5 Azacytidine treatment regulates the epigenetic reprogramming of lung cancer models followed by a substantial anti-cancer action

Neelabh Datta*

"Asutosh College" University of Calcutta, 92, Shyama Prasad Mukherjee Rd, Bhowanipore, Kolkata, 700026, West Bengal, India.

Introduction

At worldwide level lung cancer rests as 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). For both sexes and pathological subtypes combined, the overall survival is still 19% despite intensive screening programs that are recommended by the American Cancer Society to diagnose the malignancy in its early stages and allow a timely treatment to occur. 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 factors that contribute to the establishment of aberrant signaling networks with overexpressed oncogenes and downregulated tumor suppressors, along with mutated genes with modified/silent functions. 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). In addition, 50% of cases of Non-Small Cell Lung Cancer (NSCLC) (NSCLC accounts for 80% of lung cancer cases) do not have identified targetable mutations, and most of the time when patients are positive for particular mutations, therapy resistance intercedes (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. 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.
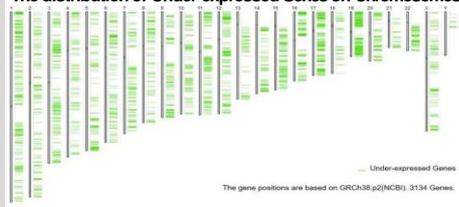
In vitro functional tests:

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. The therapeutic effect of 5-Aza in lung cancer by developing subcutaneous and orthotropic mouse models is validated. 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

### 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 Under-expressed Genes on Chromosomes

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


### 5-Aza effects upon A549 cell viability and cell cycle
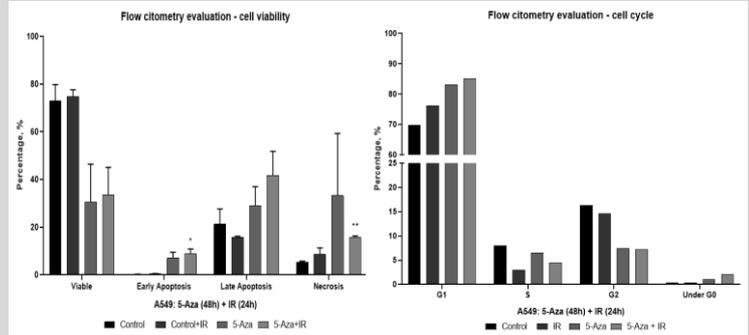
Evaluation of the effects was done upon apoptosis and cell cycle through flow cytometry as response to irradiation, two repetitive doses of 5-Aza and 5-Aza in combination with irradiation. Data showed that 5-Aza induces cell apoptosis and also necrosis and is stopping the cell cycle by blocking the cell in the G1 phase independent of the irradiation.

5-Aza inhibits the clonogenic capacity of lung cancer cells
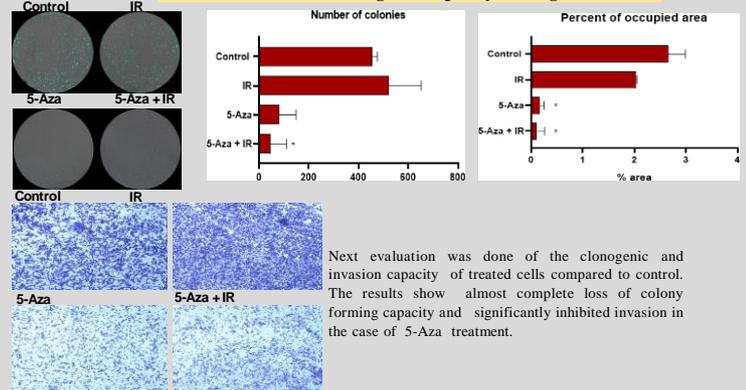

Next evaluation was done of the clonogenic and invasion capacity of treated cells compared to control. The results show almost complete loss of colony forming capacity and significantly inhibited invasion in the case of 5-Aza treatment.

In vivo evaluation of 5-Aza repositioning in lung cancer
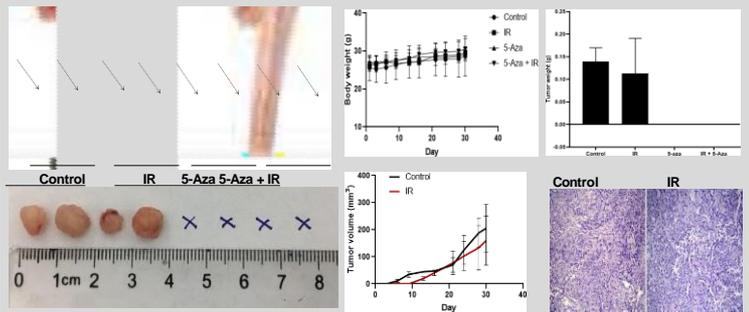
Subcutaneous xenograph model with lung cancer cells previously treated according to treatment scheme showed that under the influence of 5-Aza no tumor development was observed. All mice maintained normal body weight. Tumor volume mass weight was quantified only in the case of control and irradiated cells.
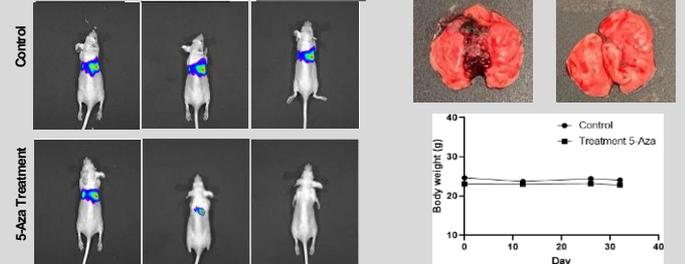
Ortothopic xenograph model with lung cancer was established to asses efficiency of sistemic 5-Aza treatment. The mice from the treatment group received bi-weekly doses of 5-Aza/3weeks. At the end of the treatment, no active tumoral sites were identified by biolumininesce-based imagistic evaluation. Macroscopic 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.

References

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