Investigating tamoxifen resistance in the luminal B estrogen receptor positive breast cancer subtype using gene expression profiling

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Abstract

Background

We recently reported that the two estrogen receptor (ER) positive breast cancer (BC) molecular subtypes can be defined by their expression of proliferation genes using a gene expression index (GGI): the luminal A and B subtypes have low and high levels respectively (JCO in press). When treated with adjuvant tamoxifen, luminal A tumors have a good prognosis, however the clinical outcome of the luminal B subtype was poor. This study aimed to explain the biological basis for these observations using global gene expression profiling and an in vitro model of ER+ BC.

Methods:

246 ER+ BC samples from women treated with adjuvant tamoxifen monotherapy were analyzed with Affymetrix gene expression arrays and evaluated using gene set enrichment analysis (GSEA). ER+ MCF-7 BC cells (control) treated with tamoxifen (TAM) and heregulin (HRG) were used to investigate molecular pathways identified using GSEA.

Results:

We found that a gene set suggesting HER2 (ERBB2) pathway activation was significantly enriched in the luminal B subtype (p=0.02). Only 10% of samples over-expressed HER2 by immunohistochemistry, suggesting that activation of HER2 signaling pathways is independent of HER2 over-expression and may contribute to TAM resistance in this subtype. To investigate this hypothesis, MCF-7 cell-lines were treated with HRG (HRG-MCF7) to create a model of ERBB2 pathway activation. HRG-MCF7 cells displayed phosphorylation of HER2/3 without HER2 over-expression. Treatment with HRG overcame TAM induced cell cycle arrest with higher S-phase fraction (p<0.01) and increased anchorage-independent colony formation (p<0.01). Gene expression profiling confirmed significant enrichment of the ERBB2 gene set (p<0.01) and higher GGI levels (p=0.02) in HRG-MCF7 cells compared with control.

Conclusions:

HRG-MCF7 cells may be useful as an in vitro model of the TAM resistant luminal B subtype. In this group, targeting activated HER2 signaling may be a helpful treatment strategy despite the lack of HER2 over-expression. Our data suggests that agents like lapatinib may be effective only in the luminal B and not the luminal A tumors, demonstrating the importance of stratifying by subtype in future clinical trials of ER+ disease.

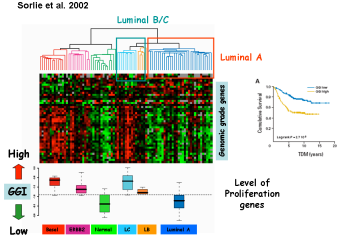
Background

In our previous research, we have shown that estrogen receptor positive breast cancer (ER+BC) can be divided into distinct molecular subtypes based on their level of expression of proliferation genes—low levels of proliferation corresponded to the luminal A subtype and high levels to the luminal B subtype. (Loi et al, JCO April 2007)

These two subtypes were associated with statistically significant different clinical outcomes in both tamoxifen (only) and systemically untreated populations.

We used Gene set enrichment analysis (GSEA) to investigate the biological basis for these observations

Looking at proliferation in breast cancer subtypes using the GGI: (Sotiriou et al, JCNI 2006): The Luminal A has low levels of proliferation and a good clinical outcome, compared with Luminal B which has high levels of proliferation and a poor clinical outcome: log rank p<0.001 (for further details refer to Loi et al, JCO, April 2007)



Materials & Methods

We used a dataset of 246 ER+ BC samples that had been treated with adjuvant tamoxifen monotherapy and hybridized using Affymetrix U133A Gene Chips. The full details are described in Loi et al, JCO 2007

GSEA was performed using software from Submramanian et al, PNAS 2005, version 1.

MCF-7 cells were grown at 37 °C, 5% CO2 in phenol-red free Improved Modified Eagle's medium (IMEM) supplemented with 5% fetal bovine serum. Before treatment, cells were serum starved for 24 hours. Cells were then treated with vehicle (ethanol), estrogen [E2- Sigma] (1 nM), tamoxifen [TAM] (4-hydroxy-tamoxifen-Sigma, 100nM), heregulin-beta 1 [HRG- CytoLab/Peprotech Asia] (10ng/mL) or tamoxifen and heregulin [TAM-HRG] (same doses) for 24 hours prior to collection.

Cell cycle analysis was performed using flow cytometry with BrDU

Antibodies against HER2 (# 2242), HER3 (# 4754), phosphorylated (P) HER2 (Tyr877; # 2241), HER3 (Tyr1289; # 4791), tyrosine (P-Tyr-100; # 9411), AKT (S473; # 9271, S6 (S235/236; # 2211) and ERK (T202/Y204; # 9101) were obtained from Cell Signaling and β actin from MP 24

Anchorage independent growth was tested by soft agar assay. In brief, 4000 cells were suspended in 0.35% bactoagar with different treatments were grown on a bottom layer of solidified 0.6% bactoagar in 35mm dishes. After 10 days the colonies on the top layer were counted. The size exclusion limit for a positive colony counting was ~60μmdiameter.

RNA was extracted from cell lines using the TriZOL (Invitrogen) method and hybridized to Affymetrix Gene Chips using standard Affymetrix protocols.

Results: GSEA of luminal A & B subtypes

The Luminal B subtype seems to have a poor outcome on adjuvant tamoxifen (Loi et al, JCO 2007)

We applied GSEA to identify gene sets correlated with the luminal B ER+ subtype.

Using a FDR ≤0.10 and p <0.05 57 gene sets were significantly associated with the luminal B subtype.

As expected most of these were related to cell cycle and proliferation

We noted the significance of the ERBB2 gene set (p=0.02, FDR=0.09) as less than 10% of samples over-expressed HER2 by immunohistochemistry (+3). Results remained significant for this gene set when the HER2+++ samples were removed.

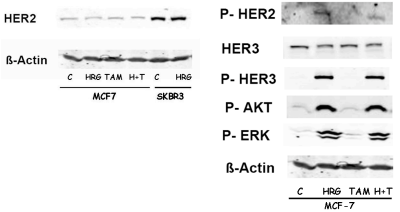
Gene Set class	Name of gene set	Nominal p value	FDR
Cell cycle related	Cell cycle	0	<0.001
	CCR5 Drives positive	0	<0.001
	CR cell cycle	0	<0.001
	Rb Pathway	0	<0.001
	Cell cycle pathway	0	<0.001
	Cell cycle checkpoint	0	<0.001
	G1 pathway	0	<0.001
	G2 pathway	0	<0.001
	Leukine down regulated	0.002	<0.001
	Dp2a2 Pathway	0	<0.001
	CBCR Pathway	0.001	<0.001
	Cell cycle regulator	0	<0.001
Metabolism related	MAPK1243 Pyrimidine metabolism	0	0.002
	MAPK1231 Purine metabolism	0	0.02
	MAPK1794 Folate biosynthesis	0.002	0.02
	MAPK1191 Sterol biosynthesis	0.002	0.02
	Glycolysis pathway	0.009	0.03
Other	ERBB2 pathway	0.02	0.09
	PAP down regulated	0.002	0.002
	IM DTCA pathway	0.002	0.006
	DNA Damage signaling pathway	0	0.002
	PI3 signaling pathway	0.002	0.02
	Mtd pathway	0.02	0.02
	Radiation sensitivity	0	0.02

Table of gene sets (selected) significant in the tamoxifen resistant subtype: C2 gene sets were used (www.broad.mit.edu/gsea) ERBB2 gene set curated from Perou et al, Nature 2000

We chose to investigate the hypothesis that ERBB2 pathway activation without over-expression contributes to tamoxifen resistance and a high proliferative state in the luminal B subtype

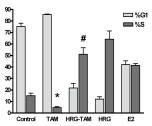
Results: Heregulin treated MCF-7 cells are resistant to tamoxifen and show activation of ERBB2 pathway without over-expression

•MCF-7 cells treated with Heregulin (HRG, a ERBB3 ligand) was used to create a model of ERBB2 pathway activation without over-expression

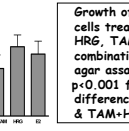


Western Blot analysis
1. **HER2 over-expression present in SKBR3 not MCF-7**
2. **HRG stimulates phosphorlyation of HER2, HER3, AKT, ERK supporting ERBB2 pathway activation**

•Normally, MCF-7 cells are highly sensitive to tamoxifen treatment. HRG induces resistance to tamoxifen in MCF-7 cells. (Error bars= 95%CI, results are representative of triplicate experiments)



Serum starved MCF-7 treated with HRG, TAM & combination for 24 hrs and analyzed with flow cytometry.
***, # p<0.001**
S% phase b/w Rx



Growth of MCF-7 cells treated with HRG, TAM & combination in soft agar assays. p<0.001 for difference b/w TAM & TAM+HRG

•Microarray analysis of cell lines reveals higher levels of proliferation (by GGI scores, p=0.02) & significant enrichment of the ERBB2 gene set (p=0.001, FDR=0.16)

Conclusions

•We have validated a biological hypothesis derived from microarray-derived breast cancer subtypes suggesting that ERBB2 activation in the absence of over-expression is an important contributor to tamoxifen resistance in the luminal B subtype.

•HRG- treated MCF-7 cells may be useful as an in-vitro model of the luminal B subtype

•This demonstrates the importance of stratifying future clinical trials by subtypes in order not to miss potentially important results that can be diluted by tumor biological heterogeneity.

•Our results suggest that agents like lapatinib and pertuzumab could be effective in the luminal B, not the luminal A subtype.

•Further research is needed into characterizing the underlying oncogenic pathways that can be targeted in the clinical setting in order to develop suitable treatment strategies for ER+ BC patients