Biochemical consequences of cancer-specific somatic mutations in Ubiquilin-1

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Background

Ubiquilin-1 (UBQLN1) has been implicated as a key player in the pathogenesis of several neurodegenerative diseases, however thus far its potential role in tumorigenesis has been overlooked. UBQLN1 acts as an adaptor molecule to mediate degradation of ubiquitinated proteins by the proteasome, engage with the aggresome pathway, aid in autophagy, and modulate receptor trafficking. Our previous work has shown that disrupting the function of UBQLN1, via siRNA-mediated loss, causes lung epithelial cells to develop many hallmarks of cancer, including increased proliferation, colony formation, and epithelial-mesenchymal transition. Also, UBQLN1 interacts with several proteins implicated in cancer development, including IGF1R, VCP, and BCLb. Nearly ten percent of human non-small cell lung cancers (NSCLC), especially adenocarcinomas, have been shown to contain non-synonymous, somatic mutations in Ubiquilin-family genes.

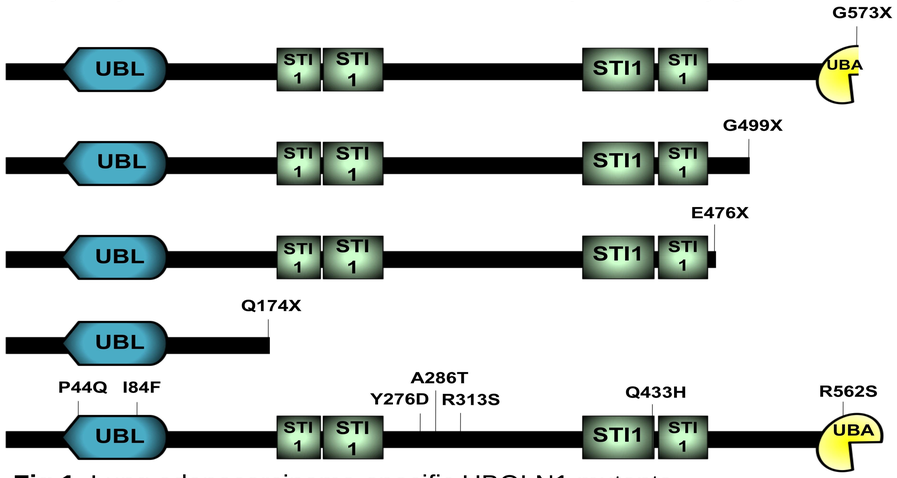

Fig 1. Lung adenocarcinoma-specific UBQLN1 mutants

Objectives

Based on our current understanding of UBQLN1 function, we hypothesize that these mutations will

1) Influence the overall stability of the UBQLN1 molecule
2) Impact the ability of UBQLN1 to interact with its substrates, specifically those with a known link to cancer
3) Alter the capacity of UBQLN1 to fulfill its purpose in various pathways

Results

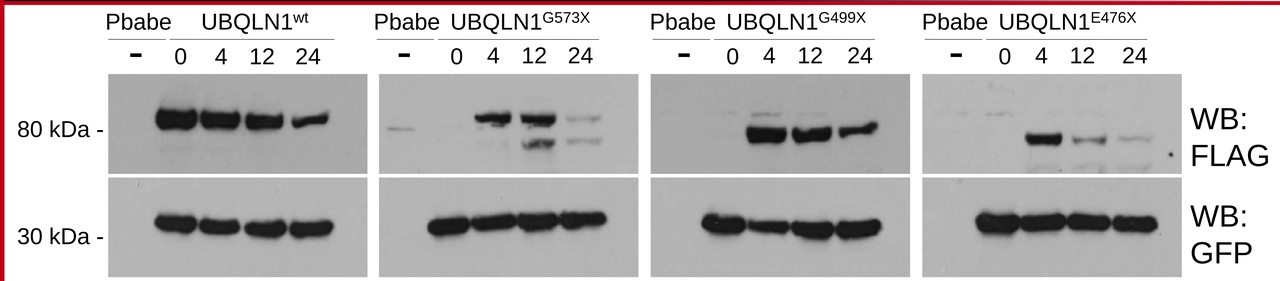

Fig 2. Cancer-specific UBQLN1 truncations are highly unstable at normal cellular conditions. Transfected 293T cells were treated with MG132 for 4, 12, and 24 hours to test the stability of UBQLN1 truncations.

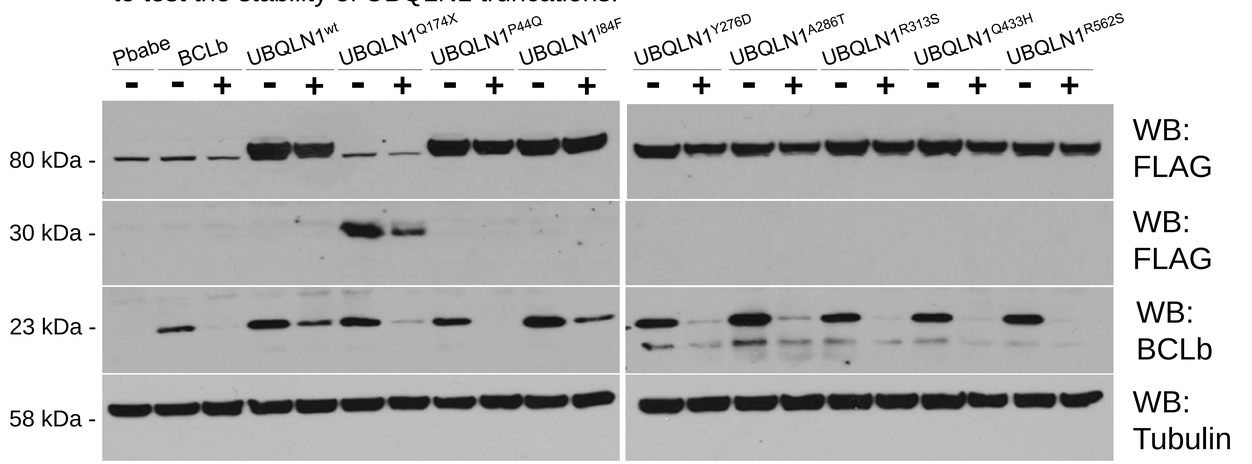

Fig 3. Cancer-specific UBQLN1 mutations alter interaction and stabilization of BCLb. 293T cells were transfected with BCLb and UBQLN1 mutants, then treated with cyclohexamide to determine the ability of each mutant to stabilize BCLb.

Future Endeavors

We will continue delineating the consequences of these mutations by observing changes in

1) Interactions with known cancer-related substrates, ubiquitin, and the proteasome
2) Activity within aggresome, degradation, and trafficking pathways
3) Dimerization and production of reported transcript variants
4) Cellular response leading to development of tumorigenic markers

Acknowledgements

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Dysregulated microRNA Expression in Colon Adenoma Tissue

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Introduction

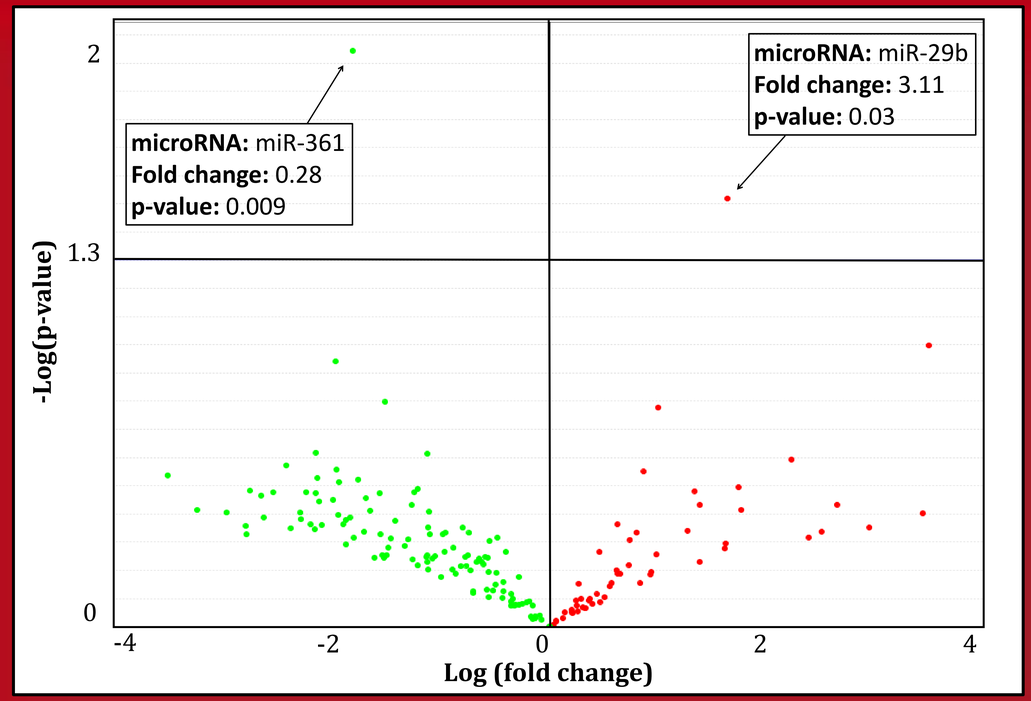
Early detection of colorectal (CR) adenomas is important in reducing colorectal cancer (CRC) mortality, therefore biomarkers used to detect CR adenomas are in great need. MicroRNAs are small non-protein-coding RNAs responsible for regulation of gene expression and whose expression is altered during the progression of CRC. The purpose of this study was to identify dysregulated expression of miRNAs in CR adenoma tissue for the potential use as biomarkers for the detection and prevention of CRC.

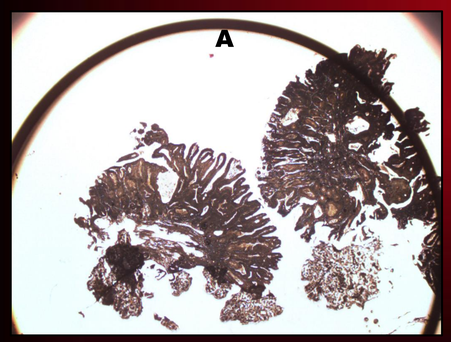
Materials and Methods

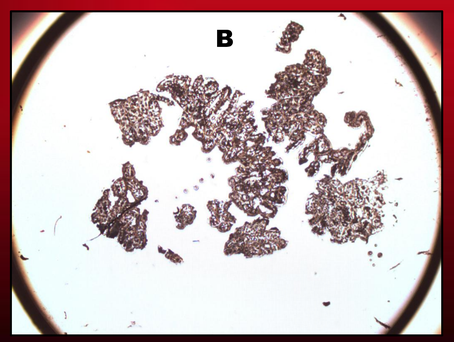
Samples of colon tissue containing both non-neoplastic and adenomatous epithelium were obtained as formalin fixed paraffin embedded blocks. Slides were prepared from these samples and examined under a microscope. Using laser capture microdissection (LCM), the two desired cell populations were separated and extracted from the tissue. These cell samples were incubated and digested with proteinase-K. RNA was isolated (Paradise® PLUS), generated into cDNA and pre-amplified (Megaplex™). The samples were loaded into 380 miRNA TaqMan® low-density array cards (TLDA) and underwent RT-PCR for analysis.

Results

FIGURE 1. Volcano plot comparing fold changes and p-values of 380 microRNAs in colon adenoma tissue with non-neoplastic colon tissue from the same patient (n=3)







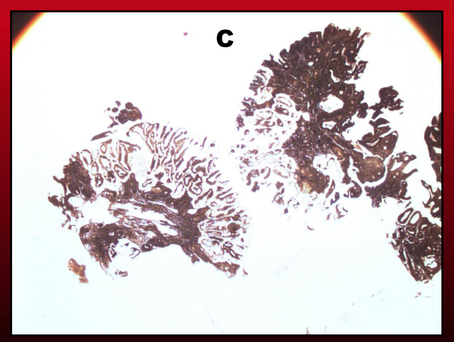


FIGURE 2. Laser capture microdissection process, transferring desired colon adenoma cells from the tissue in **A** to the cap in **B**. The remaining tissue is shown in **C** after LCM.

Key Findings

- Significant upregulation of miR-29b (p=0.03) and downregulation of miR-361 (p=0.009) in colon adenoma tissue compared to non-neoplastic tissue from same patient
- Laser capture microdissection provides enough cellular material to adequately perform RT-PCR with TLDA cards

Conclusions

- Colon adenoma tissue shows dysregulation of at least two miRNAs known to be involved in tumorigenesis.
- This miRNA expression is unique compared to that of CRC and therefore may be a reliable biomarker for early detection of CR adenomas.
- This study requires more patient samples to obtain a more accurate description of microRNA expression in colon adenomas

Acknowledgements

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Restrictive Blood Transfusion Protocol in Liver Resection Patients Reduces Blood Transfusions without Worsening Overall Outcomes

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Background:

Continued reports demonstrate the effects of hospital transfusion related to a longer length of stay, more complications, and possibly worse overall oncologic outcomes. The hypothesis for this study was that a restrictive transfusion protocol would reduce overall blood transfusions with no worsening in overall outcomes.

Methods:

A cohort study was performed using our prospective database from 1/2000 to 6/2013. September of 2011 served as the separation point for the date of operation criteria because this marked the implementation of more restrictive blood transfusion guidelines.

Acknowledgements:
Research supported by the grant R25-CA-134283 from the National Cancer Institute and the School of Medicine Summer Research Scholar Program.

Operative Data Before and After Protocol

	9/2009-9/2011 No Transfusion (n=87)	9/2009-9/2011 Received Transfusion (n=39)	After 9/2011 No Transfusion (n=46)	After 9/2011 Received Transfusion (n=14)
Blood Transfusions	69.0%	31.0%	76.7%	23.3%
Liver Procedure				
Atypical Resection	1.1% (1)	0.0%	0.0%	0.0%
Central Liver Resection	3.4% (3)	2.3% (1)	2.2% (1)	0.0%
L. Lateral Segmentectomy	21.8% (19)	4.6% (2)	19.6% (9)	14.3% (2)
L. Lobectomy	16.1% (14)	2.3% (1)	2.2% (1)	7.1% (1)
L. Trisegmentectomy	26.4% (23)	5.7% (3)	2.2% (1)	0.0%
R. Lobectomy	***2.3% (2)***	***11.5% (5)***	39.1% (18)	50.0% (7)
R. Posterior Sectorectomy	16.1% (14)	4.6% (2)	0.0%	0.0%
R. Trisegmentectomy	12.6% (11)	11.5% (5)	19.6% (9)	14.3% (2)
Segmentectomy	16.1% (14)	4.6% (2)	15.2% (7)	14.3% (2)
Number of Units Transfused	N/A	2.0	N/A	4.0
Total Blood Loss	***200.0***	***600.0***	***200.0***	***400.0***
Operative Time	***129.2***	***167.5***	***127.9***	***194.7***
Preoperative Intervention				
None	78.9% (69)	67.6% (26)	74.4% (34)	40.0% (6)
Prior Surgery	9.9% (9)	14.7% (6)	15.4% (7)	30.0% (4)
Embolization	8.5% (7)	0.0%	2.6% (1)	10.0% (1)
Endoscopic Stent	***2.8% (2)***	***11.8% (5)***	7.7% (4)	20.0% (2)
Portal Vein Embolization	0.0%	5.9% (2)	0.0%	0.0%
Preoperative Intent				
Cure	84.4% (73)	93.1% (36)	84.4% (39)	100% (14)
Palliation	15.6% (14)	6.9% (3)	15.6% (7)	0.0%
Number of Tumors	1.0	***1.0***	1.0	***1.0***
Size of Largest Tumor	***3.5***	***6.0***	***3.1***	***6.0***
Defined Tumor Margin	13.0% (11)	10.8% (4)	9.5% (4)	25.0% (4)
CVP Total	2.0	3.0	3.0	1.5

Outcome Data Before and After Protocol

	9/2009-9/2011 No Transfusion (n=87)	9/2009-9/2011 Received Transfusion (n=39)	After 9/2011 No Transfusion (n=46)	After 9/2011 Received Transfusion (n=14)
Complication	***35.1% (31)***	***61.8% (24)***	***4.5% (2)***	***80% (11)***
Intraoperative Surgical Complication				
None	98.9% (86)	94.9% (37)	97.8% (45)	85.7% (12)
Hemorrhage	1.1% (1)	2.6% (1)	0.0%	7.1% (1)
Hypotensive Episode	0.0%	2.6% (1)	2.2% (1)	7.1% (1)
Grade of Complication				
Oral Antibiotics	24.1% (7)	4.2% (1)	20.0% (1)	0.0%
IV Antibiotics	55.2% (16)	33.3% (8)	20.0% (1)	20.0% (1)
Return to ICU				
Percutaneous Drainage	20.7% (6)	58.3% (14)	40.0% (2)	60.0% (3)
Operative Correction				
Organ Diversion	0.0%	0.0%	0.0%	0.0%
Death	0.0%	4.2% (1)	20.0% (1)	20.0% (1)
Infection	0.0%	2.6% (1)	0.0%	7.1% (1)
Length of Stay	5.0	***8.0***	5.0	***8.0***
New Recurrence	23.0% (20)	12.8% (5)	9.3% (4)	21.4% (3)
Site of Recurrence				
Liver	56.5% (26)	69.2% (9)	25.0% (2)	50.0% (1)
Lung	19.6% (9)	0.0%	12.5% (1)	0.0%
Bone	6.5% (3)	0.0%	12.5% (1)	0.0%
Lymph Nodes	6.5% (3)	7.7% (1)	0.0%	50.0% (1)
Other	10.9% (5)	23.1% (3)	50.0% (4)	0.0%
Karnofsky Score	100	90	100	100
Status				
No Evidence of Disease	48.3% (42)	41.0% (16)	60.5% (26)	57.1% (8)
Alive with Disease	42.5% (37)	38.5% (15)	34.9% (15)	28.6% (4)
Died of Disease	8.0% (7)	7.7% (3)	2.3% (1)	0.0%
Died of Other Cause	***1.1% (1)***	***12.8% (5)***	***2.3% (1)***	***14.3% (2)***

Results:

- The restrictive blood transfusion guidelines reduced the percentage of patients that received blood from 31.0% before 09/01/2011 to 23.3% (0.03).
- Patients who received blood before and after the restrictive period had similar predictive factors:
  - Major hepatectomies, higher intra-operative blood loss, lower pre-operative hemoglobin, older age, prior systemic chemotherapy, and lower pre-operative nutritional parameters (all $P<0.05$).
- Patients who received blood did not have worse overall progression free survival or overall survival.

Conclusion:

A restrictive blood transfusion protocol reduces the incidence of blood transfusions and the number of packed red blood cells transfused. Patients who require blood have similar pre-operative and intra-operative factors that cannot be mitigated in oncology patients. Restrictive use of blood transfusions can reduce cost and does adversely effects patients undergoing liver resection.

Novel Therapies for BRAF-Inhibitor Resistant Melanoma

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Introduction

Most patients with metastatic melanoma carrying a BRAF-V600E mutation develop resistance to Vemurafenib (PLX) treatment after 6 months. Combination drug therapy may overcome resistance. 17-DMAG is an Hsp90 inhibitor that has shown promise in multiple cancer treatment clinical trials, but it causes significant systemic cytotoxicity. We have found that 17-DMAG effectively kills PLX-resistant melanoma cells. Furthermore, we identified a uniquely expressed receptor, extracellular matrix metalloproteinase inducer (EMMPRIN) which is highly expressed in metastatic melanoma and PLX-resistant cells, to provide a novel target for cancer cell-specific drug delivery. Using an S100A9 ligand, we have created an EMMPRIN targeted probe and liposome that binds to melanoma cells *in vivo*, thus designing a novel *in vivo* drug delivery vehicle.

Methods

Cell Culture

Human melanoma cells lines A2058, A2058PLX, A375, and A375PLX were used in culture. A2058PLX and A375PLX were maintained with 10μM and 1.0μM PLX-4032 (Vemurafenib), respectively, at each passage for one year to maintain resistance.

Protein Analysis

Whole cell protein lysates were analyzed by standard Western Blot technique for apoptosis and autophagy associated proteins.

Cytoxicity Assays

Cells were plated in black 96-well plates at $2x10^3$ cells/well and treated with specified drug concentration 24 hours after plating. Cell viability was analyzed at 24 and 48 hours and assessed using the ATPlite assay system and read on a SPECTRAL Ami imaging system. Percent cell viability was determined by dividing the treated cells' luminescence counts by the corresponding control cell luminescence.

Morphologic Studies

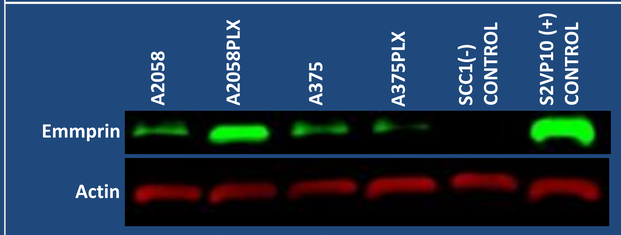
Cells were imaged at 40X after 48 hr treatment in PLX, DMAG, PLX + DMAG, and control. Identification of autophagosomes demonstrates that 17-DMAG kills cells via inhibition of autophagy.

Flow Cytometry

Cells were plated at $5X10^5$ cells/well in 6-well plates. After 24 hours cells were scraped and incubated for 2 hr. S100A9 ligand (probe) was conjugated to a CF-750 NIR dye. Liposomes have an S100A9 are encapsulated with CF-750 dye.

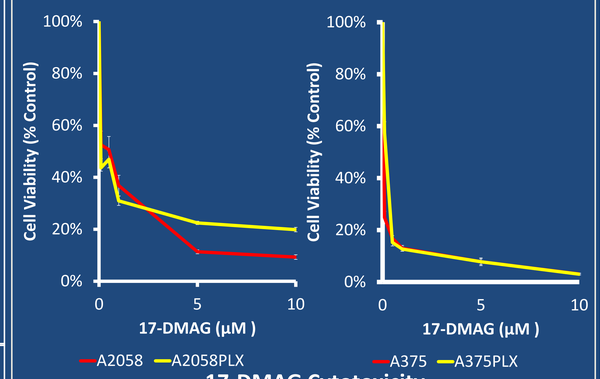
In Vivo Analysis

5 athymic mice received subcutaneous tumors of $5X10^6$ cells/tumor with either the A2058 or A2058PLX cell line. S100A9 ligand probe or liposome was injected at 5 O.D. Accumulation of probe in tumor was evaluated by the region of interest method using SPECTRAL Ami imaging system every 6 hr for 48 hrs.



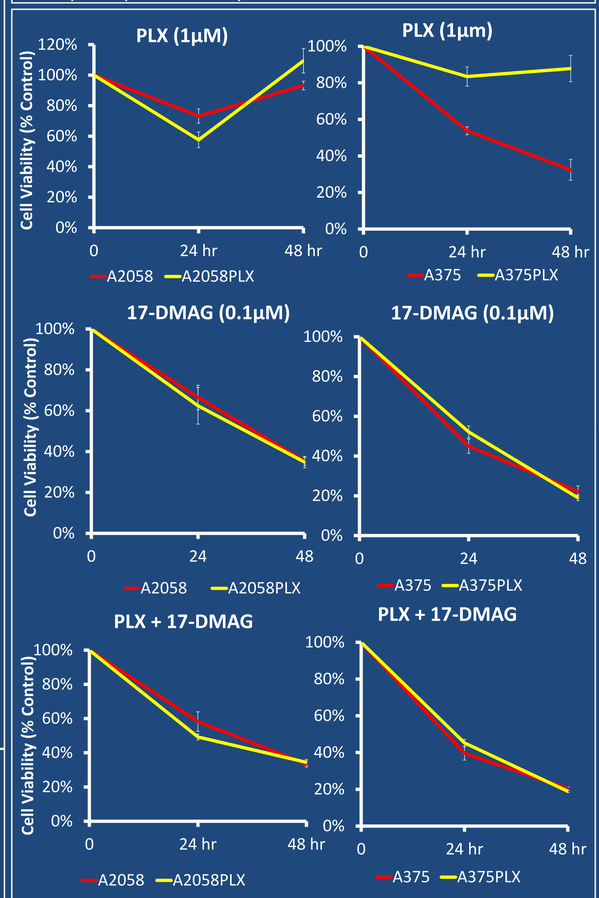
Melanoma Cells Express Emmprin

Western blot analysis confirms Emmprin expression in human melanoma cell lines. Protein quantification demonstrated Emmprin is more highly expressed in PLX-resistant cells.



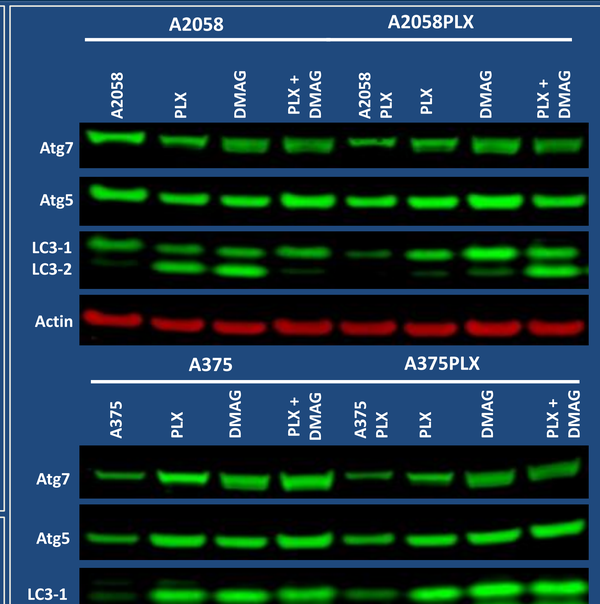
17-DMAG Cytotoxicity

17-DMAG causes equivalent cytotoxicity in both resistant and native melanoma cell lines. Cells were treated for 48 hours with increasing doses of 17-DMAG (0.1, 0.5, 1.0, 5.0, and 10.0 μM) and analyzed by ATPlite assay.



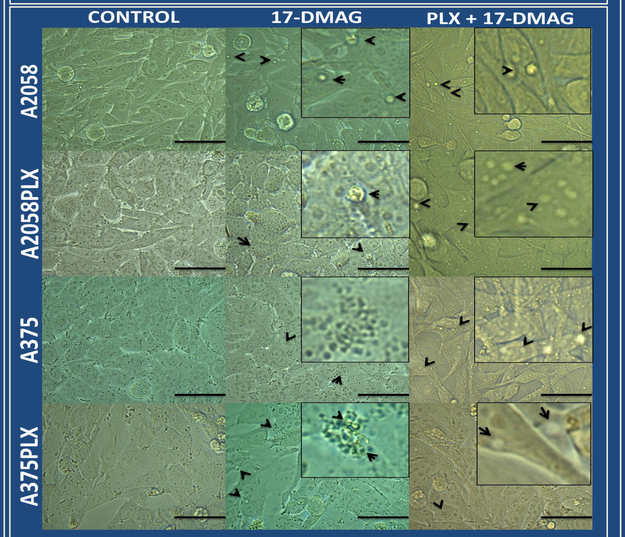
17-DMAG Sensitizes PLX-Resistant Cells

Cell viability was analyzed at 24 and 48 hours for cells treated with 17-DMAG (0.1μM), PLX (1.0 μM), or 17-DMAG (0.1μM) + PLX (1.0 μM). Significant cytotoxicity is achieved in both native and resistant cell lines at therapeutically relevant doses of 0.1μM 17-DMAG and 1.0 μM PLX at 24 and 48 hours *in vitro.*



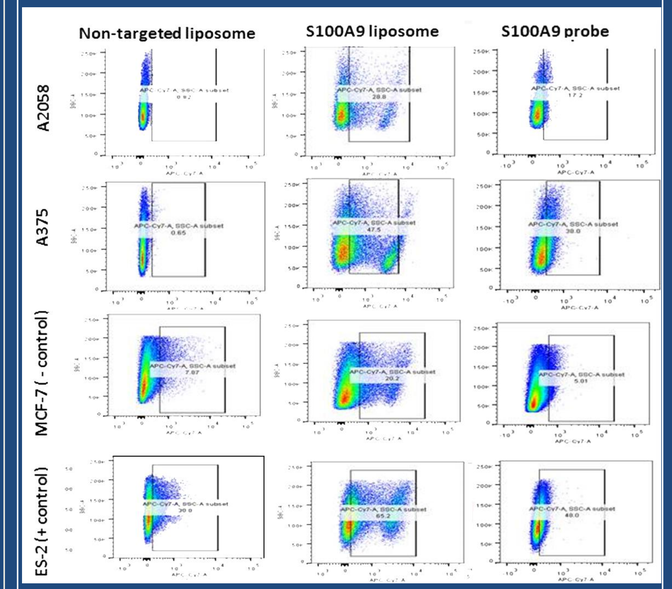
Protein Analysis of Cytoxicity Studies

Western blot analysis suggests that treatment with 17-DMAG (0.1μM) or PLX (1.0μM) + 17-DMAG (0.1μM) causes cytotoxicity via autophagy inhibition by preventing the conversion of autophagosomes to autophagolysosomes, as demonstrated by the increase of LC3-2 expression and in the ratio of LC3-2 to LC3-1. The autophagy regulator proteins Atg 7 and Atg 5 are unaltered by treatment, demonstrating that the early steps of autophagy are unaffected.



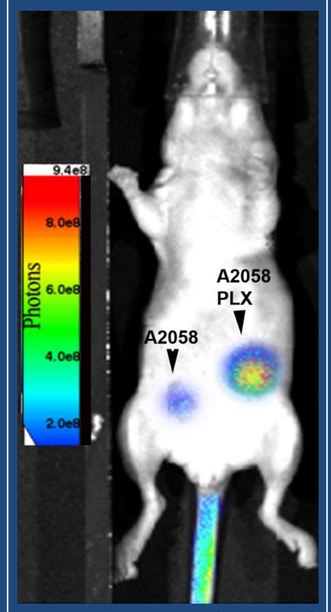
17-DMAG Inhibits Autophagy at the Autophagolysosome

Accumulation of autophagolysosomes (black arrow) indicates inhibition of the final stages of autophagy. Cells were imaged at 40X 48 hours post-treatment. Cells were plated at $5X10^5$ cells/well in 6-well plates. Scale bar equals 50μm.



Flow cytometry confirms *in vitro* binding of S100A9 probe and S100A9 liposome to melanoma cells

Probe binding was similar in both the PLX-sensitive and resistant lines.



***In vivo* Tumor Localization of S100A9-Probe**

Accumulation occurs after 6 hr in subcutaneous melanoma tumors. Mice were given tail vein injections of S100A9 probe at 5 O.D. AMIview images were taken every 6 hr. S100A9 liposome also demonstrates *in vivo* tumor accumulation from 18 hr to 48 hr.

Conclusions

The use of EMMPRIN targeted liposomes via an S100A9 ligand is a novel delivery system which could improve the concentration of drug within a tumor and reduce systemic toxicity. This method may efficaciously treat patients with BRAF-inhibitor resistant metastatic melanoma.

Future Directions

Encapsulate 17-DMAG in S100A9 liposomes to evaluate *in* vivo therapeutic efficacy.

Acknowledgements

This project was supported by grant R25-CA-134283 from the National Cancer Institute.