, 83 of which were significantly differentially expressed in TAM *vs.* control treated cells [35]. Interestingly, 60% of the genes identified in this study correlated with those found in the tumor samples from the Umar study [34].

Chromatin-immunoprecipitation (ChIP) based technologies allow for the exploration of regions of the human interactome for potentially relevant interactions and are an important resource for understanding how protein-protein and protein-DNA interactions contribute to antiestrogen resistance. Cicatiello *et al.* performed a genome-wide analysis integrating information gleaned from time-course gene expression, ChIP-seq, and microRNA expression profiling experiments. This approach allowed for the identification of primary, secondary, and downstream E2 targets in two different ER+ cell lines and implicated 11 transcription factors that signal to 33 clusters of 1270 genes [25].

We have developed a motif-guided sparse decomposition (mSD) method as one approach to integrating transcription factor binding information (based on ChIP-on-chip or motif data) with gene expression data. The general goal of applying mSD is to identify small gene regulatory modules that can be used as seeds for building gene network models. This method was validated using a breast cancer cell model of estrogen independence, where the transcription factors AP1, ETF, ER, STAT, and NFκB, along with their target genes, were

found to be distinctly regulated [36]. A number of the predicted interactions were independently confirmed in data from a genome wide ChIP-seq analysis of E2 regulated genes [25].

To decrease the excessive noise and false positives inherent in microarray and protein-DNA binding data, respectively, we recently used a Gibbs sampling method to uncover fundamental regulatory associations between transcription factors and target genes differentially regulated by E2 stimulation or withdrawal [37]. We found that ER no longer significantly modulates transcription in E2 withdrawn ER+ breast cancer cells, consistent with a decreased dependence upon, and widespread reprograming of, ER signaling during the development of E2 independence. AP1 and ER were activated by E2 whereas NFκB, p53, and CEBPA target genes correlated with E2 withdrawal. SP1 and STAT transcription factors were significantly regulated under both conditions. Further, we obtained evidence to suggest that signaling through STAT5B activates ER regulated genes associated with proliferation/survival during E2 withdrawal. Thus, STAT signaling may ultimately contribute to the acquisition of E2 independence and subsequent breast cancer progression. Our Gibbs sampling based approach offers the opportunity to identify potentially meaningful relationships between transcription factors and effector genes within the context of antiestrogen resistance [37].

Regulatory networks for ER and its associated transcription factors have been described in MCF-7 cells using three publicly available data sets from ChIP based studies (ChIP-on-chip, ChIP-seq, and ChIP-pet). Six common transcription factor hubs and 16 common transcription factors were identified among the three datasets. This ER signaling is altered in TAM resistant MCF-7 cells; ChIP-seq found only two common transcription factor hubs (RORA; PITX2) and eight common transcription factor targets in the resistant *vs.* parental cells [38]. To extend their studies, Shen *et al.* used a modulated empirical Bayes model that allows for the integration of gene expression data with data from transcription factor motif scans and ChIP-on-chip binding studies, permitting for the differentiation between genomic and non-genomic actions. The transcription factor hubs identified mediate both genomic (ERα and SP1) and putatively non-genomic (ZFP161, TFDP1, NRF1, TFAP2A, EGR1, E2F1, PITX2) actions of E2. Target genes regulating cell proliferation, cell cycle control, and cell death were controlled by genomic mechanisms while non-genomic mechanisms controlled target genes involved in DNA repair, replication, and recombination, as well as posttranslational modifications and cellular development [39].

In addition to identifying transcription factors/networks that potentially contribute to estrogen signaling and TAM resistance, ChIP-based technology has been used to establish the forkhead box protein, FOXA1, as a key component of ER binding to DNA [40,41]. Hurtado *et al.* investigated the role of FOXA1 in ER binding and endocrine response in ER+ breast cancer cell lines [42]. Using siRNA to silence FOXA1, they used ChIP-seq to map ER binding. Nearly all ER binding (90%) was significantly decreased in intensity with the suppression of FOXA1, an effect that was reversible upon re-expression of FOXA1. Cells expressing FOXA1 siRNA showed a decrease in 95% of all E2 regulated genes and resulted in significant growth arrest. These data further validate that FOXA1 is necessary for ER-DNA binding and establish its role in estrogenic signaling in breast cancer cells. FOXA1 also is required for TAM/ER binding to chromatin and TAM-mediated transcription and for ligand independent ER activity in TAM resistant cells [42].

ChIP-seq analysis of ER binding in primary breast cancer tumors with differing clinical outcomes found an inverse correlation between signal intensity of ER binding and clinical outcome, with the signal being highest in metastatic samples. ERE and FOXA1 motifs were enriched in ER binding events in poor outcome samples *vs.* ERE in tumors with good

outcomes. Further, FOX1A was coexpressed with ER in 95% of metastatic samples. Similar results were seen in TAM resistant breast cancer cell lines. These data suggest that FOXA1 contributes to the reprograming of ER binding in some TAM resistant breast cancers [43].

Methylome

In some breast cancer cells, complete loss of ER (the primary form of *de novo* resistance) can be associated with hypermethylation of the ER promoter. Demethylating agents restore ER expression, E2 responsiveness, and TAM sensitivity in ER- breast cancer cell lines [44]. In contrast, the acquired resistant phenotype usually is not associated with loss of ER expression (see [45] and reviewed in [46]). Furthermore, histone deacetylase (HDAC) inhibitors can downregulate ER and its concordant signaling in ER+ breast cancer cells and restore TAM sensitivity in preclinical models [47]. A phase II clinical trial of the HDAC inhibitor Vorinostat given with TAM showed a 40% overall clinical response (response or stable disease) in metastatic endocrine therapy resistant ER+ breast cancer [48]. Therefore, investigating epigenetic changes of hormone responsive genes may be useful in determining potential mechanisms of antiestrogen resistance. In support of this idea, differential methylation hybridization in MCF-7 cells resistant to TAM (MCF-7-T) or FAS (MCF-7-F) revealed that these antiestrogens epigenetically modify a distinct set of promoters correlating with altered mRNA expression and loss of E2 stimulation of the E2 target genes identified in sensitive cells. Thus, aberrant epigenetic modifications likely contribute to these resistance phenotypes [22,39].

Fackler *et al.* conducted a genome-wide methylation array analysis of primary invasive breast cancers. Several hypermethylated genes were identified with the ability to predict recurrence and to separate ER+ from ER- tumors. These data were validated *in silico* using a random dataset from The Cancer Genome Atlas database [49]. In patients receiving adjuvant hormonal therapies, predicting which women are most likely to recur is of major clinical importance. Methylation analysis using microarray based technology of candidate genes (chosen for possible relevance to breast cancer progression/hormone responsiveness) identified 10 genes whose methylation status predicted for TAM resistance in women with ER+ recurrent disease treated with first-line TAM monotherapy. Hypermethylation of PITX2 most strongly predicted for distant recurrence in these women [50]. These findings were subsequently validated in a multicenter independent cohort of patients [51,52]. Interestingly, PITX2 also was among the transcription factors identified by Shen *et al.* to mediate some of the non-genomic effects of E2 [39]. Therefore, methylation status of E2 regulated genes, including PITX2, may be potentially viable biomarkers for assessing which patients are likely to recur on TAM. Whether data from CpG methylation studies will adequately predict responsiveness to hormonal therapies is an interesting and potentially clinically relevant concept that warrants further study. We recently have begun to include epigenetic investigations as one component of our systems biology-based research to identify factors that can robustly predict for early *vs.* later recurrence in TAM treated ER+ breast cancer patients.

In an MCF-7 cell based *in vitro* model of TAM withdrawal, microarray experiments were used to assess gene expression changes associated with the maintenance of the resistance phenotype [53]. A subset of E2-regulated genes were found to be downregulated by promoter methylation and re-expressed following treatment with E2 and a demethylating agent. Interestingly, an antiproliferative effect was seen in the co-treated cells. Microarray analysis revealed genes upregulated by this co-treatment including genes involved in proliferation or apoptosis. Four genes, including the p53 target gene GDF15, were independently validated and found to be significantly methylated in resistant cells. These data suggest that long term exposure to TAM leads to the epigenetic silencing of a subset of

E2 responsive antiproliferative genes, which may allow for the proliferation of resistant cells. Reactivation of these genes through the combination of demethylating agents and E2 showed marked growth inhibition, providing a potentially new therapeutic approach in TAM resistant breast cancer [53].

Conclusions

Single gene/network approaches to understanding the molecular changes that drive the acquisition of an antiestrogen resistant phenotype have offered useful but somewhat limited insight into the complexity of a phenotype driven, at least initially, by activation of a multifunctional transcription factor, *i.e.,* ER. The recent revolution in "omics" technologies allows for the investigation of the previously poorly understood interactions between the genome, epigenome, transcriptome, interactome, methylome, and metabolome. Merging data garnered from these technologies with state-of-the-art computational and mathematical modeling has underscored the complexity of changes associated with antiestrogen responsiveness but also provided the opportunity for an integrated approach that could fundamentally change our understanding of endocrine signaling in breast cancer. Recent transcriptomic studies have better defined E2/antiestrogen signaling networks, helped to develop new prognostic biomarkers and gene signatures that may predict for responsiveness to antiestrogens, and revealed the potential contribution of microRNA and SNPs to endocrine resistance. Proteomics-based analyses are uncovering critical regulatory associations between transcription factors and target genes. The likely importance of how epigenetic modifications may contribute to antiestrogen signaling/resistance is beginning to be recognized. Systematic innovations in mathematical and computational modeling will facilitate improved integration/analysis of data from omics-based technologies and allow for the discovery of new therapeutic options and the ability to better inform treatment decisions in women with ER+ breast cancer.

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