



Investigating differences between tamoxifen resistant and sensitive breast cancer cells with flow cytometry

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Abstract

The active metabolite of tamoxifen, 4-hydroxytamoxifen, functions as an anti-estrogen in breast cancer cells and thus inhibits proliferation. While tamoxifen continues to be successfully used to treat estrogen-dependent breast cancer, most patients receiving treatment will develop chemoresistance over time. Two commonly reported biomarkers of tamoxifen resistance are decreased expression of insulin-like growth factor 1 receptor (IGF-1R) and increased expression of epidermal growth factor receptor (EGFR). In prior work we have shown that these receptors facilitate chemoresistance and have unique regulatory functions measurable in resistant cell lines compared with nonresistant. Thus, we hypothesized that these receptors and a newly identified biomarker, integrin $\beta 1$, may be used to search for the presence of resistant breast cancer cells within a population of cells that are sensitive to tamoxifen therapy. We tested this by designing a straightforward cell-labeling approach to measure differences in the receptor expression of resistant vs. sensitive cells cytometrically. Our results show that separation is possible when observing the expression of IGF-1R as well as integrin $\beta 1$. Interestingly, we found no detectable difference in EGFR expression between tamoxifen resistant and -sensitive cells when measured with cytometry despite the fact that EGFR is upregulated in resistant cells. Our long-term goal is to utilize sorting to isolate tamoxifen resistant subpopulations of cells by receptor expression level. Isolating rare resistant cells that reside within a population of drug-sensitive cells will offer new insights into why chemoresistance occurs.

KEYWORDS

breast cancer, chemoresistance, integrin $\beta 1$, tamoxifen

1 | INTRODUCTION

With its unique beginnings in the 1960s as a proposed contraceptive, tamoxifen has been a long-standing pillar of effective treatment for patients with estrogen receptor-positive breast cancer. The active

metabolite of tamoxifen, 4-hydroxytamoxifen, functions as an anti-estrogen in breast cancer cells and thus inhibits cancer cell proliferation. While tamoxifen continues to be successfully used to treat estrogen-dependent breast cancer, most patients receiving treatment will develop chemoresistance over time and will relapse with more

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aggressive cancer. Additionally, yet less frequently observed, is the occurrence of innate resistance to tamoxifen in patient populations.¹⁻⁴ With breast cancer as the second highest cause of cancer deaths in US women,⁵ additional insight and characterization of tamoxifen resistance in breast cancer is greatly needed to address complications associated with these outcomes.

To date there have been many biomarkers of tamoxifen resistance identified suggesting that the efficacy of tamoxifen treatment in estrogen-dependent breast cancer may be predicted based on a biomarker expression profile. Two commonly reported biomarkers of tamoxifen resistance are the membrane-spanning insulin-like growth factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR). IGF-1R expression is associated with tamoxifen sensitivity and is decreased in tamoxifen resistant breast cancer cells. Conversely, increased EGFR expression is associated with tamoxifen resistance, and lower expression is observed in tamoxifen sensitive breast cancer cells. In addition to these cell surface receptors, we identified increased expression of integrin β 1 in tamoxifen resistant breast cancer cells. Integrin β 1 is involved in focal adhesion between cells and there is increasing evidence that integrin β 1 modulates proliferation, invasion, survival, and metastasis of cancer cells via activation of multiple signaling pathways that include focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K).⁶⁻⁸

Although the exact mechanisms of tamoxifen resistance in ER-positive breast cancer cells remain unknown, the increase and/or decrease in the expression of these biomarkers suggest that delineation between resistant and sensitive cells is possible. If a subpopulation of intrinsically resistant cells survive treatment, which we hypothesize herein, then the receptor profile of these cells would enable us to identify and isolate this subtype. The different expression profiles of IGF-1R, EGFR, and integrin β 1 may be exploited in this scenario where the three biomarkers provide a new strategy to search for rare chemoresistant cells within large populations of tamoxifen sensitive cells. Once isolated, further studies can determine what factors drive resistance and if resistance is acquired or innate.

Therefore, we share in this brief report a study in which we determine the limits and confines of cell identification when comparing a tamoxifen resistant MCF-7 breast cancer cells to tamoxifen sensitive MCF-7 cells based on the aforementioned biomarkers. We simultaneously measure biomarkers, as previously described in References,⁹⁻¹¹ by fluorescently labeling IGF-1R, EGFR, and integrin β 1. We utilize flow cytometry to compare receptor expression level and determine if the differences are enough to separate tamoxifen resistant from non-resistant cells. We accomplish this by a preliminary strategy that takes mixtures of these cells to mimic a scenario in which cells that survive treatment are innately present among cells that are therapeutically affected. Breast cancer cells, when fully studied, have a significant degree of heterogeneity with respect to growth rate, chemoresistance, and metastatic ability. Understanding these underlying differences and exploiting them is a large step toward the development of more effective therapeutics.

2 | MATERIALS AND METHODS

2.1 | Cell culture

We chose this study to evaluate the common mammary carcinoma cell line, MCF-7 estrogen receptor-positive cells (ATCC[®] Manassas). These cells grow as a monolayer in culture and are known to be tamoxifen-sensitive in that their proliferation is halted when treated with tamoxifen. We then generated a tamoxifen resistant cell line (TamR). The TamR sub-line develops when MCF-7 cells have a sustained exposure to tamoxifen. Additional details about the TamR subline and other cell culture methods have been previously described in Reference.¹¹

2.2 | Cell labeling and flow cytometry

MCF-7 and TamR cells were labeled following the BioLegend[®] Cell Surface Flow Cytometry Staining Protocol with fluorophore-conjugated primary antibodies. Further antibody labeling protocol details can be found in the supplementary data. Additionally, unlabeled cells were measured as well as microspheres for control studies as described below.

The fluorescent labels chosen included phycoerythrin (PE), Alexa Fluor[®] 488, and the tandem dye, PE-Cy7. Measurements were performed with a single excitation wavelength cytometry system (NL-1000, Cytek[®] Biosciences). The conjugated antibody fluorophores included IGF-1R - PE (CD221, clone 1H7 catalog # 351806), EGFR - PE-Cy7 (clone AY13, catalog # 352910), and integrin β 1 - Alexa Fluor[®] 488 (CD29, clone TS2/16 catalog # 303016) (BioLegend[®]). For optimal labeling efficiency, titrations were performed (0.1, 1, 2, 4, and 8 μ l of antibody using $\sim 1 \times 10^6$ cells) and evaluated using standard protocols. The spectral measurements required un-mixing (SpectroFlo[®]) after measurement of single-color controls. We evaluated proper single-color controls (Figures S1 and S2) and determined microsphere use was acceptable. We thus labeled microspheres (AbC[™] Total Antibody Compensation Beads, Life Technologies) with the respective antibody-fluorophore pairs. All samples used for measurement underwent the same incubation, washing, and reagent steps as well as resuspension into 200 μ l of DPBS without calcium or magnesium prior to read-out. We acquired up to 10,000 events for every run and repeated the study $n = 3$ times. Our results were analyzed post-acquisition (FCS Express, De Novo[™] Software, Pasadena, CA) for gating analysis (supplemental Figures S2, S3, and S4), cell counts, and other statistical outcomes, for example, mean fluorescence intensity (MFI) and %CV ($SD/mean$).

2.3 | Immunoblotting

For immunoblot analysis, integrin β 1, 1:1000 (clone A-4, sc-374,429, Santa Cruz Biotechnology), β -actin, 1:2000 (clone C4, sc-47,778, Santa Cruz Biotechnology), IGF-1R, 1:1000 (polyclonal, #3027, Cell

Signaling Technology), EGFR, 1:1000 (clone D38B1, #4267, Cell Signaling Technology), anti-rabbit IgG-HRP, 1:5000 (#7074, Cell Signaling Technology), and anti-mouse IgG-HRP, 1:5000 (sc-2005, Santa Cruz Biotechnology) antibodies were used. Immunoblot analysis was performed as previously described in Reference.¹¹

3 | RESULTS AND DISCUSSION

To determine if tamoxifen resistant MCF-7 breast cancer cells (TamR) can be distinguished from tamoxifen sensitive MCF-7 cells by flow cytometry based on biomarker expression, we evaluated both cell types separately and together. The expression of three membrane spanning receptors: IGF-1R, integrin β 1, and EGFR was compared in each cell line by immunoblot and flow cytometry. Results using a 1:1 mixture were evaluated as a preliminary example of the ability to distinguish TamR cells from MCF-7 cells. A subpopulation of intrinsically resistant cells is likely to be a much smaller portion of the cells within a given culture. Overall, we found that it was possible to distinguish

TamR from MCF-7 cells using flow cytometry and labeling with integrin β 1 or IGF-1R but not EGFR.

Fluorescence labeling and flow cytometry was first optimized with a standard titration. Our measurements differed for each of the three biomarkers owing to the fact that each receptor is expressed differently depending on the tamoxifen sensitivity. To obtain optimal fluorescence signals for the TamR and MCF-7 cells, steps toward calibration (Table 1 and Figure 1) were required prior to simultaneous labeling. When evaluating repeated measurements of the mean of the fluorescence intensity for the given channel measured, we identified that a concentration range of three to 4 μ l of antibody per 10^6 cells provided optimal labeling efficiency for integrin β 1, IGF-1R, and EGFR.

Upon measuring and analyzing combinations of TamR and MCF-7 cells with all three fluorescent labels we found it was possible to distinguish tamoxifen resistant from tamoxifen sensitive cells when labeled with integrin β 1 and IGF-1R but not EGFR. (Figure 2). Scatter graphs are used to identify the cell population(s) based on side and forward scattered light detection (Figure. 2 left panel: SSC vs. FSC).

TABLE 1 Mean fluorescence intensity data captured from antibody titrations

Mean fluorescence intensity (MFI)						
Ab volume (μ l)	Integrin (Alexa Fluor [®] 488)		IGF-1R (PE)		EGFR (PE/Cy7)	
	Titration 1	Titration 2	Titration 1	Titration 2	Titration 1	Titration 2
0.1	14,738	17,823	35,584	15,122	2500	1206
1	84,828	87,517	117,601	93,259	2746	1916
2	96,815	102,604	130,830	113,761	3221	1908
4	100,616	106,030	138,348	122,968	3963	2242
8	103,554	107,741	142,063	128,037	4695	2691

Note: The values were taken by setting upper and lower limit markers for each entire population and identifying the MFI. These data correspond to the curves pictured in Figure 1.

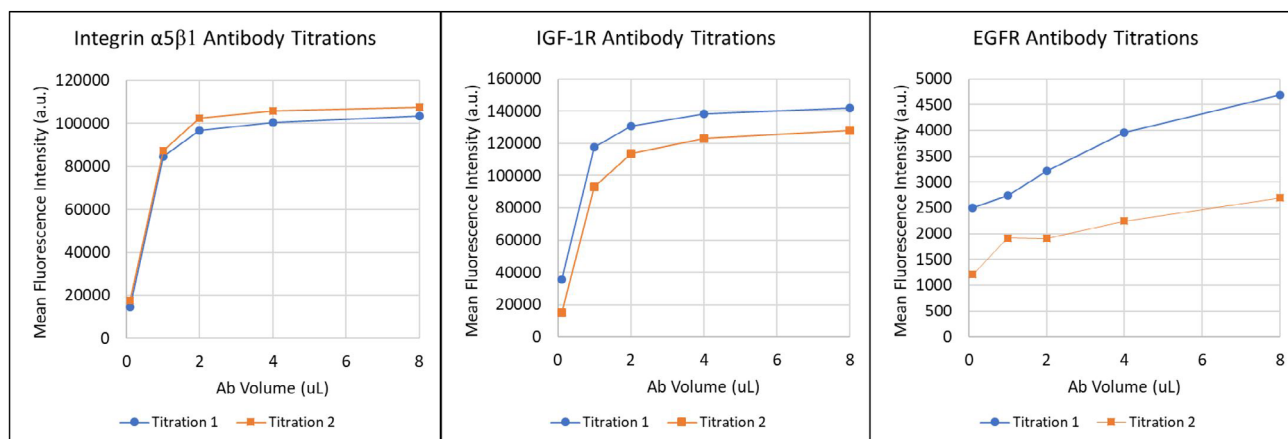


FIGURE 1 Mean fluorescence intensity (MFI) results from cytometry measurements. The MFI are graphed to visualize antibody titration curves for labeling standardization. The membrane spanning receptors EGFR, IGF-1R, and integrin β 1. Repeated and independent measurements ($n = 2$) were made to confirm the increase and saturation points with the Ab-fluorophore labels [Color figure can be viewed at wileyonlinelibrary.com]

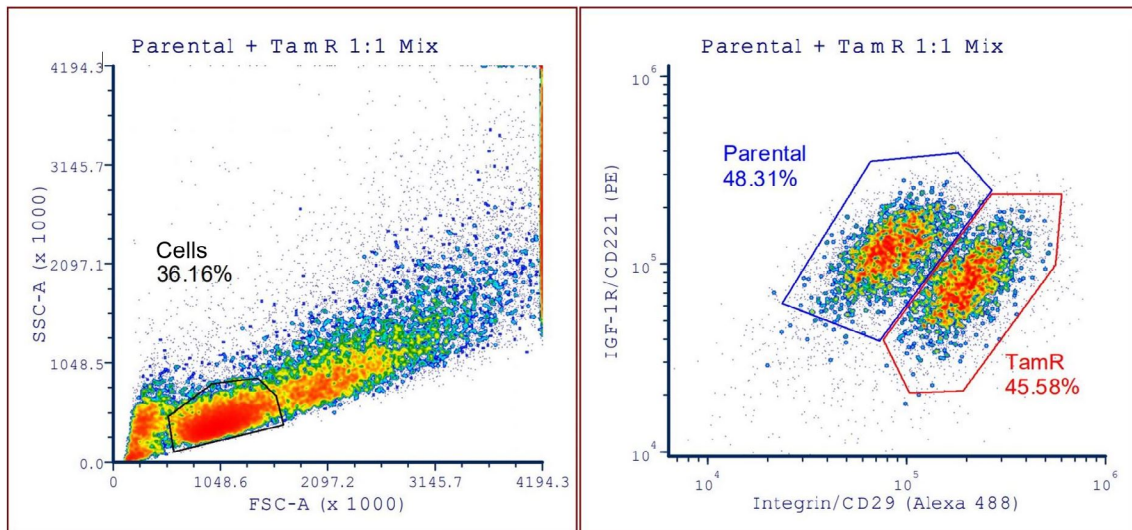


FIGURE 2 Flow cytometry results shown in the form of scatter plots. Left panel: Forward scatter versus side scatter; right panel: IGF-1R expression (PE emission) versus integrin β 1 expression (Alexa 488) [Color figure can be viewed at wileyonlinelibrary.com]

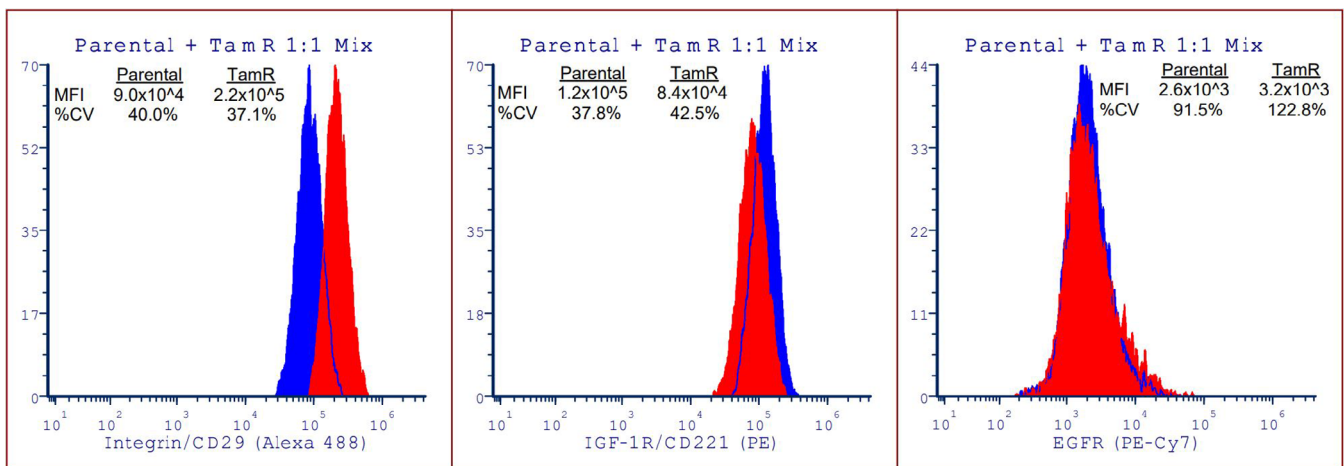


FIGURE 3 Cytometry histograms showing relative fluorescence intensity distributions when comparing the TamR cells and the MCF-7 cells. Differences or similarities in the expression of integrin β 1, EGFR, and IGF-1R are apparent using an overlay of the population data with the parental cells (blue) and TamR cells (red) [Color figure can be viewed at wileyonlinelibrary.com]

Following is a comparison of different fluorescence intensities for respective emission channels to differentiate TamR from MCF-7 cells (Figure 2, right panel). We observed a higher level of integrin β 1 expression on TamR cells (MFI = 2.2×10^5 at %CV = 37.1) when compared with MCF-7 cells (MFI = 9×10^4 for at %CV = 40.0), where % CV refers to the coefficient of variation within the sample. We also observed a lower expression of IGF-1R on the TamR cells compared with the MCF-7 cell line (MFI = 8.4×10^4 at %CV = 42.5 for TamR cells and MFI = 1.2×10^5 at %CV = 37.8 for MCF-7). No difference in EGFR expression was observed between TamR and MCF-7 when measured by flow cytometry (Figure 3).

To confirm IGF-1R, integrin β 1, and EGFR expression measured by flow cytometry, immunoblot analysis was performed. Immunoblot

analysis revealed a significant decrease in expression of IGF-1R and a significant increase in integrin β 1 and EGFR expression in TamR cells compared with MCF-7 cells (Figure 4). The immunoblot expression analysis is similar to our flow cytometry data except for the EGFR expression profile. One possible explanation for this observed difference between immunoblot and flow cytometry is the cellular localization of EGFR. EGFR is localized to subcellular compartments/organelles and to the membrane as a membrane spanning receptor.¹² The overall increase in expression of EGFR measured by immunoblot in the TamR cells does not necessarily mean the cell surface expression will increase and be detected by flow cytometry. Therefore, these results support the use of IGF-1R and integrin β 1 expression analysis by flow cytometry to distinguish TamR cells from MCF-7 cells and

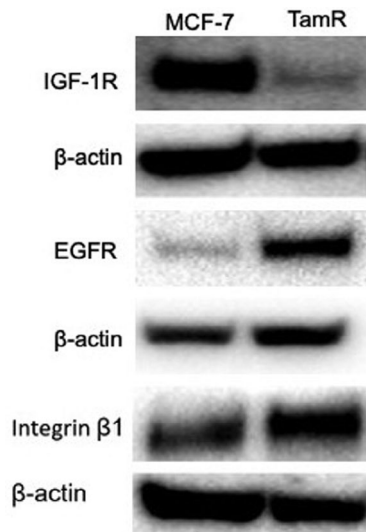


FIGURE 4 Expression of integrin $\beta 1$, EGFR, and IGF-1R for both MCF-7 and TamR cells measured by immunoblot. Measurements were performed with an anti-integrin $\beta 1$ antibody, anti-EGFR antibody, anti-IGF-1R antibody, and beta actin

suggest that EGFR is not useful for distinguishing TamR and MCF-7 cells by flow cytometry. The development of a protocol for sorting tamoxifen resistant breast cancer cells from tamoxifen sensitive breast cancer cells by flow cytometry requires the identification of cell surface proteins that have altered expression dependent on sensitivity to tamoxifen.

4 | CONCLUSIONS AND FUTURE WORK

Presented in this brief report is an evaluation of three distinct transmembrane receptors and their expression-level differences in chemoresistant cells when compared with nonresistant cells. Future studies will include additional breast cancer cell lines, primary breast cancer cells, and in vivo studies. Based on prior work by us and others, alterations in cell signaling pathways are associated with tamoxifen resistance and components of these pathways can be used as biomarkers of chemoresistance. We hypothesize that a rare subpopulation of cells within a tamoxifen sensitive cell culture or tumor have characteristics associated with tamoxifen resistance. This necessitates a method for cell sorting to isolate this subpopulation for further study. In order to determine if the chemoresistant cells can be distinguished in such a way that will allow for cell sorting, we designed a study labeling MCF-7 and TamR cells with conjugated antibodies that recognize integrin $\beta 1$, IGF-1R, and EGFR receptors. After mixing the cells to model a scenario where tamoxifen resistant cells are part of a population of tamoxifen sensitive cells, the mixture was analyzed for single-cell statistics using flow cytometry. These data demonstrate the ability to distinguish TamR cells from MCF-7 in a mixed population by expression levels of integrin $\beta 1$ and IGF-1R but not by EGFR expression. Since cytometry can be utilized as a tool to distinguish TamR cells from MCF-7 cells, the next steps will be to take cell lines

that have not been mixed and label for the same markers then utilize cell sorting to collect the rare cells with resistant receptor profiles for sequencing, metabolic profiling, and other treatment studies. If an innate tamoxifen resistant sub-population exists within a culture prior to treatment with tamoxifen, then identification and sorting such subsets of cells would lead to new understanding about how tamoxifen resistance develops in ER-positive breast cancer patients. In addition to the abovementioned biomarkers, since there is an increasing wealth of evidence to indicate that tamoxifen resistant breast cancer cells bear stem cell-like characteristics, additional biomarkers such as CD34¹³ and CD133¹⁴ can be studied using a similar approach. Finally, we envision the development of a diagnostic approach that predicts tamoxifen sensitivity or monitors tamoxifen sensitive breast cancer for the development of resistance. The overall significance is the ability to understand why ER-positive breast cancers patients become resistant to tamoxifen treatment; this might save patients from years of ultimately ineffectual treatment.

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AUTHOR CONTRIBUTIONS

Aric Bitton wrote the paper, prepared the samples, performed measurements with the flow cytometer and analyzed the data. Yan Zheng assisted in writing and data collection including western blot experimentation. Kevin D. Houston and Jessica P. Houston directed the work and its interpretation and guarantee the integrity of its results.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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