PADI inhibition overcomes the acquisition of resistance after KRAS blockage in pancreatic ductal adenocarcinoma cells

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INTRODUCTION

In pancreatic ductal adenocarcinoma (PDAC) KRAS activating point mutations are frequently observed. KRAS inhibitors specific for the G12C mutation have been developed to selectively target cancer cells. However, $KRAS^{G12C}$ cells display metabolic adaptations and rapidly acquire resistance to the treatment. Peptidyl Arginine Deiminases (PADIs) catalyze the conversion of peptidyl-arginine into citrulline residues, and thus they can influence the activity of targeted enzymes and the state of the chromatin by histone citrullination. Although there is evidence that PADIs can modulate the activity of enzymes involved in metabolism, the role of these proteins in sustaining cancer cells fitness remains to be clarified. In this project we aim to evaluate the contribution of PADIs in controlling the metabolic resetting of cancer cells that have acquired resistance to KRAS inhibition.

MIA PaCa-2 cells show development of resistance to LC-2 after repeated treatments

MIA PaCa-2 are PDAC cells characterized by biallelic $KRAS^{G12C}$ mutation. The repeated treatment of these cells with the PROTAC LC-2 caused the acquisition of resistance.

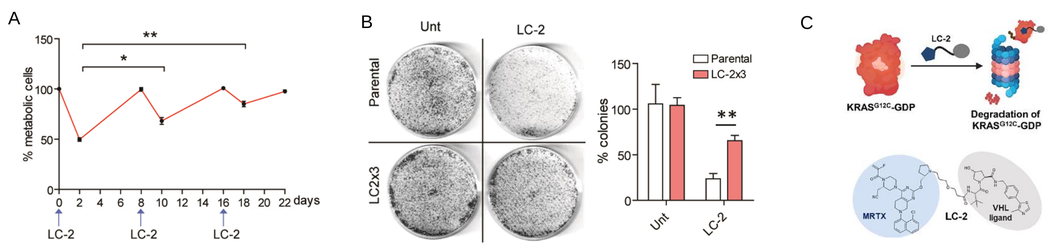

Fig. 1 Multiple administration of LC-2 induces cancer cell resistance: A) Resazurin assay result after the repeated administration of LC-2 in MIA PaCa-2 parental cells; LC-2 was used at IC_{50} concentration (2,5 μM). B) Clonogenic assay performed on parental and on resistant cells (LC-2x3, which were repeatedly treated with LC-2 for at least three times), before and after the administration of LC-2 2,5 μM. C) Structure of the PROteolysis TArgeting Chimera (PROTAC) LC-2: MRTX subunit specifically binds KRAS, while VHL ligand recruits E3 ligase allowing the ubiquitination and subsequent proteasome-mediated degradation of KRAS.

LC-2 and BB-Cl-amidine display a strong synergic anti-neoplastic effect

The combined treatment with LC-2 and BB-Cl-amidine rapidly induced apoptosis in MIA PaCa-2 parental cells, leading to a drastic decrease of ATP level and clonogenic efficacy. Notably, the combination of LC-2 10 μM and BB-Cl 5 μM brought ATP level and colony-forming ability to null levels.

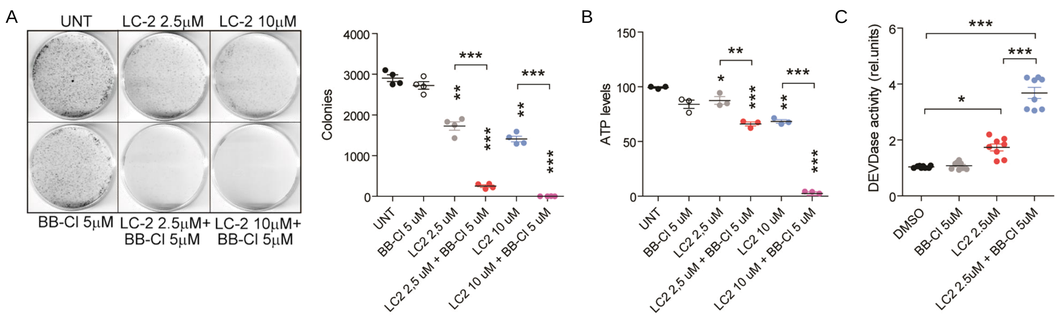

Fig. 3 The co-treatment of MIA PaCa-2 parental cells with LC-2 and BB-Cl-amidine shows a strong synergic anti-neoplastic effect: A) Clonogenic assay performed on parental cells treated as indicated for 30 hours. BB-Cl-amidine was used at IC_{50} concentration (5 μM). B) ATP level in parental cells treated as described in Fig. 3A. C) DEVDase activity, which indicates activation of caspase-3/7, in parental cells treated as indicated for 15 hours.

The depletion of PADIs with a genetic approach is similar to BB-Cl-amidine mediated inhibition

Cells knocked-down for PADI3 via shRNA and treated with siRNA for PADI1 mimicked the effect of BB-Cl-amidine when they were treated with LC-2 10 μM. Importantly, in contrast with transient inhibition of PADIs, the stable downregulation of PADI3 is detrimental for cancer cell fitness. Moreover, the engraftment efficacy of MIA PaCa-2 PADI3 knocked-down cells subcutaneously injected in nude mice was significantly compromised.

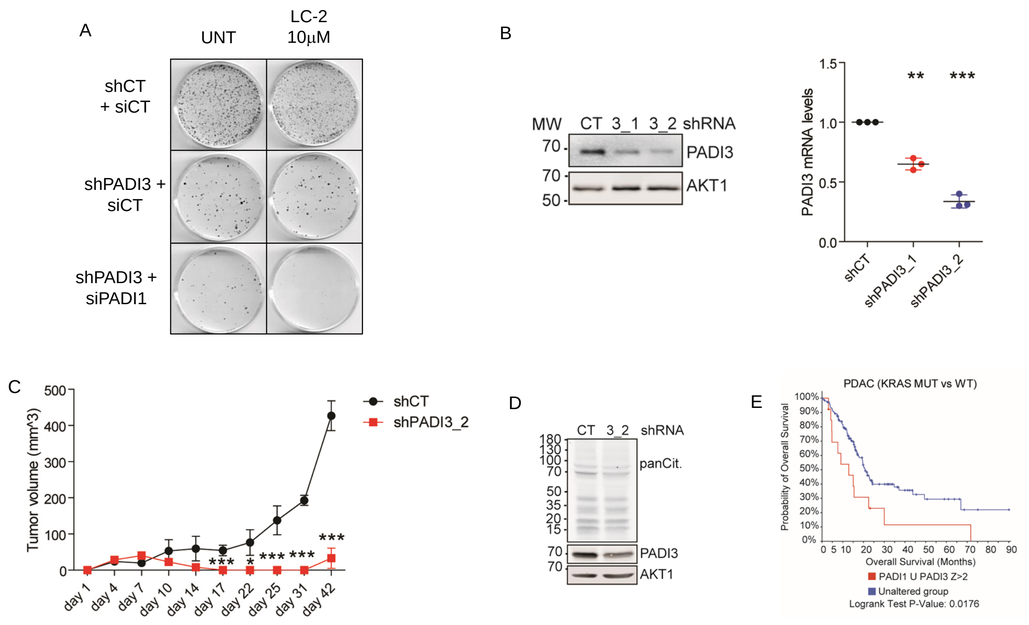

Fig. 5 Analysis of LC-2 effect in PADI-depleted cells: A) Clonogenic assay of MIA PaCa-2 shPADI3 cells transfected with siRNAs for 48 hours and treated with LC-2 10 μM for 24 hours. B) Validation of PADI3 knock-down by Western blot analysis (left) and qPCR (right). C) Growth of tumors generated by subcutaneous injection of 2.5*10^6 MIA PaCa-2 shCT and MIA PaCa-2 shPADI3_2 cells in nude mice (n = 5). At day 42 mice were euthanized and the tumors were collected. D) Western blot analysis of PADI3 and citrullinated proteins in the biggest tumor collected from mice (Anti-peptidyl-citrulline monoclonal antibody MABN328, Sigma-Aldrich). E) Higher levels of PADI1 and PADI3 (above third quartile) are negative prognostic factors for PDAC in TCGA patients.

MIA PaCa-2 cells developing LC-2 resistance become refractory to PADI1 and PADI3 LC-2 induced down-regulation

LC-2 resistance is not driven by the impairment of LC-2 efficacy, because KRAS was still efficiently degraded in resistant cells, and MAPK and AKT pathways were not involved, as they were not differentially regulated between parental and resistant cells. Conversely, in parental cells LC-2 treatment led to PADI1 and PADI3 down-regulation, which was not yet observed in resistant cells. This suggests a possible role of PADIs in the development of resistance.

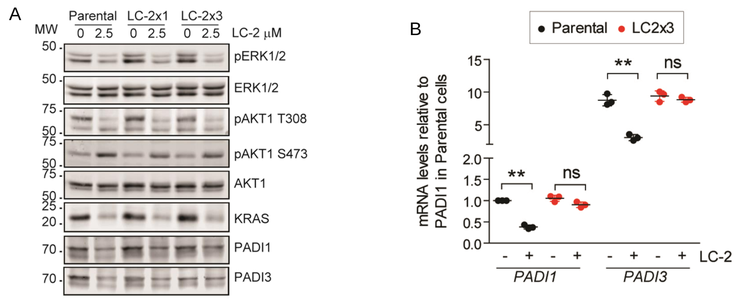

Fig. 2 In MIA PaCa-2 parental and resistant cells the expression of PADIs after LC-2 treatment is differential: A) Western blot analysis of the indicated proteins in parental and resistant cells treated with LC-2 for 30 hours (Anti-PADI1 monoclonal antibody 22997, Anti-PADI3 monoclonal antibody 24377, Cayman Chemical). B) Relative mRNA levels of PADI1 and PADI3 obtained by RNA-seq analysis of parental and resistant cells treated as described in Fig. 2A.

MIA PaCa-2 cells resistant to LC-2 are re-sensitized by BB-Cl-amidine

Although LC-2x3 MIA PaCa-2 cells are resistant to the treatment with LC-2, they are as sensitive as parental cells to the co-treatment with LC-2 and BB-Cl-amidine, suggesting an important role of PADIs in the acquisition of resistance.

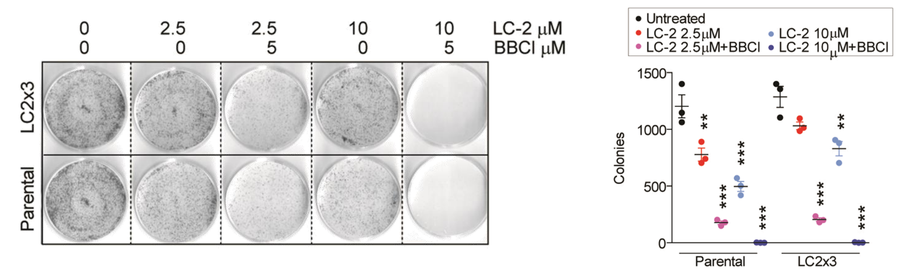

Fig. 4 LC-2 resistant cells are sensitive to the combined treatment with LC-2 and BB-Cl-amidine: Clonogenic assay performed on parental and resistant cells treated as indicated for 30 hours.

MIA PaCa-2 resistant cells upregulate fatty acid metabolism. Enzymes involved in fatty acid beta-oxidation are found enriched in citrullinated proteins enriched fractions.

RNA-seq analysis indicates that cells resistant to KRAS blockage display a metabolic rewiring and upregulate the metabolism of fatty acids. Interestingly, immunoprecipitation of citrullinated proteins reveals that in LC-2 resistant cells there is also an enrichment of citrullinated enzymes involved in fatty acid metabolism.

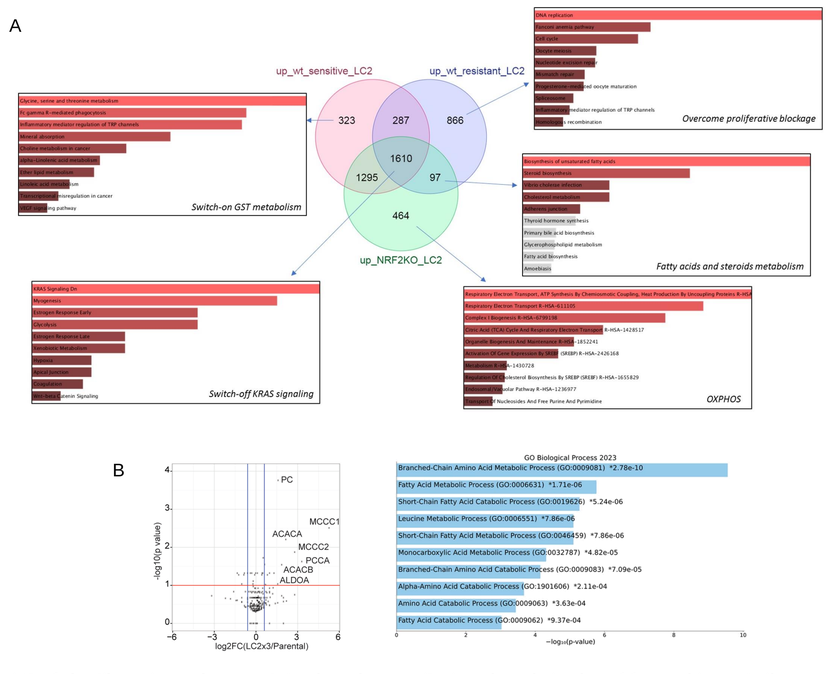

Fig. 6 RNA-seq and IP of citrullinated proteins reveal that in resistant cells there is an induction of fatty acid metabolism and that several enzymes belonging to this pathway are citrullinated: A) RNA-seq analysis of MIA PaCa-2 cells sensitive or resistant to KRAS blockage. Two models of resistance are compared: MIA PaCa-2 cells made resistant by consecutive treatments with LC-2; MIA PaCa-2 cells in which the KRAS-NRF2 axis is disrupted by knocking-out NRF2. B) Immunoprecipitation of citrullinated proteins followed by mass spectrometry analysis in MIA PaCa-2 parental cells and resistant to LC-2 (Anti-Citrulline-Biotin monoclonal antibody SAB5202285, Sigma-Aldrich). Enrichment is calculated by comparing IP efficacy between resistant and sensitive cells. Enrichr (https://maayanlab.cloud/Enrichr) and Appyters were used to compute functional enrichment.

CONCLUSIONS

In PDAC, the blockage of $KRAS^{G12C}$ often leads to resistance of cancer cells, which display metabolic adaptations to overcome the effects of chemotherapy. Our results suggest that PADIs play a fundamental role in the metabolic resetting observed in LC-2 resistant cells. Omics data indicate that the most important pathway involved in LC-2 resistance is fatty acid metabolism, whose main enzymes are both upregulated at RNA level and citrullinated. However, further studies are needed to understand the role of citrullination on the activity of these enzymes. Importantly, these experiments show that a combined approach which targets $KRAS^{G12C}$ and PADIs appears to be promising in PDAC therapy.