Downregulation of Argininosuccinate synthase 1 confers Tamoxifen Resistance in Invasive Lobular Breast Cancer

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Lay abstract

Invasive Lobular Carcinoma (ILC) is an understudied unique subtype of breast cancer that faces late detection, higher stage at diagnosis and late recurrence. Patients with ILC are treated as patients with invasive ductal cancers (IDC) of the breast. ILC is estrogen dependent but develops resistance to anti-estrogen therapy leading to recurrence. We study why ILC cells become Tamoxifen-resistance to develop combined therapy for ILC patients.

Abstract

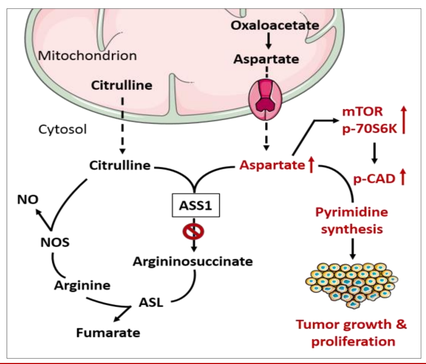
ILC is an estrogen receptor (ER) positive subtype of breast cancer. The comparative effectiveness of tamoxifen (TAM) in IDC is better than in ILC[1]. Our overall goal is to determine drivers of tamoxifen resistance and targeted therapy to improve efficacy of anti-estrogen therapy. We have developed tamoxifen resistant (TAMR) ILC cell lines. Metabolomics and RNA-seq analyses of parental and TAMR cells revealed differential expression of multiple genes and pathways in TAMR cells. Of the 15 common dysregulated genes between these pathways, downregulation of *ASS1* was associated with poor disease-free survival in TAM-treated patients.

Our goal is to investigate how ASS1 downregulation promotes TAM resistance and explore avenues to increase ASS1 level to enhance TAM efficacy.

Hypothesis

➢**Increasing *ASS1* expression in TAMR cells may improve the efficacy of tamoxifen treatment.**

Background: ASS1 function and metabolic flux of aspartate towards synthesis of arginine and pyrimidine [2-3]

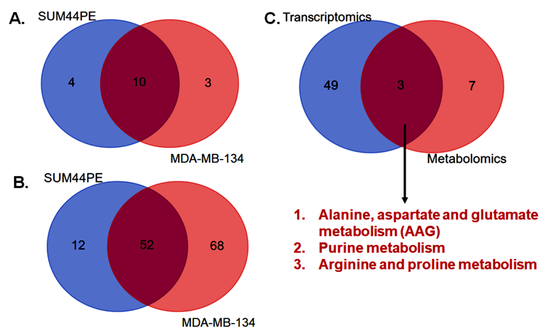


METHODOLOGY

1. Developed tamoxifen resistant (TAMR) ILC cells (MDA-MB-134-VI and SUM44PE), by prolonged exposure to 100-500nM tamoxifen (TAM).
2. Subjected four cell lines (MDA-MB-134-VI , MDA-MB-134-VI-TAMR, SUM44PE, SUM44PE-TAMR) to a. RNA-seq and b. LC-MS.
3. Validated differentially expressed genes by qPCR and western blot analysis in TAMR and LTED (Long Term Estrogen Deprived) ILC cells.
4. Analyzed promoter methylation by methylation specific PCR (MS-PCR).
5. Treated cells with 5-Aza-2′-deoxycytidine (dAZA) to demethylate genes.
6. Studied effect of drug combination by MTT assay.

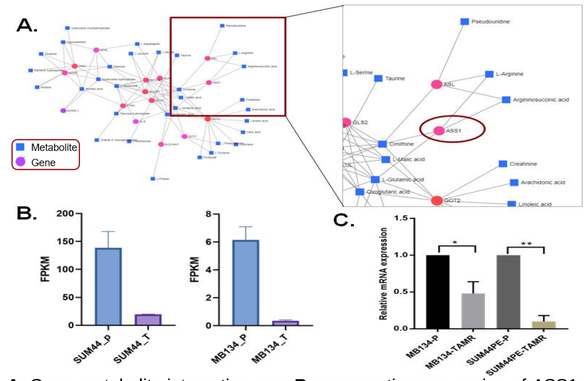
RESULTS

Omics profiling revealed alteration of overlapping pathways in TAMR ILC



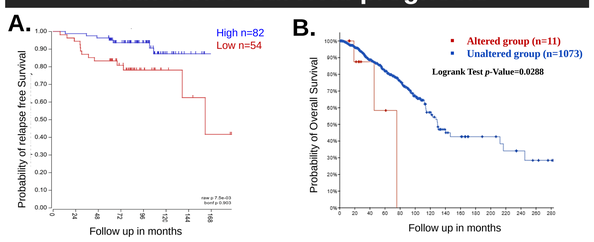
Significantly and mutually altered **A.** metabolic process and **B.** de-regulated gene sets in parental *vs.* TAMR cells. **C.** Overlap of both -omics

ASS1 is downregulated in TAMR cell lines



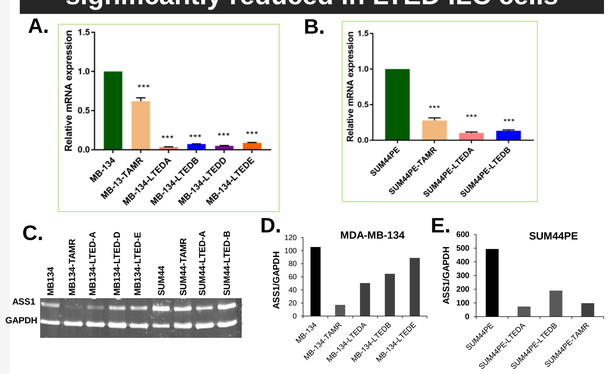
A. Gene-metabolite interaction map **B.** comparative expression of *ASS1* in parental *vs.* TAMR cells. **C.** qPCR validation of *ASS1* expression.

Decreased expression of *ASS1* associates with worse survival prognosis



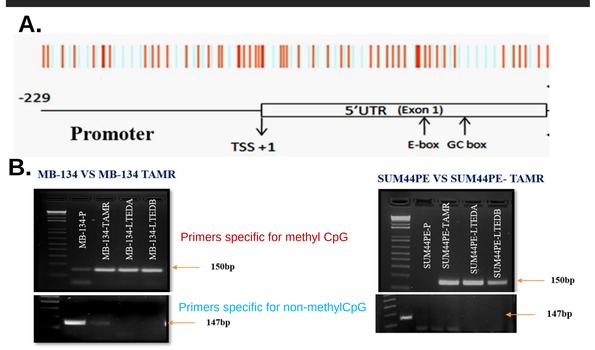
A. Relapse free survival w.r.t *ASS1* expression in breast cancer patients who has received adjuvant tamoxifen therapy [4]. **B.** Overall survival of breast cancer patients related to *ASS1* expression.

ASS1 RNA and protein levels are significantly reduced in LTED ILC cells



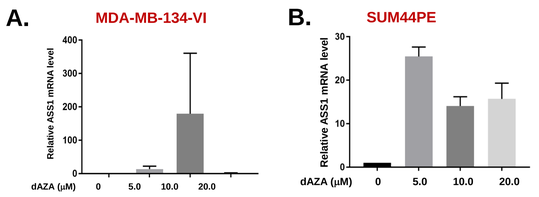
qPCR analysis of **ASS1** in **A.** MDA-MB-134 cells and its derivatives **B.** SUM44PE cells and its derivatives. **C.** Western blot analysis of ASS1, **D & E**. Densitometric Analysis of Western Blot for ASS1.

CpG islands in the *ASS1* promoter is methylated in ILC-TAMR and LTED cells



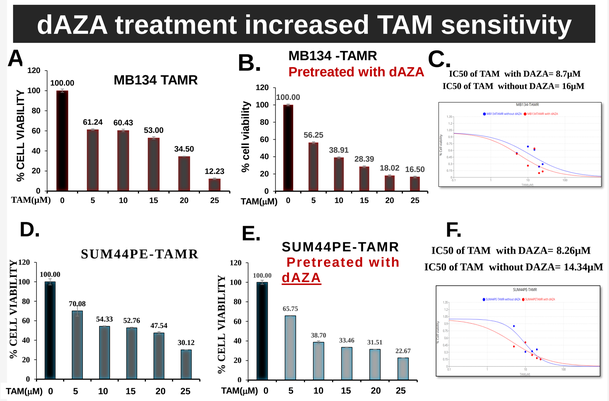
A. Schematic showing CpG island in *ASS1* promoter *(red bars- CpG)* [5]. **B.** MS-PCR of *ASS1* promoter region using primers specific for methyl-CpG and non-methyl CpG in MB-134 and **C.** SUM44 cells.

5-Aza-2′-deoxycytidine (dAZA) treatment reactivates *ASS1* expression



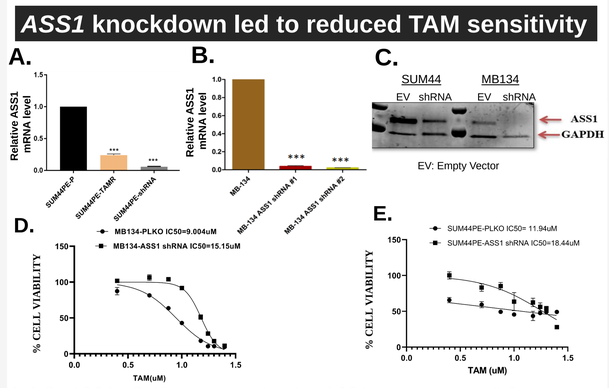
TAMR-ILC cell line **A.** MDA-MB-134-VI-TAMR and **B.** SUM44PE-TAMR cells were treated with 5, 10, or 20 μM of the demethylating agent 5-Aza-2′-deoxycytidine (dAZA) for 120 hours. Total RNA was analyzed for *ASS1* expression by qPCR.

dAZA treatment increased TAM sensitivity



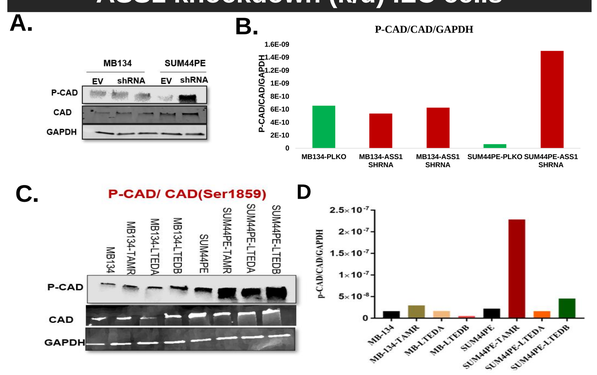
TAMR-ILC cell line were untreated (**A&D**) or pre-treated with 5μM dAZA (**B&E**) for 120 hours. Cells were then treated with increasing concentration of TAM. Cell viability was measured by MTT assay. **C & F.** IC50 TAM of dAZA untreated and treated cells.

ASS1 knockdown led to reduced TAM sensitivity



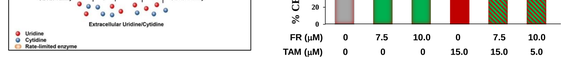
A & B. *ASS1* mRNA expression level in ASS1 knocked down ILC cell lines (parental)**. C.** Western blot for ASS1 protein level expression in ASS1 KD vs. PLKO. **D & E**. IC50 of TAM for the ILC-ASS1 KD vs PLKO cells treated for 120 hrs.

Level of p-CAD is increased in TAMR and ASS1 knockdown (k/d) ILC cells



A&B. pCAD level in ASS1 k/d and control cells quantitated in the bar diagram. **C&D.** pCAD level in ILC sensitive vs. TAMR and LTED cell lines quantitated in the bar diagram.

Inhibition of Dihydroorotate dehydrogenase by Farudodstat increased TAM sensitivity



A. Schematic of Pyrimidine metabolism [6] B. ILC-TAMR cell viability treated with Farudodstat (FR, IC50: 5-9 μM)) alone and in combination with TAM using MTT Assay. * p<0.05.

SUMMARY

➢ **Metabolomics and transcriptomics studies revealed downregulation of *ASS1* in TAM- resistant ILC cell lines.**

➢**Detection of hypermethylation in the ASS1 promoter region in ILC-TAMR cell lines suggests methylation-mediated silencing of *ASS1* transcription, which is reflected in ASS1 protein deficiency.**

➢ **Observed increased expression of phospho-CAD in ILC-TAMR cells that can potentially divert the metabolic influx of aspartate towards pyrimidine synthesis via CAD activation.**

➢ **Based on our recent data, we can postulate that activation of pyrimidine biosynthesis pathway due to the *ASS1* loss might be responsible for TAM resistance and tumor aggression.**

CONCLUSION & FUTURE STUDIES

- ❑ **This study reveals novel insights into TAM resistance in ILC, with the first-time demonstration of ASS1 downregulation in TAMR-ILC cells.**
- ❑ **Restoring ASS1 expression through demethylation or by targeting DHODH reduced TAMR cell growth and enhanced TAM sensitivity.**
- ❑ **These findings offer potential therapeutic strategies to overcome TAM resistance in ILC patients.**

Future Studies:

- ✓ ***In vivo* drug testing in mice injected with TAMR cell lines.**
- ✓ **Mass Spectrometric analysis of pyrimidine biosynthesis.**
- ✓ **Human ILC Tissue microarray to investigate ASS1 expression, and correlation with disease –free survival.**
- ✓ **We plan to expand our research to include other breast cancer subtypes, such as invasive ductal carcinoma, to evaluate the broader relevance of our findings.**

References

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Acknowledgement

This work is supported by Anderson ILC fund to Dr. Bhuvaneswari Ramaswamy, MD MRCP. We are grateful to Steffi Oesterreich, PhD, University of Pittsburgh for providing LTED cells.