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Heterogeneous estrogen receptor expression in circulating tumor cells suggests diverse mechanisms of fulvestrant resistance

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ABSTRACT

Fulvestrant is a dose dependent selective estrogen receptor (ER) down-regulator (SERD) used in ER-positive metastatic breast cancer (MBC). Nearly all patients develop resistance. We performed molecular analysis of circulating tumor cells (CTC) to gain insight into fulvestrant resistance.

Preclinical studies were performed with cultured breast cancer cells spiked into human blood and analyzed on the CellSearch® system. Clinical data are limited to a subset of patients with ER-positive MBC from a previously reported pilot trial whose disease was progressing on fulvestrant (N = 7) or aromatase inhibitors (AIs) (N = 10). CTCs were enumerated and phenotyped for ER and B-cell lymphoma (BCL2) using the CellSearch® CXC kit.

In preclinical modeling, tamoxifen and AIs resulted in stabilized ER expression, whereas fulvestrant eliminated it. Five of seven patients progressing on fulvestrant had ≥5CTC/7.5 ml WB. Two of these five, treated with 500 mg/month fulvestrant, had no detectable CTC-expression of ER and BCL2 (an ER regulated gene). Three patients had heterogeneous CTC-ER and BCL2 expression indicating incomplete degradation of the ER target by fulvestrant. Two of these patients received 250 mg/month whereas the third patient received 500 mg/month fulvestrant. Her cancer harbored a mutation (Y537S) in the estrogen receptor alpha gene (*ESR1*). All seven ER positive patients progressing on AIs had heterogeneous CTC-ER expression.

Abbreviations: AI, aromatase inhibitor; BCL2, B-cell lymphoma 2; CCS, charcoal stripped calf serum; CTC, circulating tumor cells; EpCAM, epithelial cell adhesion molecule; ER, estrogen receptor; *ESR1*, estrogen receptor alpha gene; ETs, endocrine therapies; E2, 17β-estradiol; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; IM, intramuscular; LBD, ligand binding domain; MBC, metastatic breast cancer; oSERD, oral selective estrogen receptor down-regulator (SERD); OS, overall survival; pt-DNA, circulating plasma tumor DNA; SERD, selective estrogen receptor down-regulator; SERMs, selective estrogen receptor modulators; WB, whole blood.

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These results suggest heterogeneous mechanisms of resistance to fulvestrant, including insufficient dosage, *ESR1* mutation, or conversion to dependence on non-ER pathways. CTC enumeration, phenotyping, and genotyping might identify patients who would benefit from fulvestrant dose escalation versus switching to alternative therapies.

1. Introduction

Multiple endocrine therapies (ETs) are effective in hormone receptor (HR)-positive metastatic breast cancer (MBC). These drugs include selective estrogen receptor (ER) modulators (SERMs: tamoxifen, toremifene, or raloxifene), aromatase inhibitors (AIs: anastrozole, letrozole, exemestane), and selective ER down-regulators (SERDs), such as fulvestrant.

Several mechanisms of resistance to ET have been proposed (Osborne and Schiff, 2011), including absence of ER expression by deletion or suppression, alteration of ER signaling pathway genes, or upregulation of multiple growth factor receptor pathways. Another possible mechanism of resistance includes mutations in the ligand binding domain (LBD) of estrogen receptor alpha gene (*ESR1*) (Jeselsohn et al., 2015; Robinson et al., 2013; Toy et al., 2013), which confers ligand-independent ER signaling and therefore a relative and context-specific resistance to ET.

Importantly, simple pharmacokinetic considerations are also important mechanisms of resistance. Clinical trials have shown little, if any, evidence of dose response to SERMs or AIs (Hayes et al., 1995; Jonat et al., 1996; Tormey et al., 1976, 1983). In contrast, the activity of fulvestrant is clearly dose dependent. The initially recommended dose of fulvestrant was 250 mg intramuscular (IM) once monthly, after a brief loading period, for all patients (Osborne et al., 2002). In the CONFIRM trial, Di Leo et al. demonstrated that 500 mg IM is superior to 250 mg IM, and the former has become the standard dose (Di Leo et al., 2010). Recently, fulvestrant 500 mg has been shown to improve overall survival (OS) when compared to AI in patients with HR positive MBC (Ellis et al., 2015). Nonetheless, even at the higher dose (500 mg/month), most HR-positive MBC develop resistance and progress. Currently, there is no way to predict which patients, if any might benefit from even higher doses of fulvestrant, or how to monitor if dose adjustments have been effective. Further, there is also no means to predict if a patient on a SERD might be better treated with an alternative form of ET, addition of complementary treatments to ET, such as everolimus or palbociclib, or even proceed to chemotherapy for palliation.

ER is clearly the target of ET, and ER is highly predictive of ET response or not (Davies et al., 2011). While not completely controlled by ER, BCL2 expression is strongly correlated with ER, and it is presumed to be, at least in part, an estrogen-responsive gene (Teixeira et al., 1995). Thus, monitoring ER and downstream genes such as BCL-2 might provide baseline and pharmacodynamic monitoring tools to predict response to any ET and to optimize the dose of fulvestrant, or other, newly developed SERDs. However, serial biopsies of metastatic disease to demonstrate loss of ER expression or changing expression of other markers is invasive, expensive, and logistically difficult. In this regard, circulating tumor cells (CTC) are currently being investigated as a type of "liquid biopsy" that might substitute for cancer tissue biopsy (Alix-Panabieres and Pantel, 2013). We have recently reported the analytical validity of measuring CTC expression of markers of endocrine sensitivity (ER, BCL2) or resistance (HER2, Ki-67) using the CellSearch® system (Janssen Diagnostics, LLC) (Paoletti et al., 2015). In that study, a subset of patients with ER-positive MBC were progressing on fulvestrant or an AI, giving us the opportunity to examine expression of these biomarkers when the cancer had developed resistance to these agents. We report variable expression of CTC-ER and CTC-BCL2, suggesting multiple inter-patient mechanisms of resistance to SERD therapy.

2. Materials and methods

2.1. *In vitro preclinical studies of CTC-biomarker expression*

MCF-7 and SKBR3 cells were originally obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center (LCCC; Georgetown University, Washington, DC) and routinely maintained as previously described (Rae et al., 2005). For assays in defined hormone-free conditions, cells were repeatedly washed and grown in steroid depleted media (phenol red-free IMEM supplemented with 10% charcoal-stripped calf serum—CCS), and on the fifth day the cells were treated *ex vivo* for 24 h with the following ET drugs: tamoxifen (5×10^{-8} M); 17β-estradiol (E2) (10^{-10} M); tamoxifen (5×10^{-8} M) + E2 (10^{-10} M); fulvestrant (5×10^{-8} M); fulvestrant (5×10^{-8} M) + E2 (10^{-10} M). MCF-7 and SKBR3 cells cultured in steroid hormone-free conditions were used as positive and negative controls for cellular ER expression, respectively. Approximately 150 of these treated cells were then spiked into 7.5 ml of normal human blood and processed using the CellSearch® System. The identity of the cells was confirmed by standard Short Tandem Repeat profiling (February 2011) and cultures were subjected to routine testing for mycoplasma contamination.

2.2. *Patient accrual, blood collection, and processing, and CTC analysis*

Fifty patients with progressive MBC scheduled to start a new therapeutic regimen of any type (ET or chemotherapy or other) were enrolled into a prospective single-institution pilot study to