Integrated omics-based approach to the identification of specific expression profiles associated with PTEN-loss

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Abstract

Introduction PTEN is a protein that restrains cells from growing unchecked; when it is absent or not functioning (as it often happens in prostate and colorectal cancer - CRC), cancer develops more easily and grows in a more aggressive and therapy-resistant fashion. However, just measuring PTEN mutational status or gene/protein levels in clinical samples may not be sufficient to assess its function, as its regulation is complex. Therefore, we analyzed miRNA, transcriptomic, and proteomic profiles of isogenic CRC models differing for PTEN status, in order to identify a set of genes or proteins that are stably modulated by the presence or absence of a functional PTEN.

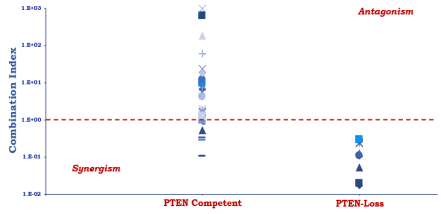
Background PTEN is a protein that restrains cells from growing unchecked; when it is absent or not functioning (as it often happens in prostate and colorectal cancer - CRC), cancer develops more easily and grows in a more aggressive and therapy-resistant fashion. However, just measuring PTEN mutational status or gene/protein levels in clinical samples may not be sufficient to assess its function, as its regulation is complex. Therefore, we analyzed miRNA, transcriptomic, and proteomic profiles of isogenic CRC models differing for PTEN status, in order to identify a set of genes or proteins that are stably modulated by the presence or absence of a functional PTEN.

Methods To identify biomarkers of PTEN loss of function and functional downstream effectors of PTEN activity, the isogenic CRC cell lines HCT116 (PTEN+/+) and HCT116 PTEN-/-(Horizon Discovery Ltd) were subjected to comprehensive miRNA profiling (Human miRNA Microarray Release 21, Agilent), transcriptomic analysis (NextSeq 500, Illumina), and semi-quantitative phosphoproteomic analysis (Antibody Microarray Service, KinexTM).

Results miRNA profiling found 18 miRNAs differentially expressed in HCT116 PTEN+/+ and their HCT116 PTEN-/- counterpart: only miR-196a-5p was up-regulated in HCT116 PTEN-/-, whereas the other 17 miRNAs were all down-regulated; several of these target miRNAs have been validated by qRT-PCR. RNA-Seq data were analyzed via the RAP pipeline available at CINECA that relies on the Tuxedo suite (Tophat-Cufflinks-Cuffdiff) for read mapping, transcript abundance estimation and transcript or gene-based differential expression. RNA-Seq analysis showed 247 differentially expressed genes in HCT116 PTEN+/+ cells as compared to HCT116 PTEN-/- (159 up-regulated and 88 down-regulated in HCT116 PTEN-/-) and clustering into 9 subgroups (including: tissue morphogenesis, negative regulation of phosphatase metabolic process, negative regulation of hydrolase activity, regulation of sequence-specific DNA binding transcription factor activity). qRT-PCR assay of 10 dysregulated genes was performed to validate the RNA-Seq data set. Proteomic analysis showed that 92 proteins (25 total proteins and 67 phospho-proteins) were differentially expressed in HCT116 PTEN+/+ and their HCT116 PTEN-/- counterpart, some of which have been validated by western blot.

Conclusions Our data identify differentially expressed sets of miRNA/genes/proteins between HCT116 PTEN+/+ and HCT116 PTEN-/-. Bioinformatics analysis to cluster these sets into functional PTEN-loss "signatures" is ongoing.

PTEN loss is a crucial determinant of synergism between MEK and PI3K/mTOR inhibitors

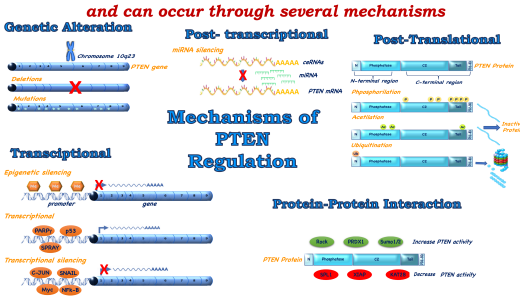


Growth inhibitory interactions between trametinib and getadolisib were assessed in a panel of **32 tumor cell lines** using a fixed-ratio (1:1) with a wide range of concentrations (0.1–1000 nM). Viability was then assessed after 72 h by Crystal violet assay and pharmacologic interactions were evaluated using the Calcusyn software. By this method, an average combination index at the ED_{50}, ED_{75}, and ED_{90} < 1 indicates synergism, = 1 indicates additivity, and > 1 indicates antagonism.

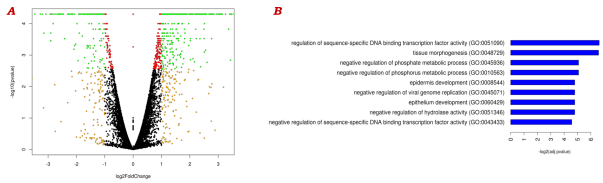
Measuring *PTEN* mutational status or gene/protein levels in clinical samples maybe cumbersome and difficult to obtain, because it may be not sufficient to represent whether PTEN is present or not, due to its complex regulation.

For this reason we are proposing to identify "***PTEN-loss of function signature***", a set of genes or proteins that are stably modulated by the presence or absence of a functional PTEN.

PTEN inactivation is a frequent event in many cancer types and can occur through several mechanisms

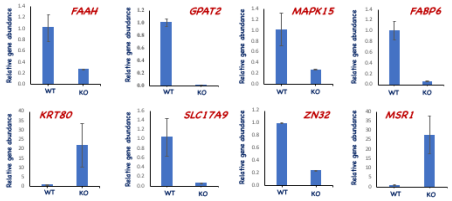


RNA-Seq analysis showed 247 differentially expressed genes in HCT116 PTEN-/- cells as compared to HCT116 PTEN+/+



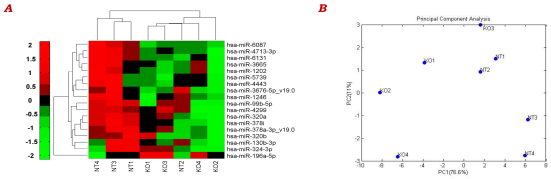
A. Volcano Plot of gene-based differential expression for all Cuffdiff-assembled gene comparisons. Green dots represent genes both significantly (qvalue < 0.01) and strongly (abs(log2fc) > 1) modulated. **B.** Biological process enrichment for PTEN-/- regulated genes.
RNA-Seq analysis showed **247 differentially expressed genes** in parental HCT116 cells as compared to HCT116 PTEN-/-: **159 up-regulated** and **88 down-regulated** in HCT116 PTEN-/- and clustered into 9 subgroups.

Validation of RNA-Seq analysis in HCT116 PTEN+/+ (WT) cells as compared to HCT116 PTEN-/- (KO)



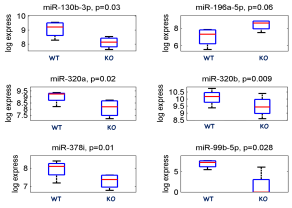
The mRNA was detected by quantitative real-time PCR in HCT116+/+ and HCT116 PTEN-/- cells. Results represent the average±SEM of three independent experiments and are expressed as mRNA abundance relative to control (WT).

Analysis of miRNA in HCT116 PTEN+/+ (NT) and HCT116 PTEN-/- (KO)



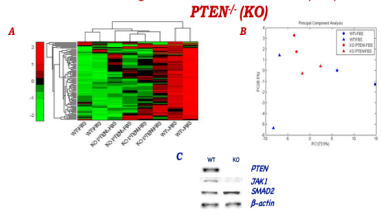
A. Hierarchical Cluster of all modulated miRNA in biological quadruplicates miRNA profiling. **18 miRNAs** are differentially expressed in HCT116 (PTEN+/+) and their HCT116 PTEN-/- counterpart. Paired-Student's test and a permutation test were used to identify potential deregulated miRNAs **B.** Unsupervised Principal Component Analysis of miRNA modulated in HCT116 (PTEN+/+) and and their HCT116 PTEN-/-.

Validation of miRNA profiling differentially expressed in HCT116 PTEN+/+ (WT) and HCT116 PTEN-/- (KO)



The presence of miR-130b, miR-196a-5p miR-320a, miR-320b, miR-378i, miR-99a-5p was detected by real-time PCR in HCT116 and HCT116 PTEN-/- cells. Among them, **one miRNA** (miR-196a-5p) is up-regulated in HCT116 PTEN-/-,as compared to HCT116 PTEN+/+, whereas the other **5 miRNAs** are all down-regulated.

Proteomic Analysis in HCT116 PTEN+/+ (WT) and HCT116 PTEN-/- (KO)



A. Hierarchical Cluster of all deregulated proteins, differentially expressed in HCT116 PTEN+/+ and their HCT116 PTEN-/- counterpart. **B.** Unsupervised Principal Component Analysis of protein modulated in HCT116 PTEN+/+ and their HCT116 PTEN-/-. Proteomic analysis shows that **92 proteins** are differentially expressed in HCT116 (PTEN+/+) and their HCT116 PTEN-/- counterpart: **25 total proteins** and **67 phospho-proteins**. **C.** Isogenic HCT116 cell lines was lysed and analyzed by western blotting using antibodies specific of the indicated proteins. Western blot with antibodies specific for β- actin are shown as protein loading and blotting control.

Material and Methods

- For RNA-Seq, total RNA was isolated using a suitable kit. RNA libraries for sequencing were generated according to the total stranded Illumina TruSeq RNA samples preparation protocol and only the adapter-ligated fragments that are appropriate for sequencing were quantified. Sequencing was performed on NextSeq 500 (Illumina). RNA-Seq data were analysed via the RAP pipeline available at CINECA (8) that relies on the Tuxedo suite (Tophat-Cufflinks-Cuffdiff) (9) for read mapping, transcript abundance estimation and transcript or gene-based differential expression. Cuffdiff output was filtered by taking into account only significant comparisons (adjusted p-value < 0.05).
- For microRNA profiling analysis, total RNA was labelled and hybridized to Human miRNA Microarray Release 21 (Agilent) containing probes for 2549 human microRNAs. Scanning and image analysis was performed using the Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System with miRNA Complete Labelling and Hyb Kit Protocol manual. Signals from miRNA arrays were verified for quality control and extracted by Agilent Feature Extraction 10.7.3.1 software. All values lower than 1 were considered below detection and the threshold was fixed at 1. The arrays were normalized dividing by the median intensity. All data were log2-transformed and entirely processed by MATLAB (The MathWorks Inc.) in house-built routines. Paired-Student's test and a permutation test were used to identify potential deregulated miRNAs.
- The commercial KinexTMAntibody Microarray Service was used for phosphoproteomic analysis. In particular the KAM-900P antibody microarray tracks 613 phosphosite-specific antibodies (for phosphorylation) and 265-pan-specific antibodies (for expression levels of total proteins) in duplicate measurements. The analysis is based on the average of the individual signals for each antibody spot (n=4) for each sample, standard deviations, and p-values, using a 2-tailed, paired Student T test. Standard deviations and the percentage change from controls (%CFC) between HCT116 (PTEN+/+) and HCT116 PTEN-/- were calculated; at least one of the samples had to have an Averaged Globally Normalised Intensity value that exceeded 1000.Proteins, that will be validated in this project by immunoblotting analysis, were classified into: "priority" with %CFC ≥ 60, p value ≤0.07, Globally Normalized intensity value ≥1500 and "possible" with %CFC ≥ 50, p value ≤0.05 and Globally Normalized intensity value ≥1000.

Conclusions

Completion of the proposed research activities will lead to:

- the validation of functional PTEN-loss signature(s) as prognostic biomarkers in CRC;
- the identification of novel molecular targets that may be exploited therapeutically in PTEN-insufficient tumors and potential biomarkers to prospectively select groups of patients at the highest chance of benefiting from specific therapeutic interventions for future clinical trials;
- the identification of genetic aberrations that are "functionally equivalent" to PTEN-loss, thereby providing novel potential prognostic and predictive markers. This, in turn, should pave the way for successful clinical trials and a more rational usage of both human and economical resources.

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