JBUON 2021; 26(6): 2360-2367
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Lidocaine for ovarian cancer. Preclinical *in vitro* data

Razvan Popa[1,2,3], Diana Cenariu[1], Ancuta Jurj[4], Noemi Dirzu[1], Sergiu Pasca[5], Tiberiu Tat[3], Daniela Ionescu[6], Alexandru Irimie[2]

[1]Medfuture Research Center for Advanced Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. [2]Department of Surgery, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. [3]Department of ICU, Ion Chiricuta Clinical Cancer Center, Cluj-Napoca, Romania. [4]Research Center for Functional Genomics, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. [5]Department of Hematology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. [6]Department of ICU, Octavian Fodor Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania.

Summary

Purpose: *To explore the influence of long non-coding ribonucleic acid (lncRNA) small nucleolar RNA host gene 1 (SNHG1) on the proliferation and apoptosis of gastric cancer cells.*

Methods: *Data from the TCGA ovarian cancer cohort was downloaded using cBioPortal. The results shown in this section are in whole based upon data generated by the TCGA Research Network. The ovarian cancer cell lines used in this study were A2780 and A2780 cis.*

Results: *Only cases which had a documented sensitivity or resistance to platinum were selected, resulting in the inclusion of 287 patients with ovarian cancer. According to previous results, we investigated the mode of action of lidocaine in ovarian cancer cells. The obtained data for both cell lines and selected concentrations suggested a slight inhibition of relative gene expression, but not statistically significant when compared to the control group.*

Conclusions: *Local anesthetics administration has proved to be protective against tumor invasion and cell growth inhibition on different cancer cell lines. Even if our results suggest a higher lidocaine dose to be administered to patients, we also demonstrated that we could modify the biology of tumor cells.*

Key words: *lidocaine, ovarian cancer, gynecologic malignancies, anesthesia*

Introduction

Ovarian cancer is one of the most frequent types of cancer in women with high incidence and mortality rates [1]. It usually has a high impact on females right after menopause (over 50 years old), but sometimes it can affect younger women as well. Its high mortality rates are due to rapid primary dissemination within the peritoneal cavity. Aggressive surgery, usually pelvic exenteration is the most efficient procedure and treatment in such tumors. Nevertheless, surgery presents its own risks for spreading the disease intraperitoneally. It is known in clinical practice that right surgical technique, the resected section size with clear margins and careful manipulation of the tumor in order not to disseminate cells elsewhere are the primary steps in eluding a recurrence. However, in ovarian cancer this is not often possible as patients usually present to the clinician when the tumor has already enclosed the peritoneum, the reproductive organs, the omentum and the sigmoid colon. Even if the tumor is localized, the micrometastases might be invisible macroscopically, but they can already be present at the intervention time and by manipulating the solid tumor, a re-

Corresponding author: Razvan Popa, MD. Department of Surgery, Iuliu Hatieganu University of Medicine and Pharmacy, 34th-36th Republicii street, 400124, Cluj-Napoca, Romania.
Tel: +04-0753636029; Email: p.razvan@gmail.com
Received: 13/02/2021; Accepted: 02/04/2021

lease of tumor cells will occur, which can metastasize locally or through the vascular and lymphatic systems [2]. The circulating tumor cells, surgical stress, metabolic response, and subsidiary surgical inflammation will inhibit the cell-mediated immunity [3]. As a consequence of surgical stress, proinflammatory cells, mainly activated macrophages, are present in large numbers at the site releasing cytokines and vascular endothelial growth factor (VEGF), a well-known pro-angiogenic factor, thus promoting neovascularization and cancer spread. Inflammation occurs perioperatively as a natural response of the body to the surgical trauma. The anti-inflammatory properties of local anesthetics will attenuate the overstimulation of the inflammatory pathways response through their mediators: interleukins, tumor necrosis factor, lipopolysaccharide, by weakening leukocyte adherence and migration through the intercellular adhesion molecule-1 (ICAM-1) [4]. Lidocaine is a local anesthetic from the amide group, widely used in regional anesthesia and postoperative pain management. The abovementioned studies showed the anti-inflammatory effect of lidocaine by inhibiting the T-cell lineage, the secretion of IL-2, TNF-α and interferon γ. The anti-inflammatory properties of lidocaine were previously demonstrated by different research teams in acute lung injury through NF-kappa-B signaling [5], reduced cancer cell migration mediated by VEGF [6], reduced tumorigenicity of ovarian cancer ES-2 cells and human breast cancer cells *in vitro* and *in vivo* [7,8], SRC inhibition on metastasis was shown in a murine model [9], inhibition of human bladder cancer cell proliferation [10] and induced apoptosis of cervical and thyroid cancer cells [11,12].

The role of anesthetics in preventing tumor recurrence is still assiduously investigated. Anesthesiologists interact with cancer patients through anesthesia, delivery of perioperative analgesics, and by treating postoperative or chronic pain. The present research demonstrated that anesthesia techniques and postoperative pain management could influence the patient's oncological evolution after surgery. The beneficial effect of propofol, nonsteroidal anti-inflammatory drugs and lidocaine were already proved as well as locoregional anesthesia techniques [13]. Lidocaine is one of the commonly used drugs for local anesthesia. The *in vitro* experimental data from the literature suggests that besides local anesthesia effect, postoperative nausea (GVPO) reduction, less opioid consumption and lidocaine might have cytotoxic, antiproliferative and apoptotic effects on tumor cells and also the ability to potentiate the effect of certain cytotoxic drugs such cisplatin [14].

The purpose of the present study was to determine the *in vitro* sensitivity of different types of ovarian cancer cell lines (A2780 and A2780cis) to lidocaine treatment. Although local anesthetics are usually used in perineural injections, intraperitoneal administration was documented in rats and in humans [15]. In the literature the *in vitro* doses of lidocaine used in researching the antitumor effect were higher than the therapeutic doses used in local anesthesia [16].

In this study we wanted to see if higher doses are more effective or smaller doses are enough to eliminate residual tumor cells. Injecting lidocaine directly into the tumor for size reduction preoperatively, or peritoneal lavage after surgery may be a better approach to capitalize on the antitumor effects than only local and regional anesthesia techniques.

Methods

TCGA gene expression analysis

Data from the TCGA ovarian cancer cohort was downloaded using cBioPortal.

The results shown in this section are in whole based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga.

Cell culture

The ovarian cancer cell lines used in this study were A2780 and A2780 cis (cell lines resistant to cisplatin) purchased from ECACC (European Collection of Authenticated Cell Culture). All cells were grown in a humidified atmosphere at 37°C, supplemented with 5% CO_2. Cell cultures were maintained in RPMI 1640 (Gibco™) culture medium, supplemented with 10% fetal bovine serum (FBS) (Gibco™), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco™).

Lidocaine

Dilution was performed with ultrapure water. We established the doses between 1-75μg/ml relaying on the literature, as in normal intravenous bolus and plasma lidocaine levels were found to be between 1.6-4.6 μg/ml [16], but for VEGF inhibition doses 11.5-46μg/ml concentrations were effective [6].

Cell viability assay

Cell viability was evaluated using MTT assay (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, Germany). Both cell lines, A2780 and A2780 cis respectively, were cultured sub-confluence before being seeded for the experiments. At a seeding density of 10^4 cells/well, cells were seeded in 96-well plates and after 24 h incubation were treated with stepwise lidocaine concentrations. The cytotoxic and antiproliferative activity of lidocaine on the cells was evaluated after 24 and 48 h using MTT assay. After the incubation period, treated cells were incubated for 2 h with 1 mg/mL MTT solution in the dark at 37°C. The metabolized formazan salt was resolubilized

in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) by incubation for 15 min in the dark at room temperature under gentle shaking. The absorbance at 570 nm was read using spectrophotometer. The results were expressed in optical density (OD) units and then transformed in percent of viability compared to the control group. The experiments were performed in triplicate.

Apoptosis evaluation by fluorescence microscopy

The ovarian cancer cell lines were cultured at a seeding density of 12×10^4 cells/well in 24-well plates for 24 h in a CO_2 incubator at 37°C, after which cells were exposed to lidocaine in stepwise concentrations. Forty-eight h post-therapy, the apoptotic effects of lidocaine were evaluated with Olympus IX71 inverted microscope using the Multiparameter Apoptosis Assay Kit (Cayman cat no 600330, Estonia). To evaluate the different apoptotic events the kit uses Hoechst dye to stain the nuclei, FITC-labeled Annexin V for the staining of the outer membrane of the apoptotic cells, RedDot™2 to reveal the plasma membrane permeability and TMRE (Tetramethyl rhodamine ethyl ester) for the mitochondrial membrane activity potential. Cells were analyzed in UV light. The experiments were performed in triplicate.

Total RNA extraction and RT-PCR

Total RNA extraction from A2780 and A2780 cis cells was performed using TriReagent (Invitrogen, Waltham, USA) according to the manufacturer's instruction. RNA concentration and quality were assessed using Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, USA). 500 ng of total RNA were reversed-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression evaluation was

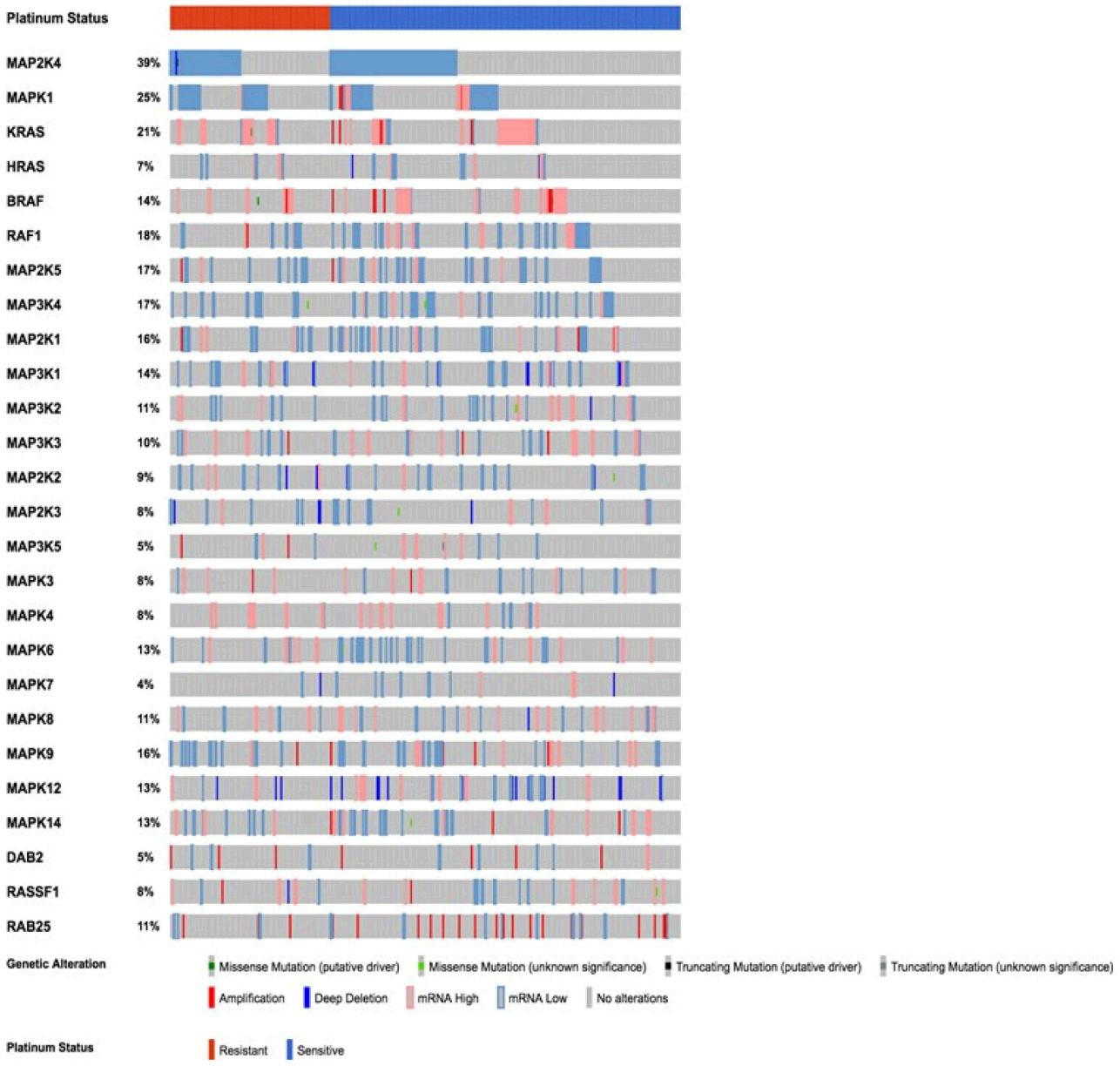

Figure 1. Oncoprint of the overlap between sensitivity or resistance to platinum compounds and the occurring mutations.

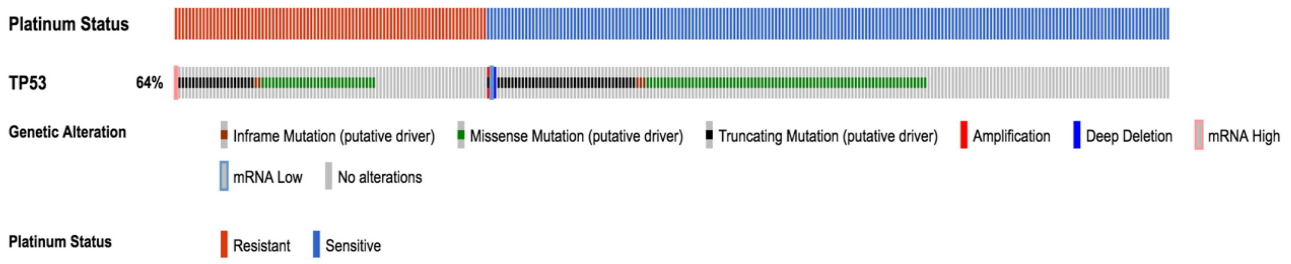

Figure 2. Oncoprint representation of *TP53* mutation distribution between platinum sensitive and resistant patients in the cohort.

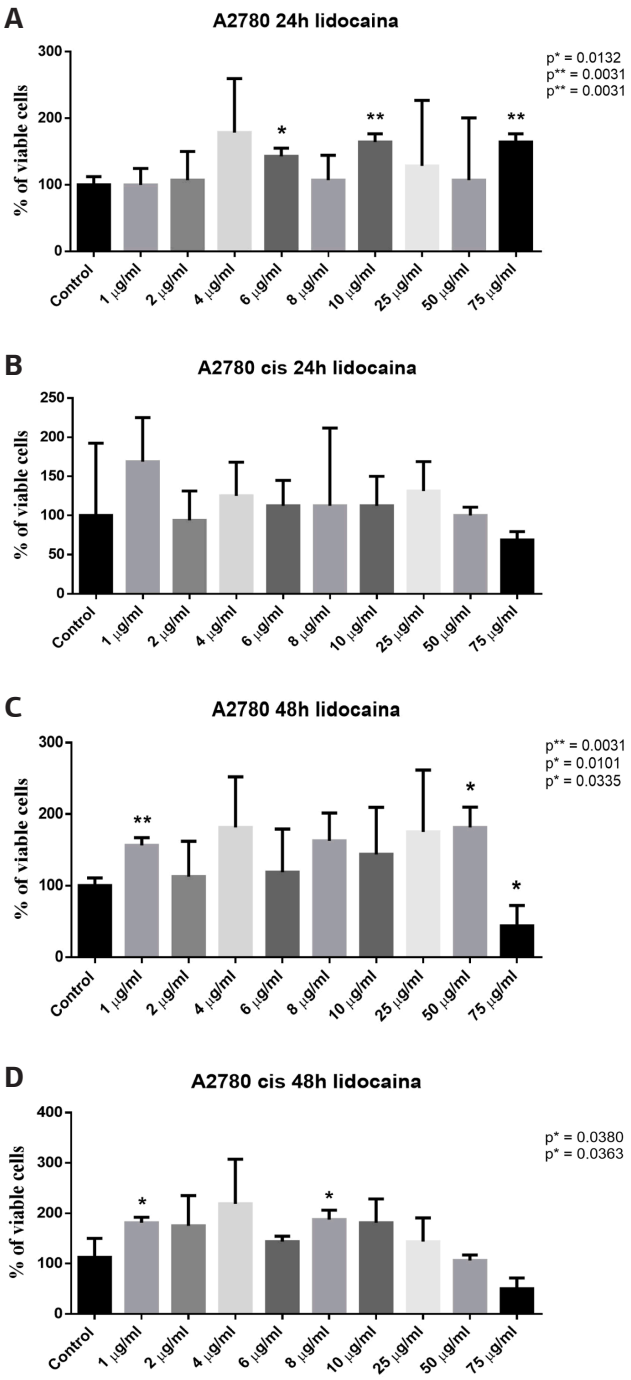

Figure 3. The antiproliferative effect of lidocaine evaluated at 24 **(A, B)** and 48 h **(C, D)** on A2780 and A2780 cis cell lines using MTT assay.

conducted using SYBR Select Master Mix (Applied Biosystems) and qRT-PCR was performed on ViiA™7 System (Thermo Scientific) in 10 μl volume using a 384-well microplate. GAPDH was used as internal control. Relative quantification was conducted using the $2^{-\Delta\Delta CT}$ method.

Statistics

Data were expressed as mean ± standard deviation. The difference between experimental conditions and controls was analyzed using Student's t-test (statistically significance was considered at $p<0.05$). Image generation was carried out using GraphPad Prism version 6 software.

Results

TCGA

Only cases which had a documented sensitivity or resistance to platinum were selected, resulting in the inclusion of 287 patients with ovarian cancer. Because of the EGFR target of lenalidomide documented through drugs.com, we decided to analyze only the MAPK pathway offered by default by cBioPortal, being represented by KRAS, HRAS, BRAF, RAF1, MAP3K1, MAP3K2, MAP3K3, MAP3K4, MAP3K5, MAP2K1, MAP2K2, MAP2K3, MAP2K4, MAP2K5, MAPK1, MAPK3, MAPK4, MAPK6, MAPK7, MAPK8, MAPK9, MAPK12, MAPK14, DAB2, RASSF1, RAB25 (Figure 1).

Mutual exclusivity between the mentioned genes revealed 2 co-occurring instances represented by MAP2K1 and MAPK9 (p=0.003) and MAP2K1 and MAP2K5 (p=0.023). Because of the aforementioned co-occurrences, we decided to explore the effect of MAP2K1 expression on the other mRNA signatures. Due to the high frequencies and known importance, we included MAP2K4 and KRAS. Moreover, because it is an important and frequent gene, we decided to take a look at TP53 also. The oncoprint for this is represented in Figure 2.

Lidocaine activity in ovarian cancer cell lines

To determine chemosensitivity of ovarian cancer cell lines A2780 and A2780cis to lidocaine, cell lines were treated with stepwise drug concentra-

tions: 1μg/ml, 2 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, and 75 μg/ml. Unexposed cells were used as controls. The cell viability and the cytotoxicity of lidocaine were assessed using MTT assay. According to the obtained results, the cytotoxic effects of lidocaine and cell viability for both ovarian cancer cell lines was established. In Figure 3 are presented the results at 24 and 48 h on both cell lines, A2780 and A2780cis, for lidocaine exposure. At 24 h, cell destruction was not yet activated in neither cell line. Statistically significant results were not obtained. At 48 h the cytotoxic effect of the lidocaine was visible at larger concentrations.

Lidocaine reduces the number of viable cells and promotes apoptosis in ovarian cancer cell lines

In order to evaluate the impact of lidocaine in the progression of ovarian cancer cells, we performed functional analysis on both cell lines, A2780 and A2780cis, respectively. Forty-eight h post-therapy, apoptosis was evaluated through fluorescence microscopy after performing staining with TMRE and Hoechst dyes. We observed that control cells stained with TMRE dye exhibited undisrupted mitochondrial membrane potential. In this regard, cells were not affected by any stimulus that might alter their morphology and

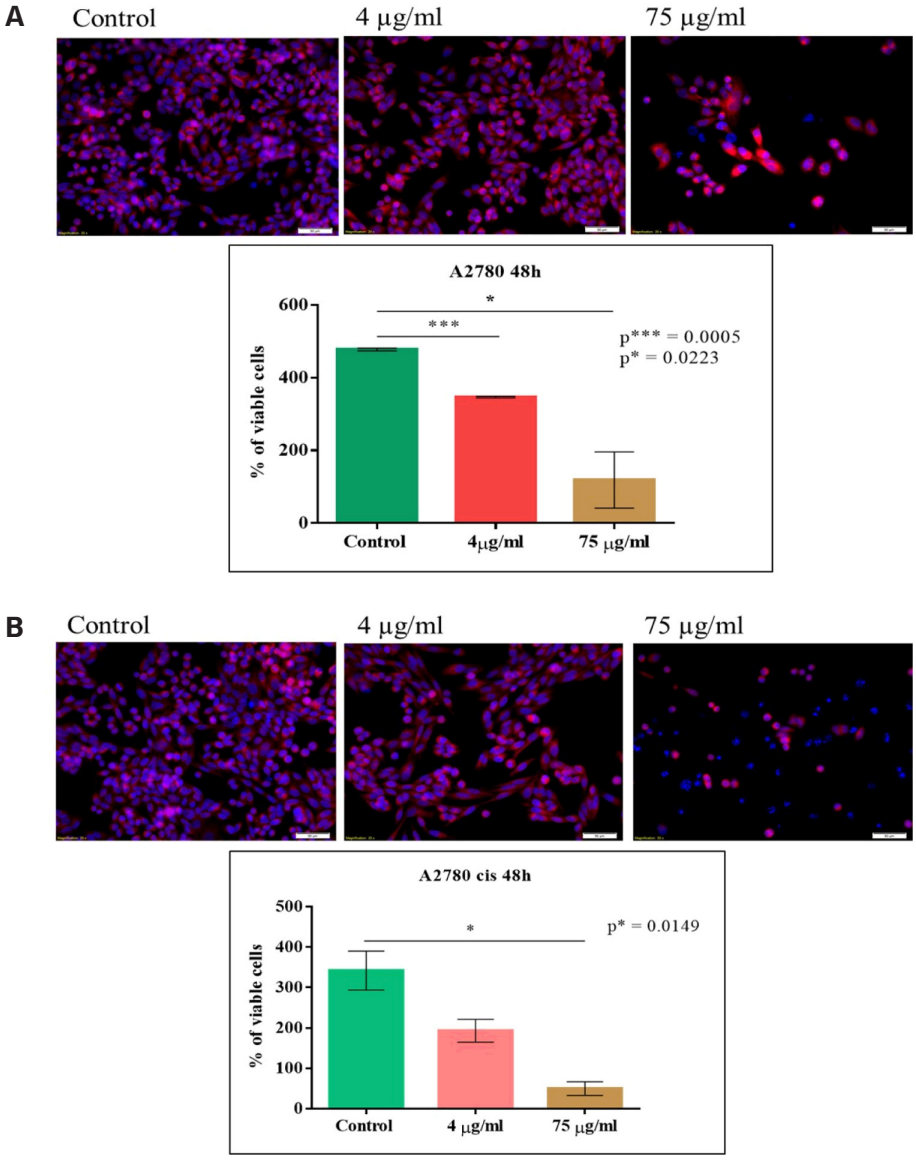

Figure 4. Assessment of apoptosis through fluorescence microscopy following exposure to 4μg/ml and 75μg/ml lidocaine on **(A)** A2780 and **(B)** A2780cis cell lines (20x magnification). Forty-eight h post therapy, a decreased number of viable cells was observed compared to control cells. Also, in the treated group disrupted mitochondrial membrane potential, and irregular and fragmented nuclei were observed which suggests that the cells are undergoing apoptosis (data presented as mean ± SD; ***p=0.0005 for 4μg/ml and *p=0.0223 for 75μg/ml for A2780 cell line; data presented as mean ± SD; *p=0.0149 for 75μg/ml for A2780cis cell line (two-sided t-test).

trigger apoptosis. Meanwhile, the first concentration of lidocaine, 4µg/ml, presented no significant differences related to cellular morphology compared to control group. In this regard, the observed effect was related to a decreased number of viable cells compared to control cells, which suggested that cellular growth and proliferation rate was affected post-therapy. The last concentration used, 75µg/ml, generated changes in the cellular morphology compared to control cells. Thus, cell membrane asymmetry was affected, and cell shrinkage and nuclear fragmentation triggering apoptosis was observed. Irregular and fragmented nuclei were highlighted through Hoechst staining on both cell lines. Mitochondrial membrane potential was disrupted and highlighted through TMRE staining. Meanwhile, we performed a statistical analysis on both ovarian cancer cell lines in order to determine if cell death inhibition was correlated with decreased cell number. Lidocaine led to loss of cell viability, which proved a strong effect on ovarian cancer cell viability (Figure 4).

TP53 profiling in ovarian cancer cell lines

According to previous results, we investigated the mode of action of lidocaine in ovarian cancer cells. The obtained data for both cell lines and selected concentrations suggested a slight inhibition of relative gene expression, but not statistically significant when compared to the control group (Figure 5).

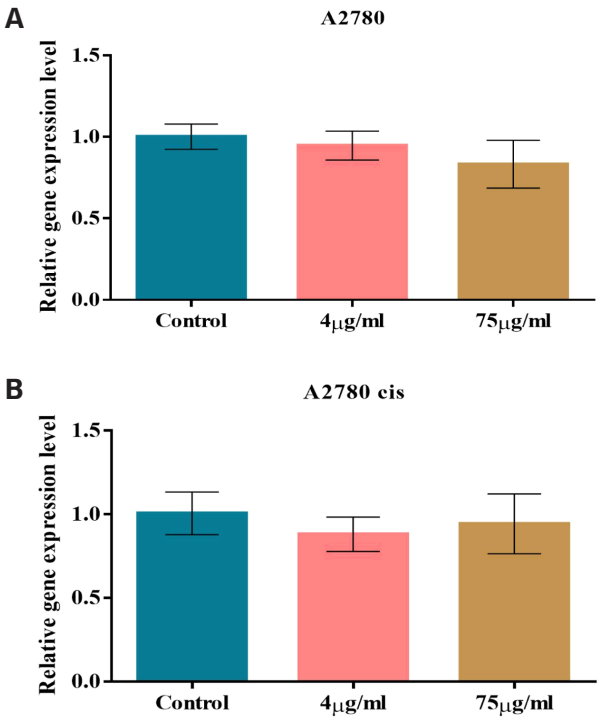

Figure 5. Validation of the effect of lidocaine treatment by qRT-PCR on *TP53*. Relative gene expression levels are shown for ^{TP53} in treated and control group (untreated cells). The data were normalized to GAPDH and B2M using ΔΔCT method, for A2780 **(A)** and A2780cis **(B)** cell lines comparing lidocaine-treated group versus control group.

Discussion

Our study demonstrated that 4 µg/ml lidocaine did not significantly affect the cellular morphology compared to the control group. In exchange, the number of viable cells after lidocaine treatment was lower compared to the control, thus validating a decrease in cell growth and proliferation. At a 75 µg/ml lidocaine concentration, the cellular morphology had been modified compared to the control. Apoptosis was triggered indicated by cell membrane modifications, cellular contraction and nuclear fragmentation. Such modifications as well as reorganization of the cytoskeleton, reorganization of actin filaments, induced cytoprotective autophagy detected by TEM as large vacuoles were observed by Izdebska et al [17]. Similar data of suppressed cell viability by inhibition of proliferation and triggered apoptosis were obtained for cervical cancer cell line, human breast tumor cells, tongue cancer cells [18], human hepatocarcinoma HepG2 cells [19], colorectal cancer SW480 and HCT116 cells [20].

Apoptosis is characterized by nuclear DNA degradation as a response to different apoptotic stimuli in a huge variety of cells.

The lidocaine's cytotoxicity mechanism seems not to be in connection with the local anesthetic's primary activity. Lidocaine directly inhibits the tyrosine kinase activity of EGFR receptor with retinoblastoma cells [21], sublingual tumor cells and human colorectal cancer cells. There might be another mechanism by which the local anesthetics influence the growth of tumor cells through direct interaction with the tumor epigenome. Genome stability and normal gene expression is maintained by a precise DNA methylation pathway. Tumor progression expresses itself once the activity of tumor suppressor genes is reduced as a result in methylation growth (progression). It was already proven that lidocaine, the most used local anesthetic, induces DNA demethylation and inhibits breast cancer development. There are some cytotoxic mechanisms involved in lidocaine therapy, such as reduction of glycolysis and adenosine triphosphate levels, a rise in intracellular calcium concentration, mitochondrial dysfunction by mitochondria depolarization and inhibition of mitochondrial respiration.

The effect of lidocaine on the TP53 protein was investigated as well. In our research, the two ovarian tumor cell lines showed a minimum but statistically insignificant difference in the expression of TP53.

TP53 encodes the suppressor tumor protein p53. At a cellular level, both for tumor and normal cells, it was proven that mutant p53 will suppress the majority if not all the cellular responses mediated by wild type p53 such as arresting the cell cycle, apoptosis, DNA repair and reduced cell motility. The absence of a functional p53 allows the cells to tolerate and accumulate altered DNA fragments, leading to genomic instability and extra genetic mutations [22]. As a result, TP53 mutations predispose to cancer initiation and development, surviving of tumor cells and metastasis, and can be associated with an inefficient response to cancer therapy and reduced survival in 11 cancer types [23]. New therapeutic strategies include restoring normal p53 protein activity through targeted treatments [24] designed to decrease mutant P53 levels at which tumor cells undergo apoptosis more rapidly.

Conclusion

Local anesthetics administration has proved to be protective against tumor invasion and cell growth inhibition on different cancer cell lines. Even if our results suggest a higher lidocaine dose to be administered to patients, we also demonstrated that we can modify the biology of tumor cells.

Acknowledgements

Razvan Popa and Diana Cenariu contributed equally to the manuscript and are both considered as first authors. Razvan Popa was funded by an internal grant of the Iuliu Hatieganu University-School of Doctoral Studies (PCD 2018-2021). This work was supported by an institutional PNCDI III 2015–2020 grant "Increasing the performance of scientific research and technology transfer in translational medicine through the formation of a new generation of young researchers"-ECHITAS, no. 29PFE/18.10. 2018.

Conflict of interests

The authors declare no conflict of interests.

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