TUMOR-STROMA INTERACTIONS INFLUENCE THE RESPONSE TO PI3K TARGETED AGENTS IN PRECLINICAL MODELS OF COLORECTAL CANCER (CRC)

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Abstract:

Background: Identification of the precise mechanisms of drug action in relationship to molecular disease drivers is crucial to the successful development of new therapeutic strategies. Recently highthroughput molecular analysis is being used to identify putative biomarkers to provide personalized cancer therapy. However, such analysis is usually focused on tumor cell-autonomous molecular determinants of sensitivity to drug treatment and overlooks microenvironmental interactions. The aim of the study is to investigate how PTEN expression/function in CRC cells modulates the response to signaling inhibitors in the context of complex microenvironmental interactions.

Methods: Isogenic CRC cell lines (X-MAN™ HCT116 and HCT116 PTEN$^{-/-}$) were treated with MAPKi and PI3K/mTORi alone or in combination, in the presence or absence of stromal fibroblasts or fibroblast/endothelial cell conditioned medium (CM). Cytofluorimetric analysis and Crystal Violet assay were used to analyze functional response to targeted agents; pathway activation and cytokine/chemokine profile were analyzed using Western blot (WB) and ELISA assay respectively.

Results: While response of CRC cells to MEKi is dictated mainly by the tumor genetic background, response to PI3K/mTORi is strongly influenced by CRC/TME interactions. Fibroblast CM selectively increases sensitivity of PTEN-competent HCT116 cells to PI3K/mTOR double inhibitor by modulating PTEN function. Indeed, despite increased levels of total PTEN protein, phosphorylation its C-terminal tail delocalizes PTEN from plasma membrane and makes it inactive, resulting in increased PIP_3 levels. PTEN inactivation, in turn, shifts the balance towards the formation of Raptor-containing mTORC1 complexes, resulting in downstream activation of mTORC1, but not mTORC2. The same results were obtained with different fibroblast and endothelial CM; stromal CM, indeed, upregulate PI3K pathway leading to greater response to PI3K/mTOR double inhibitor. The analysis of cytokine/chemokine production revealed a similar pattern in CM from different stromal cells, however this could be not sufficient to explain the observed response.

Conclusions: Understanding the mechanisms underlying microenvironmental interactions (tumor, stroma, soluble factors) may be of fundamental importance to overcome therapeutic resistance and develop more effective therapies for patients affected by CRC.

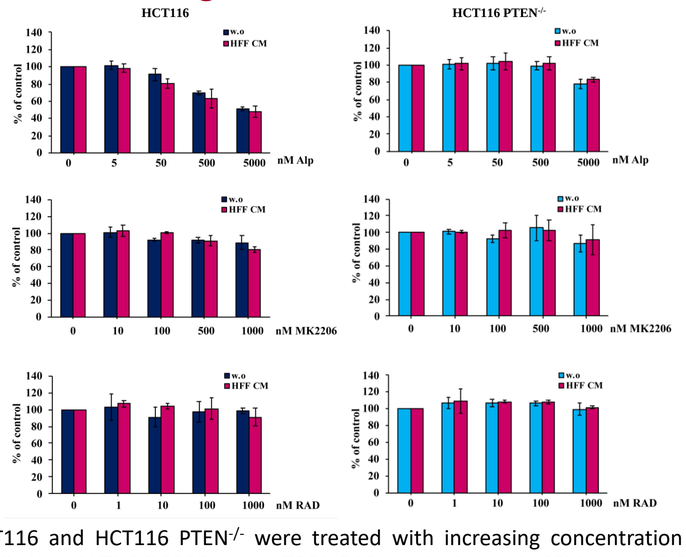
Response to MEKi is dictated by genetic background and response to PI3Ki in dictated by microenvironmental interactions…

Genetic Background	w.o FBS		Direct Co-colture		HFF CM	
Response to MEKi (IC_{50})						
HCT116	< 1		1.03	↓	< 1	↔
HCT116 PTEN$^{-/-}$	68.62		6.35	↑	215.44	↓↓
Response to PI3K/mTORi (IC50)						
HCT116	13.65		83.90	↓	< 1	↑
HCT116 PTEN$^{-/-}$	13.65		322.04	↓↓↓	11.96	↔
Response to combination (CI)						
HCT116	23.089	A	0.001	S	1	A
HCT116 PTEN$^{-/-}$	0.530	S	0.993	A	0.301	S

Legend: A: Additivity/Antagonism **S:** Synergism

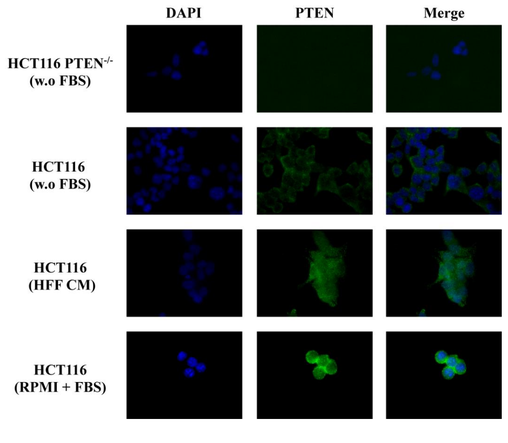
The effects of single drugs and combination treatment were analyzed with half maximal inhibitory concentration (IC_{50}) and Combination Index (CI), respectively. The arrows represent the variation of IC_{50} and CI in direct Co-colture or HFF CM colture with respect to serum-free medium (SFM; w.o FBS). Response to MEKi seems to be independent of microenvironmental interactions, but dependent on the genetic background of tumor cells; on the contrary, response to PI3K/mTOR double inhibitor is mainly influenced by the microenvironment, and in opposite ways with respect to PTEN status. Combination treatment, instead, is synergistic in direct co-culture in HCT116, specular to data obtained in serum-free conditions. A: Additivity/Antagonism S:Synergism

Single step along PI3K pathway was not sufficient to result in drug sensitization with fibroblast CM



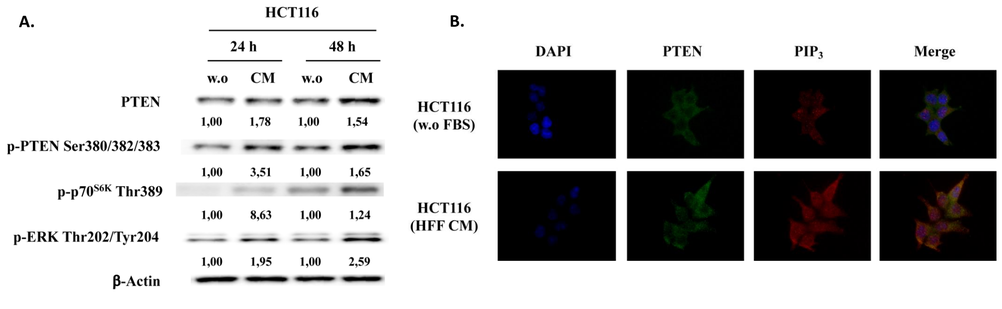
HCT116 and HCT116 PTEN$^{-/-}$ were treated with increasing concentration of PI3Ki (Alp), AKTi (ML2206) and mTORi (RAD), as indicated in SFM and HFF CM. Different from double PI3K/mTOR inhibition, no differences were observed in the response of isogenic CRC cells in presence of CM, as compared to SFM.

Stromal cells upregulates PTEN…



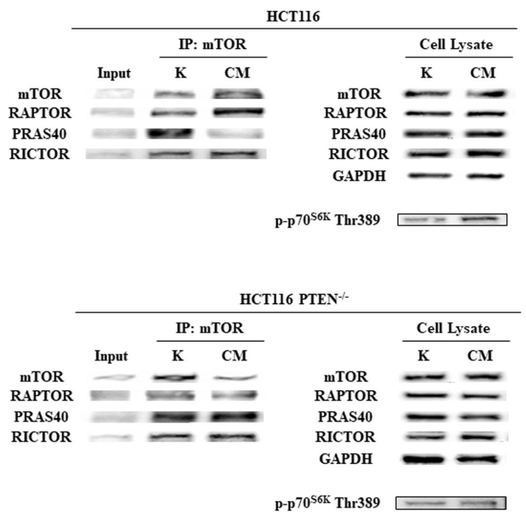
Immunofluorescence assay of PTEN in X-MAN™ isogenic HCT116 cell lines in different cell culture condition.
HCT116 in SFM show PTEN localization near the plasma membrane. HFF CM increases PTEN concentration by showing a spread of the tumor suppressor in the cell.
Immunofluorescence of PTEN in HCT116 PTEN$^{-/-}$ is shown as PTEN negative control.

…but PTEN is retained in the cytosol and inactivated by phosphorylation!!!



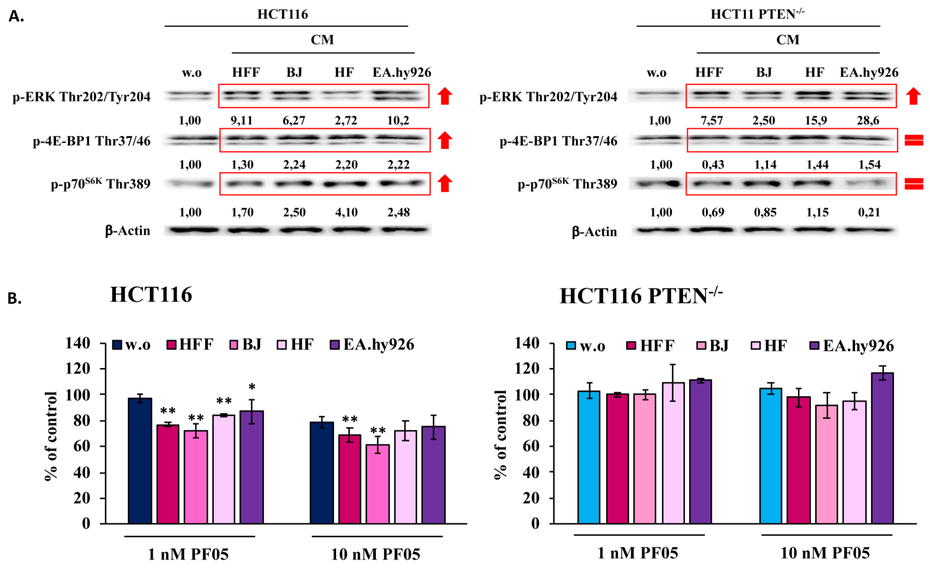
A. HCT116 cell lines were cultured in SFM (w.o); or HFF CM for 24, 48 and 72 hours. Cells were lysed and analyzed by WB using antibodies specific PTEN; p-PTEN (Ser 380/ Thr 382/383); p-p70 (Thr 389) and p-ERK. WB with antibody specific for β-actin are shown as protein loading and blotting control. CM derived from HFF cells make PTEN phosphorylated and less active, despite PTEN total proteins levels is upregulated, as demonstrate the upregulation of p-p70 (Thr 389) until 48 h.
B. Immunofluorescence assay of PTEN in X-MAN™ isogenic HCT116 cell lines in different cell culture condition. PIP_3 upregulation demonstrates PTEN inactivation

Stromal cells activate PI3K/mTOR pathway in PTEN wt context leading to mTORC1, but not mTORC2, complex activation



Immunoprecipitation (IP) studies of mTOR in X-MAN™ isogenic HCT116 cell lines (w or w/o PTEN expression). No differences in association with RAPTOR, PRAS40 or Rictor, while no association of PRAS40 subunit and a striking association with RAPTOR was detected only in PTEN competent CRC HCT116 with HFF CM. The activation of mTORC1 complex results in upregulation of p-p70 visible in cell lysate.

Different stromal cells CM upregulates PI3K pathway and PI3K/mTOR double inhibitor response



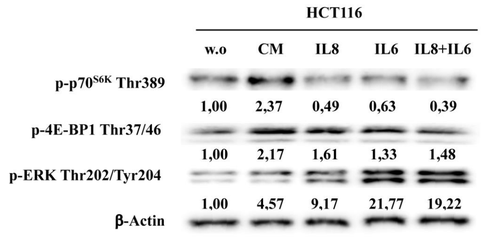
A. Isogenic CRC cells were cultured with SFM and stromal cells CM (fibroblasts: HFF, HF and BJ; endothelial cells: EA.hy926), as indicated, for 24 h. PI3K pathway was upregulated with all stromal cells CM only in PTEN-competent CRC cell line.
B. HCT116 and HCT116 PTEN$^{-/-}$ were exposed to double PI3K/mTOR pathway inhibitor, as indicated, for 72 h in SFM and stromal cells CM. CM selectively increased sensitivity to PI3K/mTORi in HCT116 cell line. In particular fibroblast derived CM had greater influence on response to PI3K/mTORi, as compared to endothelial cell-derived CM.

Analysis of cytokine/chemoline production by stromal cells reveals similar patterns of expression

HFF	BJ	HF	EA.hy 926
Angiogenin	Angiogenin	Angiogenin	Angiogenin
Angiopoietin-1	Angiopoietin-1	Angiopoietin-1	
DKK-1	DKK-1	DKK-1	DKK-1
Emmprin	Emmprin	Emmprin	Emmprin
Endoglin	Endoglin	Endoglin	Endoglin
FGF-19	FGF-19		FGF-19
			GDF-15
			IGFBP-2
IL-6	IL-6	IL-6	IL-6
IL-8	IL-8	IL-8	IL-8
			IL-17A
			Lipocain
MCP-1	MCP-1	MCP-1	
MIF	MIF	MIF	MIF
Osteopontin	Osteopontin	Osteopontin	Osteopontin
			PDGF-AA
Pentatraxin-3	Pentatraxin-3	Pentatraxin-3	
SDF-1	SDF-1	SDF-1	
Thrombospondin-1	Thrombospondin-1	Thrombospondin-1	Thrombospondin-1
UPAR	UPAR	UPAR	UPAR
	VEGF		VEGF

CM from different fibroblast (HFF, HF and BJ) and endothelial cells (EA.hy926) were analyzed by Proteome Profiler Array after 24 hours of culture in SFM. Similar pattern of cyto/chemokines were predicted, some of them are commonly produced by both fibroblast and endothelial cells (e.g. IL-8, IL-6, UPAR), while other were relatively specific for fibroblasts (e.g. MCP-1, SDF-1)

IL-8 and IL-6 do not mediate mTORC1 activation in the HFF CM



HCT116 cells were cultured with SFM, HFF CM, and SFM with recombinant Human protein of IL-8,IL-6 and the combination of two, as indicated, for 24 h. Both cytokines stimulate, at least in a small part, p-4EBP1, while p-p70 was selectively induced by HFF CM. IL-8 and IL-6 were produced, albeit at variable levels, by all fibroblast cells, but were enable to mediate mTORC1 activation in PTEN-competent CRC cells.

Conclusion:

- Sensitivity to MAPKi is dictated mostly by the tumor genetic background(PTEN status) while sensitivity to PI3K/mTOR inhibition and pharmacological interactions with combined MAPK/PI3K inhibition is strongly influenced by interaction with the TME
- In PTEN-competent cells soluble factors released by stromal elements paradoxically impair PTEN function, leading to downstream mTORC1 complex formation and pathway activation
- Paradoxical mTORC1 activation upon exposure to stroma-derived soluble factors results in functional hypersensitivity of PTEN CRC cells to the growth inhibitory effects of double PI3K/mTOR inhibitors

Future prospects:

- Isolate autologous fibroblasts and cancer cells from patients affected by CRC at different stages.
- Evaluate the putative role of miRNAs in stromal CM. miRNA analysis will be carried out also through the isolation of exoRNA. Indeed, exosomes are cell derived vesicles abundant in biological fluids.

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