REGULATION OF INTERLEUKIN (IL)-8 PRODUCTION IN COLORECTAL CANCER (CRC): INFLUENCE OF THE TUMOR GENETIC BACKGROUND

15

F. Conciatori[1], C. Bazzichetto[1,2], I. Sperduti[3], C. A. Amoreo[4], M. G. Diodoro[4], I. Falcone[1], F. Cognetti[1], M. Milella[1], L. Ciuffreda[1]

[1] Medical Oncology 1, IRCCS Regina Elena National Cancer Institute, Rome, Italy; [2] University of Rome "La Sapienza", Rome, Italy; [3] Bio-Statistics, IRCCS Regina Elena National Cancer Institute, Rome, Italy; [4] Pathology, IRCCS Regina Elena National Cancer Institute, Rome, Italy.

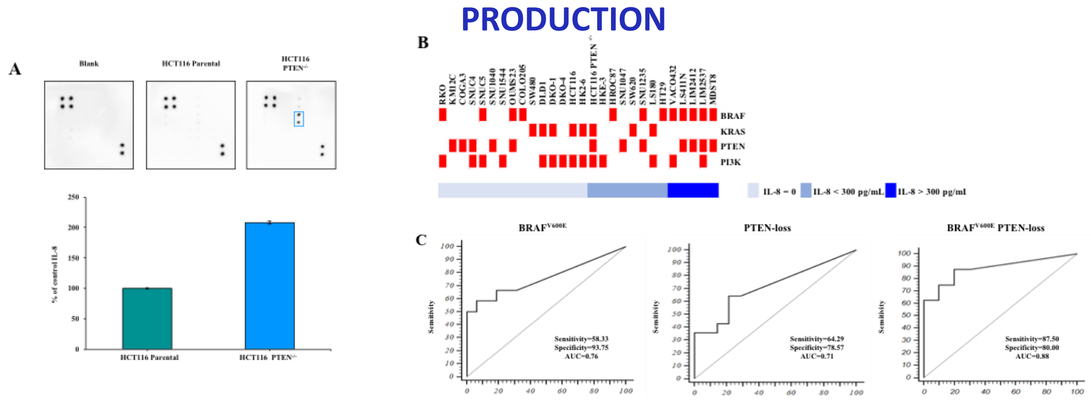
Background: Mutational status in CRC is a strong predictor for overall survival; furthermore, increasing evidence shows that the genetic background of tumor cells influences not only the response to the targeted therapy, but also the surrounding tumor microenvironment (TME) and tumor-stroma interactions (TSI), thus increasing tumor progression and cancer cells' drug resistance. In a panel of CRC cell lines, we recently demonstrated that a ROC curve-based prediction algorithm based on BRAF^{V600E} and PTEN-loss has 68% accuracy in predicting high levels of IL-8 production (p=0.002); moreover, IL-8 production was mostly regulated through activation of the MAPK, rather than the PI3K/AKT/mTOR, pathway.

Methods: Production of IL-8, IL-6 and vascular endothelial growth factor (VEGF) was determined by ELISA assay under standardized culture conditions in a panel of 28 CRC cell lines with different genetic backgrounds (e.g. KRAS, BRAF, PI3K, and PTEN loss). Modulation of cytokine production after exposure to selective inhibitors of the MAPK and PI3K pathways was assessed, by both ELISA assay and real time-PCR. BRAF, MEK1, ERK1 and ERK2 expression were modulated using siRNAs specifically targeting these genes. CRC tissue microarrays (TMA) were used for IHC analysis of IL-8 and PTEN protein expression; analytical methods were standardized to ensure reproducibility across different CRC case series.

Results: We previously demonstrated that CRC cell lines harboring both BRAF^{V600E} and PTEN-loss expressed the highest levels of IL-8, but not of VEGF and IL-6. In order to verify that data gathered *in vitro* are actually predictive of the *in vivo* clinical situation, we have analyzed IL-8 production by either cancer cells or the immune infiltrate in a case series of CRC patients characterized for a panel of relevant genes/proteins. Preliminary results show that PTEN-loss portends high IL-8 expression by cancer cells and low expression by infiltrating immune cells and *viceversa* in PTEN-competent CRC (p=0.006). IL-8 is tightly and transcriptionally controlled by activation of the MEK/ERK pathway, as the MEK inhibitor trametinib downregulated ERK Thr202/Tyr204 phosphorylation and CHOP expression and profoundly suppressed IL-8 production regardless of the genetic background; conversely, the selective BRAF inhibitor dabrafenib downregulated IL-8 only in BRAF^{V600E} contexts, but upregulated its production in parallel with ERK Thr202/Tyr204 and CHOP expression in BRAFwt CRC cells. PI3K/mTOR inhibitors did not significantly modulate IL-8 production, confirming that in CRC IL-8 release is downstream the BRAF/MEK/ERK pathway. In order to better investigate the role of the MAPK pathway in IL-8 regulation, components of the MAPK-signaling pathways (BRAF, MEK, ERK1 and ERK2) were silenced by siRNA: IL-8 production was significantly downregulated after BRAF, MEK, or ERK2 silencing, regardless of the BRAF mutational status; conversely, no effects on IL-8 expression were observed after ERK1 silencing.

Conclusions: Understanding the regulation of IL-8 production, according to the genetic background of tumor cells, and function in the context of TSI may lead to novel therapeutic strategy to restrain CRC progression, to predict pharmacological responses and to potentially determine overall patient benefit. A meta-analysis of published clinical data on the impact of IL-8 production on CRC outcome is currently ongoing.

BRAF^{V600E} AND PTEN-LOSS ARE SIGNIFICANTLY ASSOCIATED WITH IL-8 PRODUCTION

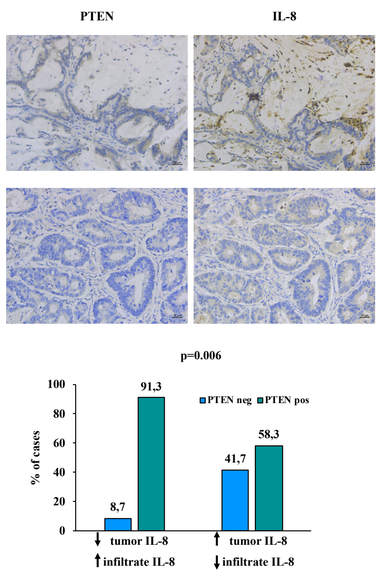


A. The different expression of cytokine was assessed by Human Angiogenesis Antibody Array (RayBiotech, Inc.): membranes were incubated with serum-free medium (Blank) or with cell culture media of HCT116 Parental and the isogenic HCT116 (PTEN$^{-/-}$) and then analyzed according to the manufacture's protocol. Specific IL-8 expression of the isogenic model was evaluated after 24h of serum-free medium, by Enzo Life IL-8 Elisa Kit. Results represent the average of three independent experiments; error bars indicate the standard deviation. Isogenic cell line was generated by Horizon from homozygous knock-out of PTEN by deleting exon 5, which encodes the active site of the protein in the colorectal cancer cell line HCT116 (Horizon Discovery).

B. 28 CRC cell lines were analyzed for their relative IL-8 expression (shade of blue) and their genetic background of BRAF, KRAS and PI3K or PTEN lack of protein expression (reported in red). All cell lines were plated to have about 1x10^6 cells after 48 hours of plating; after 24 hours from plating, culture medium was replaced by serum-free medium, and after 24 hours media were collected and cells were counted.

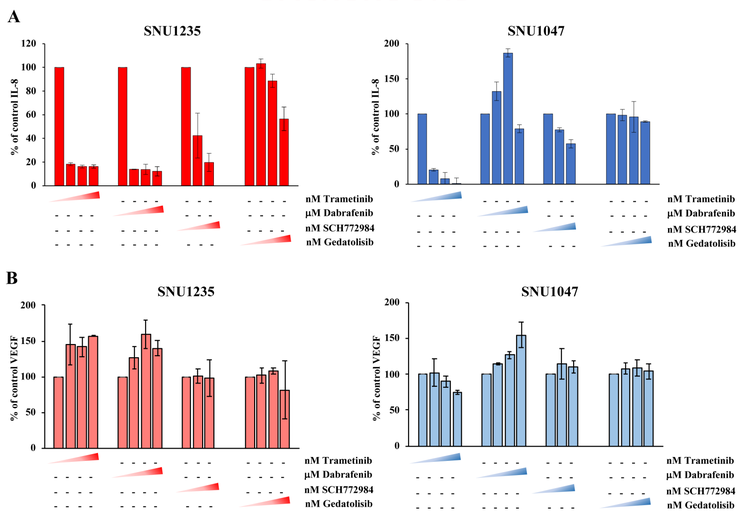
C. Statistical analysis of IL-8 production in a panel of 28 CRC cell lines showed a significant correlation between IL-8 production and either BRAF mutations (p=0.004) or PTEN-loss (p=0.05); the highest IL-8 levels were observed in cell lines carrying both BRAF mutations and PTEN-loss. Combined BRAF/PTEN analysis predicted IL-8 levels>46 pg/mL with high sensitivity and specificity (AUC=0.88) and the ROC-based prediction algorithm had 68% accuracy in predicting IL-8 production (p=0.002).

CORRELATION BETWEEN PTEN AND IL-8 IN CRC TISSUE MICROARRAY



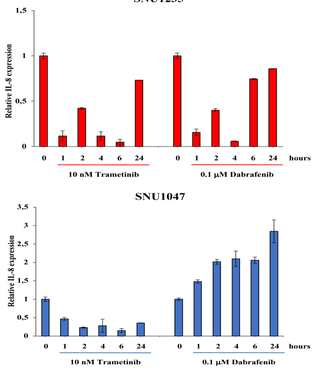
Correlations established *in vitro* are now being validated in a series of 184 clinically annotated primary/metastatic CRC samples, analyzed for PTEN and IL-8 expression by immunohistochemistry. Images were obtained at 20x magnification using a light microscope equipped with a software able to capture images (DM2000 LED,Leica) **(A)**. Preliminary statistical results show that PTEN-loss portends high IL-8 expression by cancer cells and low expression by infiltrating immune cells and *viceversa* in PTEN-competent CRC (p=0.006) **(B)**.

MEK-/BRAF-INHIBITORS REGULATE IL-8, BUT NOT VEGF, EXPRESSION, ACCORDING TO THE GENETIC BACKGROUND



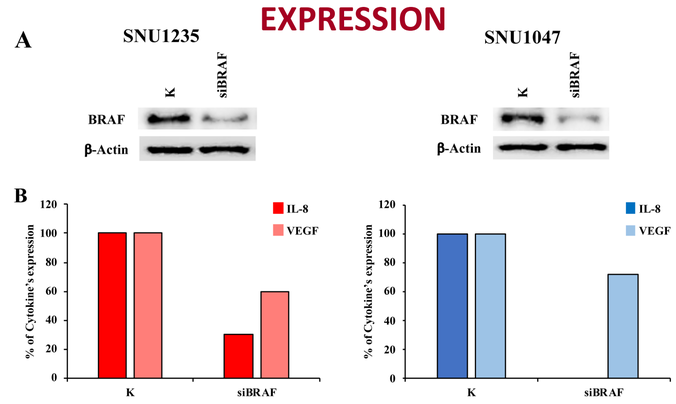
Results represent the effects of increasing concentration of the indicated drugs on IL-8 (A) and VEGF (B) protein expression in BRAF^{V600E} (left panel) and BRAF-wt (right panel) contexts. Cells were treated with increasing concentrations of trametinib (MEK inhibitor), dabrafenib (BRAF inhibitor), SCH772984 (ERK1/2 inhibitor) and gedatolisib (PI3K/mTOR inhibitor) for 24h, as indicated. IL-8 **(A)** and VEGF **(B)** were measured after 24h of treatment, using Enzo Life IL-8 and R&D VEGF ELISA, respectively. IL-8/VEGF levels were measured as pg/mL and results are expressed as % of untreated control levels. Results represent the average of three independent experiments.

MEK-/BRAF-INHIBITORS TRASCRIPTIONALLY REGULATE IL-8 EXPRESSION



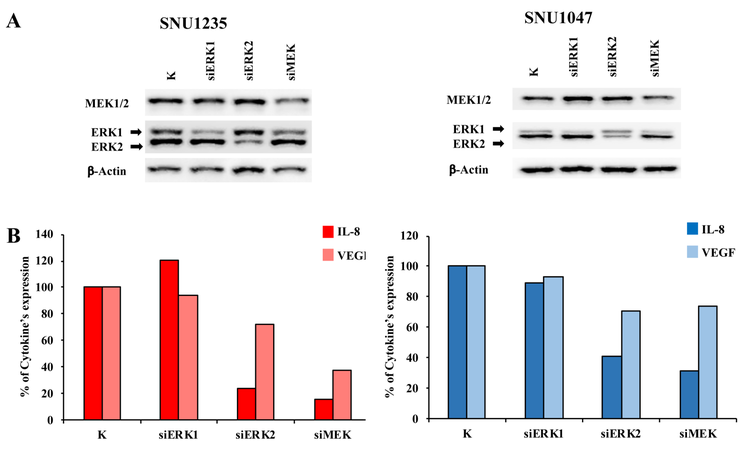
Results represent the effects of increasing concentration of the indicated drugs on IL-8 mRNA expression in BRAF^{V600E} (left panel) and BRAF-wt (right panel) contexts. Cells were treated with the indicated concentration of drugs, for 1, 2, 4 and 6 hours. The presence of IL-8 was detected by real-time PCR in all cell lines. Results were evaluated as ΔΔct of IL-8 tested relative to RPL19 and expressed as the ratio assuming the levels in the control 0 h as 1.0. Results are representative of an experiment of three independent experiments.

BRAF-SILENCING DOWNREGULATES IL-8 EXPRESSION



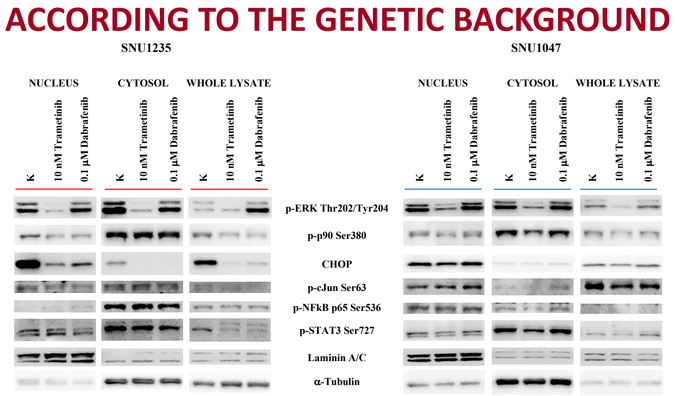
Results represent the effects of BRAF silencing on IL-8 and VEGF protein expression in BRAF^{V600E} (left panel) and BRAF-wt (right panel) contexts. BRAF was knocked down by transient transfection of RNA interference for 24h, according to the manufacture's protocol. **(A)** Molecular effect on BRAF expression was analyzed by Western Blotting using specific antibodies (β-Actin is shown as protein loading and blotting control). **(B)** IL-8 and VEGF were measured after 24h of treatment, using Enzo Life IL-8 and R&D VEGF ELISA, respectively. IL-8/VEGF levels were measured as pg/mL and results are expressed as % of untreated control levels. Results are representative of an experiment of three independent experiments.

ERK2-, BUT NOT ERK1-SILENCING DOWNREGULATES IL-8 EXPRESSION



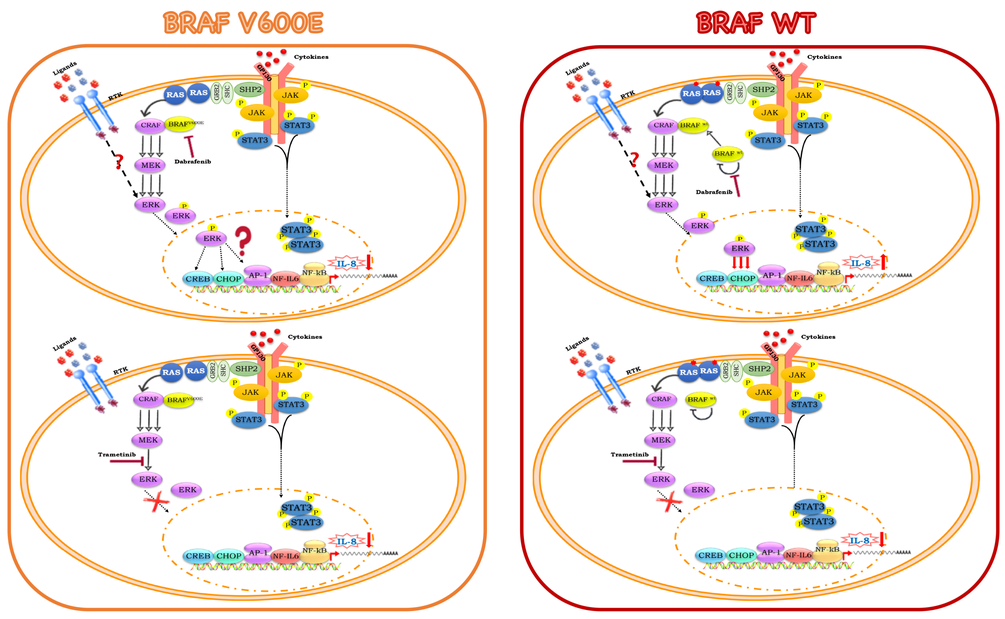
Results represent the effects of ERK1, ERK2, MEK silencing on IL-8 and VEGF protein expression in BRAF^{V600E} (left panel) and BRAF-wt (right panel) contexts. ERK1, ERK2, MEK were knocked down by transient transfection of RNA interference for 24h, according to the manufacture's protocol. **(A)** Molecular effect on BRAF expression was analyzed by Western Blotting using specific antibodies (β-Actin is shown as protein loading and blotting control). **(B)** IL-8 and VEGF were measured after 24h of treatment, using Enzo Life IL-8 and R&D VEGF ELISA, respectively. IL-8/VEGF levels were measured as pg/mL and results are expressed as % of untreated control levels. Results are representative of an experiment of three independent experiments

DIFFERENT TRANSCRIPTIONAL REGULATORS COULD BE INVOLVED IN IL-8 REGULATION, ACCORDING TO THE GENETIC BACKGROUND



Cells were treated with trametinib (MEK inhibitor) and dabrafenib (BRAF inhibitor) for 24h, as indicated. Cytoplasmic and nuclear fractions and whole lysate of cell lines were isolate. Molecular effects was analyzed by Western Blotting using specific antibodies (Laminin A/C and α-Tubulin are shown as protein loading and blotting control).

…WORKING HYPOTHESIS!



After dabrafenib treatment, signaling output from BRAF^{V600E} is blocked and there is a transient suppression of ERK activation and MAPK signaling; however, EGFR and other RTKs drive feedback activation of RAS and CRAF, leading to reactivation of ERK and restoration of MAPK signaling. In this genetic context, other transcriptional factors, such as CHOP could regulate IL-8 transcription, even in presence of p-ERK in the nucleus. On the contrary, after dabrafenib treatment, in BRAF-wt contexts BRAF forms a complex with CRAF, thereby driving paradoxical hyper-activation of both MEK and ERK. p-ERK translocates in the nucleus and IL-8 expression is upregulated (top panels).

Trametinib inhibits MEK activity, thus in turn downregulating p-ERK levels: ERK remains in the cytoplasmic compartment and IL-8 gene is not transcribed, regardless of the genetic status of BRAF (bottom panels).