Receptor status and drug independent breast cancer mRNA signature in cell lines undergoing autophagy flux.

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Background

Autophagy is a complex process of recycling endogenous proteins and or organelles in response of stress stimuli such as starvation, redox stress and cancer therapy.

Autophagy has been linked to the safety and efficacy of chemotherapy in several types of cancers.

Chloroquine, a lysosome fusion inhibitor, is currently used as an aspecific autophagy inhibitor, both in experimental and clinical settings, to prevent resistance through reduction of the autophagy mechanism.

Tamoxifen and trastuzumab are target-specific (ER/HER2 respectively) drugs highly used in clinical setting for subsets of cancer patients with the ideal receptor expression. Resistance through autophagy for both these drugs has been discussed in the literature.

Bortezomib and rapamycin are strong autophagy inducing agents that are used in combination with other chemotherapeutics in oncology clinical trials.

Currently, there is no quantitative biomarker to monitor the autophagy process *in-vivo* and no specific inhibitors to modulate the autophagy response during cancer therapy.

Aim

Identify a drug- and receptor-status agnostic autophagy signature to monitor and measure autophagy during breast cancer treatment.

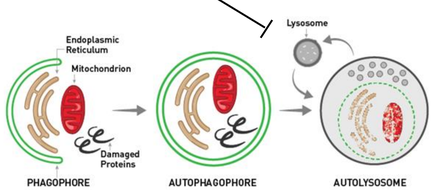
Drugs and breast cancer cell lines used in the study

	ER	PR	HER2	
MCF-7	+	+	-	Tamoxifen
MDA-MB-231	-	-	-	Rapamycin Bortezomib
SKBR3	-	-	+	Trastuzumab

RNA Sequencing

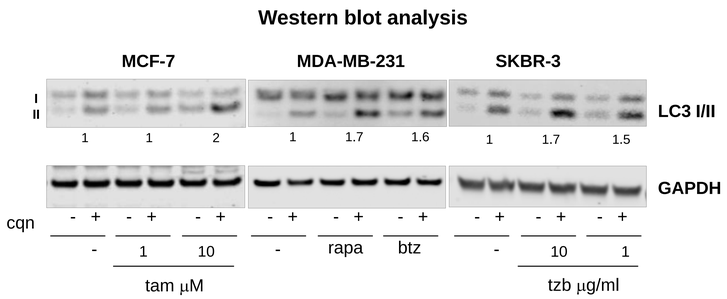
with or without Chloroquine

Western Blot



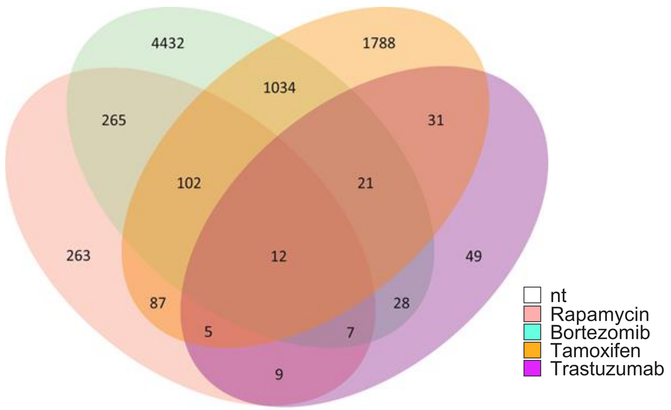
The table describes the selected pool of breast cancer cell lines and drug subsets. The selection criteria was the concomitance of clinical relevance and autophagy inducing properties. The schematic shows the mechanism of action of chloroquine, a non-selective inhibitor of lysosomal fusion.

Drug induced autophagy flux



The autophagy flux is measured by detecting the difference of LC3II levels in presence and absence of chloroquine 10μM. MDA-MB-231 cells were treated for 8 hours with rapamycin (rapa 200nM) or bortezomib (btz 1μM) while MCF-7 and SKBR3 were treated for 16 hrs with tamoxifen (tam) and trastuzumab (tzb) respectively. Cells were treated with the minimal dose of drug to achieve the earliest measurable signs of autophagy flux. All untreated and drug treated cells in absence of chloroquine were selected for total RNA isolation for further analysis.

Drug induced transcriptional changes during the autophagy flux



Total RNA from samples treated as described above was used for Illumina exome sequencing (RNA seq) with HiSeq 2500 sequencer. Differential expression analysis compared non treated cells and drug treated cells for each cell line and drug treatment. Numbers in the Venn diagram show the number of differentially expressed genes that are significantly different (adjusted p≤0.05 n=3) after each treatment. Only 12 mRNAs are in common between all treatments of the cell lines while undergoing autophagy flux.

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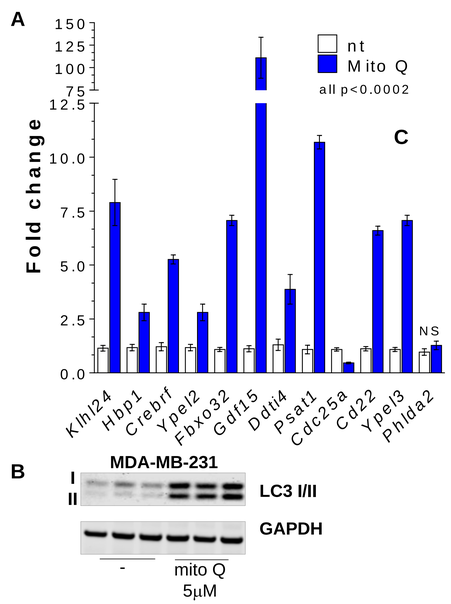
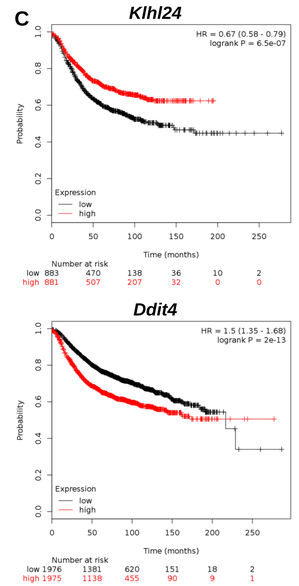
Mining autophagy candidate mRNAs *in vitro and in vivo*





The mRNA autophagy signature was verified with mitoquinone (mito Q), a known autophagy inducer. MDA-MB-231 were treated for 16 hours with mito Q and total RNA and cell extracts were used for Real Time PCR (A) and western blot (B). Eleven mRNAs responded to mito Q with upregulation or downregulation of the transcripts as the autophagy signature predicted. When sequencing data were verified with Real Time PCR, Phlda2 mRNA was not confirmed as part of the signature. Indeed, during mitoQ induced autophagy, the levels of this mRNA do not change significantly. We examined the mRNA expression levels of the 11 autophagy signature genes in sequenced samples from breast cancer patients on the database KMplotter. Risk free survival (RFS) and/or overall survival (OS), are associated with high or low expression of the mRNAs from the autophagy signature. In panel C, an example of KM plotter data shows that high levels (red line) of Klhl24 or low levels of Ddit4 (black line) are associated with better risk-free survival.

Conclusions

The 11 mRNAs autophagy signature was verified in an independent set of RNA samples isolated from cells undergoing autophagy flux. Given the presence of autophagy inhibitors and stimulators in clinical practice and the challenges of quantitative methods to verify the safety and efficacy of this class of drugs, this list may represent a simplified tool to measure the autophagy process *in vivo*.

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