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5-Azacytidine treatment determines the epigenetic reprogramming of lung cancer models followed by a significant anti-cancer activity

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Introduction

At worldwide level lung cancer remains the leading malignant pathology in terms of incidence (11.6% of the total diagnosed cases for both sexes combined), but also for mortality rates (18.4% of the total mortality cases for both sexes combined) (Global cancer statistics, 2018). Although, the American Cancer Society recommends intensive screening programs in order to diagnose the malignancy in early stages and obtain a good treatment evolution, the overall survival is still at 19% for both sexes and pathological subtypes combined (American Cancer Society, 2020). Even the ones diagnosed in their early stages are prone to develop acquired resistance to the treatment (Holohan et al., Nat Rev Cancer, 2013).

The installation and development of lung cancer brings together **genetic**, **epigenetic** and **environmental factor**s that contribute to installation of aberrant signaling networks with overexpressed oncogenes and downregulated tumor suppressor ones combined with mutated genes with modified/silenced function. The discovery of such aberrations has translated in numerous funds invested in development of targeted therapeutic agents that match such molecular alternations at gene or protein level (e.g. EGFR, ALK, BRAF, RET, MET inhibitors) (American Cancer Society, 2020). However, the main issue is that **50%** of Non-Small Cell Lung Cancer (NSCLC) cases (NSCLC accounts for 80% of lung cancer cases) **have no identified targetable mutations** and, in the case of those who are positive for specific mutations, the **development of therapy resistance** intervenes most of the times (Ansari et al., Transl Lung Cancer Res, 2016).

Study objective

In the current context of lung cancer heterogeneity, epigenetics could become an advantageous field of study. These types of modifications are reversible and are now considered an individual cancer hallmark. The most recognized epigenetic alteration consists in DNA methylation with impact on gene silencing and chromatin structure. DNA methyltransferases (DNMTs) mediate this process through covalent addition of methyl groups to a cytosine, yielding 5-methylcytosine (5mC). Lung cancer is no exception, where epigenetic modifications are standing at the base of tumor installation and development through inhibition of tumor suppressor genes and dysregulation of oncogenes (Shi et al., J of Oncol, 2019). **Although most probably these changes are dynamic and not constantly the same between patients or lung cancer stages, the malignant cell will not invest the energy in a process that is not in its favor**. Therefore, demethylation strategies may circumvent issues like tumor heterogeneity and patient ineligibility, reversing the global DNA methylation that sustains the malignant processes.

Materials and methods

The present study investigated the repositioning of 5-Azacytidine (in combination or not with irradiation) for the treatment of lung cancer in both *in vitro* and *in vivo* models.

Cell lines and treatment scheme

A549 and A549-Luc2 cell line were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37 °C with 5% CO2. Cells were treated with 5-Azacytidine (5-Aza) (4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one) acquired from Tocris, catalog number 3842, 98.9% purity at HPLC analysis. Cell irradiation (IR) was performed with doses of 2 Gy gamma radiations using a Co60 source (Theratron 1000, Theratronics, Inc., Ottawa, Ontario, Canada); dose rate of the applied radiation source was 1,98 Gy/min. The following treatment scheme was applied: Multiple dose of 5-Aza (2X) combined with IR: a. Control group that did not receive any form of treatment; b. IR group exposed to 2 Gy gamma radiations; c. 5-Aza group treated with 2 doses of 5-Aza (IC50 concentration); d. 5-Aza + IR group treated with two doses of 5-Aza (IC50 concentration) and exposed to 2 Gy gamma radiations.

***In vitro* functional tests:**

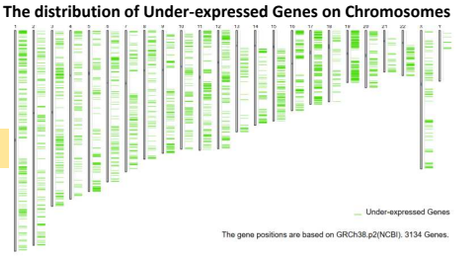
To investigate the therapeutic role of 5-Aza in A549 cell line, we performed the following functional tests: cell viability assay , flow citometry for apoptosis and cell cycle, transwell assay and wound healing assay, colony formation and spheroid formation .

***In vivo* evaluation:**

Eight week-old female atymic nude were included in the study. All experimental protocols were approved by the Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy and were conducted in accordance with the EU Directive 63/2010. We validated the therapeutic effect of 5-Aza in lung cancer by developing subcutaneous and orthotropic mouse models. The mice were injected subcutaneously in the right flank with $2x10^6$ A549 cells previously treated according to treatment scheme mentioned above. Tumor measurements and also animals weighing were done once in 3 days for 30 days. In the case of the orthotropic model, mice were injected into the right lung with $2x10^6$ A549-Luc2 cells through an open survival surgery procedure. the animals were divided in treatment group and control group (two animals/group): the treatment group received consecutive biweekly doses for 2 weeks of 5-Aza (5mg/kg) dissolved in 150ul of saline solution (intraperitoneally administration) and the control group received the same treatment scheme but only with 150 ul of saline solution. The efficiency of the treatment was monitored with the IVIS Imaging System once a week and at the end of the experiment.

Results

The distribution of Under-expressed Genes on Chromosomes

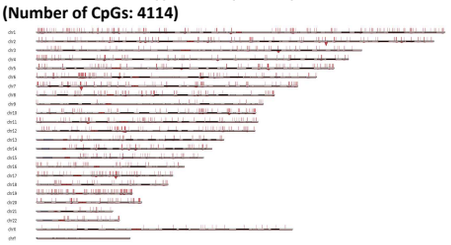


Methylation profile of lung cancer

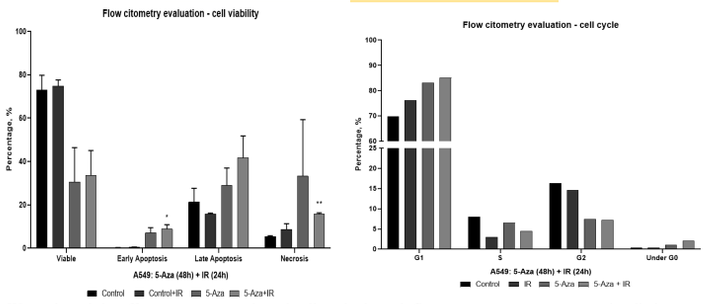
Lung cancer is mainly based on mechanisms of downregulation with 3134 underexpressed genes (green) and 1111 overexpressed ones – (data not shown) distributed among chromosomes.

Moreover, data shows that there are 4114 hyper-methylated CpGs and only 1220 hypo-methylated ones (data not shown) in LUAD compared to paired normal tissue.

The distribution of Hyper-methylated CpGs on Chromosomes (Number of CpGs: 4114)

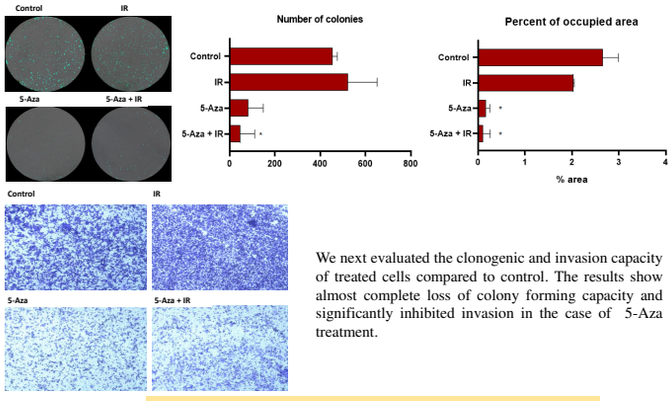


5-Aza effects upon A549 cell viability and cell cycle



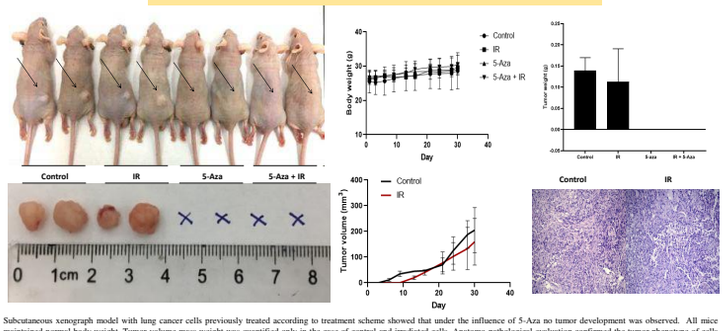
We evaluated the effects upon apoptosis and cell cycle through flow cytometry as response to irradiation, two repetitive doses of 5-Aza and 5-Aza in combination with irradiation. Data showed that 5-Aza induces cell apoptosis and also necrosis and is stopping the cell cycle by blocking the cell in the G1 phase independent of the irradiation.

5-Aza inhibits the clonogenic capacity of lung cancer cells

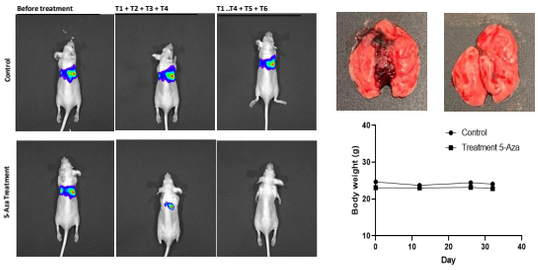


We next evaluated the clonogenic and invasion capacity of treated cells compared to control. The results show almost complete loss of colony forming capacity and significantly inhibited invasion in the case of 5-Aza treatment.

In vivo evaluation of 5-Aza repositioning in lung cancer



Subcutaneous xenograph model with lung cancer cells previously treated according to treatment scheme showed that under the influence of 5-Aza no tumor development was observed. All mice maintained normal body weight. Tumor volume mass weight was quantified only in the case of control and irradiated cells. Anatomo-pathological evaluation confirmed the tumor phenotype of cells from the control and irradiated cohorts.



Ortothopic xenograph model with lung cancer was established to asses efficiency of sistemic 5-Aza treatment. The mice from the treatment group received bi-weekly doses of 5-Aza/3weeks. At the end of the treatment, no active tumoral sites were identified by bioluminesce-based imagistic evaluation. Macroscopic evaluation confirmed the presence of a tumor mass in the lung of control mice (as seen by bioluminesce) while the treated mouse was tumor free. All mice maintained normal body weight.

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Acknowledgement

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