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Gene network signaling in hormone responsiveness modifies apoptosis and autophagy in breast cancer cells☆

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ABSTRACT

Resistance to endocrine therapies, whether *de novo* or acquired, remains a major limitation in the ability to cure many tumors that express detectable levels of the estrogen receptor alpha protein (ER). While several resistance phenotypes have been described, endocrine unresponsiveness in the context of therapy-induced tumor growth appears to be the most prevalent. The signaling that regulates endocrine resistant phenotypes is poorly understood but it involves a complex signaling network with a topology that includes redundant and degenerative features. To be relevant to clinical outcomes, the most pertinent features of this network are those that ultimately affect the endocrine-regulated components of the cell fate and cell proliferation machineries. We show that autophagy, as supported by the endocrine regulation of monodansylcadaverine staining, increased LC3 cleavage, and reduced expression of p62/SQSTM1, plays an important role in breast cancer cells responding to endocrine therapy. We further show that the cell fate machinery includes both apoptotic and autophagic functions that are potentially regulated through integrated signaling that flows through key members of the BCL2 gene family and beclin-1 (BECN1). This signaling links cellular functions in mitochondria and endoplasmic reticulum, the latter as a consequence of induction of the unfolded protein response. We have taken a seed-gene approach to begin extracting critical nodes and edges that represent central signaling events in the endocrine regulation of apoptosis and autophagy. Three seed nodes were identified from global gene or protein expression analyses and supported by subsequent functional studies that established their abilities to affect cell fate. The seed nodes of nuclear factor kappa B (NFκB), interferon regulatory factor-1 (IRF1), and X-box binding protein-1 (XBP1) are linked by directional edges that support signal flow through a preliminary network that is grown to include key regulators of their individual function: NEMO/IKKγ, nucleophosmin and ER respectively. Signaling proceeds through BCL2 gene family members and BECN1 ultimately to regulate cell fate.

1. Introduction

Over 40,000 American women die of breast cancer each year [1]; incidence is broadly similar across the European Union when considered as a percentage of the population. In 2008, over 178,000 women will be diagnosed with invasive breast cancer in the U.S., almost 70% of which will be estrogen receptor-α positive (ER+; HUGO Gene Symbol = ESR1) [2,3]. The percentage of ER+ sporadic breast cancers increases linearly with age but even in premenopausal cases the proportion is high; 62% at age ≤35 and 72% by age 49 [2–4]. Data from randomized trials and meta-analyses clearly show that all breast cancer patients derive a statistically significant survival benefit from adjuvant chemotherapy, and that all hormone receptor positive breast cancer patients benefit from adjuvant endocrine therapy [5–9]. For postmenopausal women, the benefit from adjuvant Tamoxifen (TAM) is comparable to that seen for cytotoxic chemotherapy. While 5 years of adjuvant TAM produces a 26% proportional reduction in mortality [8], many ER+ tumors eventually recur [10]. Since advanced ER+ breast cancer largely remains an incurable disease, resistance to endocrine therapies is a significant clinical problem.

Endocrine therapy is administered as an antiestrogen (AE) like Tamoxifen (TAM) or Fulvestrant (FAS; Faslodex; ICI 182,780), or as an aromatase inhibitor (AI) such as Letrozole or Exemestane. It is less toxic and potentially more effective therapy in the management of hormone-dependent breast cancers. Antiestrogens, and TAM in

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particular, have been the "gold standard" first line endocrine therapy for over 30 years [11], clinical experience with this drug likely exceeding over 15 million patient years [10]. TAM increases both disease free and overall survival from early stage breast cancer, and it also reduces the incidence of invasive and noninvasive breast cancer in high-risk women [8,9]. Raloxifene, another antiestrogen, is effective in reducing the rate of postmenopausal bone loss from osteoporosis as well as the rate of invasive breast cancer [12]. Newer antiestrogens such as FAS show significant activity relative to TAM and some AIs [13,14]. Third generation AIs are now widely accepted as viable alternatives to AEs for first line endocrine therapy in postmenopausal women with metastatic disease; overall response rates are generally greater for AIs [15]. Importantly, Tamoxifen is the only single agent with demonstrated efficacy in both premenopausal and postmenopausal women with invasive breast cancer. Other AEs and all of the AIs require the complete cessation of ovarian function.

Of current interest is identification of the optimum choice and scheduling of AEs and AIs. Evidence clearly shows improvements in disease free survival for combined adjuvant therapy (an AI and an AE usually given sequentially) over single agent TAM [16–20]. However, the ability of AIs to induce a significant improvement in overall survival compared with 5 years of TAM alone is uncertain [15]. In terms of metastatic disease, recent data imply that response rates with an AI are either equivalent with or higher than with TAM [21,22]. Given the increasing number of endocrine treatment options, there is a clear need to optimize the selection and scheduling of agents for both early stage and advanced disease. Whichever way these controversies are eventually resolved, it is clear that both AIs and AEs will remain as key modalities in the management of ER+ breast cancers. Unfortunately, the inability of endocrine therapies to cure many women with ER+ disease will also remain.

1.1. Endocrine resistance: receptor phenotypes

Several resistance phenotypes are evident from both experimental models and clinical observations. The two primary receptor phenotypes are ER+ and ER−. These receptor-based phenotypes have been further stratified by addition of the estrogen-regulated receptor for progesterone (PGR; HUGO Gene Symbol = PGR). The degree of treatment benefit from endocrine therapy varies according to receptor phenotype. For example, approximately 75% of ER+/PGR+, 33% of ER+/PGR−, and 45% of ER−/PGR+ cases of metastatic breast cancer respond to TAM [10]. Endocrine responses in truly ER− tumors are probably relatively rare and of uncertain relevance, as they most likely reflect incorrect assessments of what may be very low ER and/or PGR expression values. Data from the Early Breast Cancer Trialists' Collaborative Group meta-analyses show that TAM therapy generates a non-significant 6% reduction in the 10-year risk of recurrence. A non-significant increase in the risk of death from any cause in patients with ER− breast cancer also was reported [8,9]. The real value of PGR, which is the only modification to this clinical prediction scheme for directing endocrine therapy to become routine in over 30 years (the value of directing endocrine therapy based on HER2 is still controversial), is largely limited to ER− tumors. It is general practice in the United States to treat all ER+ and/or PR+ invasive breast tumors with endocrine therapy. However, it remains impossible to predict whether an individual patient will receive benefit from treatment and the magnitude or duration of any benefit. Better predictors of each individual patient's endocrine responsiveness are clearly needed.

1.2. Endocrine resistance: pharmacological phenotypes

Several pharmacological phenotypes have been identified in experimental models of either human breast cancer cells growing *in vitro* or of xenografts in immune-deficient rodents [10]. These phenotypes include (i) estrogen-independent (which appears equivalent to AI resistance but is not so for antiestrogen resistance [23]—some breast cancers can become resistant to an AE but still respond to an AI and *vice versa*); (ii) estrogen-inhibited (recently identified in MCF-7 models [24]); (iii) TAM-stimulated (identified first in MCF-7 xenografts [25,26]); TAM-unresponsive but FAS sensitive [27] (identified first in MCF-7 models and subsequently observed in clinical trials [13]); TAM and FAS crossresistant [28] (perhaps this is truly antiestrogen crossresistant and it is seen both clinically in patients and experimentally in MCF-7 models [13,29]). Other variations on these phenotypes likely occur but are beyond the scope of our discussion.

1.3. Clinical evidence for the prevalence of pharmacological resistance phenotypes

Obtaining direct clinical evidence for the prevalence of each of the pharmacological resistance phenotypes is challenging. We have previously noted the utility of applying clinical responses to TAM withdrawal in metastatic breast cancer as one means to define, at least in broad terms, the likely relevance of a series of pharmacological phenotypes [29]. This approach is somewhat limited, as the number of cases across all studies is modest ($n = 241$). Furthermore, TAM withdrawal responses cannot readily distinguish between TAM-stimulation and estrogen-inhibition because each should predict for a clinical benefit. The latter would induce a benefit because many breast cancers contain significant concentrations of 17β-estradiol, independent of both menopausal and ER/PGR status [10], sufficient to produce the estrogen-inhibited phenotype [24]. Indeed, the superiority of AIs over TAM in inducing clinical response strongly implies that over 75% of ER+/PGR+, at least 50% of all ER+ breast cancers irrespective of PGR expression, and 45% or more of ER−/PGR+ breast tumors are probably driven by adequate access to estrogen.

In our prior assessment, almost 9% of patients received an overall clinical response to TAM withdrawal (partial responses + complete responses). When disease stabilizations were included we estimated that less than 20% of patients received clinical benefit [29], suggesting that the sum of TAM-stimulated plus estrogen-inhibited clinical phenotypes may not account for the majority of resistant phenotypes in women. Of course, given the number of ER+ breast cancers arising every year, these phenotypes are relevant to a notable number of women. The major response to TAM withdrawal was clinically detectable disease progression – greater than 80% of cases – strongly implicating unresponsiveness as the primary clinical resistance mechanism to TAM. Whether these breast cancers are fully crossresistant to all endocrine therapies, or retain sensitivity to AIs, cannot be determined from this simple analysis.

Nomura et al. [30] took a different approach and assessed the responsiveness to estrogen and TAM in short-term primary cell cultures of $n = 153$ ER+ breast cancer biopsies. This approach allowed the authors to separate the various pharmacological phenotypes; approximately 7% of ER+ primary cultures were stimulated by TAM and almost 3% were inhibited by physiological concentrations of estradiol—notably close to our estimate of 9% for the sum of these two clinical phenotypes.

It is important here to separate responses to physiological estrogens from those produced by pharmacological estrogen therapy. High dose estrogen therapy was used prior to the advent of TAM. As with all endocrine therapies, approximately 30% of all breast cancers (receptor status was not available when most of these studies were done) responded [31,32]. Side effects were unfavorable, probably explaining the switch to TAM that also induces responses in approximately 30% of all breast cancers (when receptor status is not considered). It is also likely that the mechanisms of action of pharmacological and physiological dose estrogens differ. Over 15 years