

Lithium chloride increases sensitivity to photon irradiation treatment in primary mesenchymal colon cancer cells

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Received July 25, 2019; Accepted November 26, 2019

DOI: 10.3892/mmr.2020.10956

Abstract. Colorectal cancer (CRC) is the third most prevalent type of cancer worldwide. It is also the second most common cause of cancer-associated mortality; it accounted for about 9.2% of all cancer deaths in 2018, most of which were due to resistance to therapy. The main treatment for CRC is surgery, generally associated with chemotherapy, radiation therapy and combination therapy. However, while chemo-radiotherapy kills differentiated cancer cells, mesenchymal stem-like cells are resistant to this treatment, and this can give rise to therapy-resistant tumors. Our previous study isolated T88 primary colon cancer cells from a patient with sporadic colon cancer. These cells exhibited mesenchymal and epithelial features, high levels of epithelial-to-mesenchymal transition transcription factors, and stemness markers. In addition, it was revealed that lithium chloride (LiCl), a specific glycogen synthase kinase (GSK)-3 β inhibitor, induced both the mesenchymal-to-epithelial transition and differentiation, and also reduced cell migration, stemness features and cell plasticity in these primary colon cancer cells. The aim of the present study was to investigate the effect of LiCl treatment on the viability of primary colon cancer cells exposed to 7 Gy delivered by high-energy photon beams, which corresponds to 6 megavolts

of energy. To achieve this aim, the viability of irradiated T88 cells was compared with that of irradiated T88 cells pre-treated with LiCl. As expected, it was observed that LiCl sensitized primary colon cancer cells to high-energy photon irradiation treatment. Notably, the decrease in cell viability was greater with combined therapy than with irradiation alone. To explore the molecular basis of this response, the effect of LiCl on the expression of Bax, p53 and Survivin, which are proteins involved in the apoptotic mechanism and in death escape, was analyzed. The present study revealed that LiCl upregulated the expression of pro-apoptotic proteins and downregulated the expression of proteins involved in survival. These effects were enhanced by high-energy photon irradiation, suggesting that LiCl could be used to sensitize colon cancer cells to radiation therapy.

Introduction

Colorectal cancer (CRC) is one of the main causes of cancer deaths and the fourth most frequent cancer worldwide, with about 1,096,601 newly diagnosed cases and 551,269 deaths in 2018 (1). Despite the increase in the incidence of CRC over the last 20 years, CRC mortality has decreased in many countries, probably due to prevention strategies, early detection and more effective treatment (1). Surgery is the gold standard treatment for early colorectal tumors: Indeed it is associated with 5-year cancer-specific survival rates of 91.4 and 70.2% in stage II and III forms, respectively (2). Treatment of CRC is defined based on tumor stage. Very early tumors can be treated using local excision, while neoadjuvant chemora-diotherapy is indicated in locally-advanced rectal cancer (3,4). Metastatic disease (stage IV) is usually treated with chemotherapy with a combination of 5-fluorouracil and leucovorin (e.g. oxaliplatin-FOLFOX, irinotecan-FOLFIRI). In addition, two monoclonal antibodies against the epidermal growth factor receptor are now used in combination with well-established CRC-treatment schedules (3,5-7). According to the American Cancer Society Guidelines, radiation therapy can be considered for colon cancer therapy to promote cancer reduction before surgery. It can also be used after surgery in cases in which the tumor

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Key words: lithium chloride, high energy photon irradiation, mesenchymal colon cancer cells, cancer drug resistance, apoptosis

adheres to other organs and cannot be totally removed surgically. Moreover, the so-called intraoperative radiation therapy can be used during surgery to kill cancer cells in their location. Lastly, radiotherapy combined with chemotherapy can be used for unresectable cancer or to attenuate symptoms in advanced cancers and in case of metastases (8,9). Although anticancer therapies have yielded a good success rate, in terms of overall survival, they fail to kill cancer cells in over 90% of patients with advanced CRC due to the development of therapy resistance. Metastatic cancer cells are characterized by mesenchymal and stemness features conferring them aberrant survival capacity and evasion of apoptosis that represent the major mechanisms responsible for cancer resistance to therapy (10).

LiCl is the most well studied GSK-3 β inhibitor. It exerts its effect through a direct and indirect mechanism. In the first case, it competes with the GSK-3 β cofactor Mg²⁺, thereby inhibiting the enzyme's activity, whereas in the second case, it increases the inhibition of phosphorylation of GSK-3 β Ser9 (11). In addition to its role in the regulation of GSK-3 β , LiCl emerged as a promising drug for various diseases, such as neurological diseases, cancer, and inflammation (12-14). We previously demonstrated that LiCl induces the differentiation and the mesenchymal-to-epithelial transition (MET) of primary colon cancer cells, thereby reducing migration and stemness features (15,16). Since mesenchymal and stemness features are the main causes of aberrant survival capacity and evasion of apoptosis during cancer progression, we suggested that LiCl could sensitize colon cancer cells to chemo-radiotherapy. To address this issue, we investigated the effects of LiCl treatment on the viability of primary mesenchymal colon cancer cells in combination with radiation therapy. We observed that LiCl and high-energy photon irradiation had an additive effect both on the viability of mesenchymal colon cancer cells, and on the induction of apoptosis. Finally, at molecular level, we found that LiCl induced strong Survivin down-regulation and p53 and Bax up-regulation. We believe that these molecular changes could contribute to LiCl-mediated sensitization to high-energy photon irradiation in CRC.

Materials and methods

Sample collection. T88 primary CRC cells were previously isolated and characterized (15,16). The patient study was approved by the ethics committee of the University of Naples Federico II, 'Comitato etico per le attività Biomediche-Carlo Romano', with protocol no. 35/17. The patient provided written informed consent to the study. All methods were performed in accordance with the relevant guidelines and regulations.

Cell culture and treatments. Primary T88 cells were cultured as reported elsewhere (15,16). Subsequently, cell suspensions (500 μ l containing 240x10³ cells) were plated on 100 mm tissue culture treated plates and cells were alternatively incubated with LiCl (30 mmol/l) for 48 h, irradiated with 2 or 7 Gy of high-energy photon beams or irradiated and pretreated with LiCl. Untreated cells were compared with treated cells for subsequent cell analysis. RKO cells were from ATCC and grown in Eagle's Minimum Essential Medium (M22791L,

Sigma) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin.

MTT assay. Untreated cells, LiCl-treated cells, irradiated cells and cells irradiated after LiCl pretreatment were analyzed immediately after (T0) and 48 hours after (T48 h) treatments. Cells were washed and incubated for three hours in 100 μ l DMEM (ECB7501L; Euroclone) supplemented with 0.45 mg/ml MTT reagent; the medium was then replaced by 100 μ l of 0.1 M HCl in isopropanol and cells were incubated 30 min for lysis. Insoluble formazan was resuspended, and optical densities were measured at a wavelength of 570 nm with a Microplate Reader (Biotek Synergy Microplate Reader), according to the MTT manufacturer's protocol. Data are expressed as mean \pm SEM of three experiments.

Western blot analysis. Total protein extracts were isolated from untreated T88 cells, LiCl treated cells, irradiated cells and cells irradiated after LiCl pretreatment; all cells were lysed at time T48h. Cell lysates were prepared as reported previously (17), proteins were separated by SDS-polyacrylamide gel electrophoresis, and blots were prepared as reported previously (18). Primary antibodies against Survivin (rabbit polyclonal anti-human; cat. no. 2803; dilution 1:1,000), β -catenin (rabbit polyclonal anti-human; cat. no. 9562; dilution 1:1,000) p53 (rabbit monoclonal anti-human; cat. no. 2527) and Bax (rabbit monoclonal antihuman; cat. no. BK2772T) were from Cell Signaling Technology; the anti-GAPDH (monoclonal anti-mouse; sc-393358) antibody was from Santa Cruz Biotechnology. Membranes were probed with peroxidase-conjugated secondary antibodies against rabbit or goat IgG, and immunoreactive bands were detected as described before (19). The experiment was repeated three times with similar results. Densitometric analyses were performed using Image J software.

Flow cytometry analysis. Untreated cells, LiCl-treated cells, irradiated cells and irradiated cells pretreated with LiCl, were harvested at time T48h, fixed and stored in ice-cold ethanol at 20°C. Propidium iodide (PI) (Appllichem) was used for cell cycle analysis, as described previously (20). Briefly, cells were washed twice in ice-cold PBS and then resuspended at a concentration of about 1 million/ml of cells in 0.1% Na-citrate, 50 mg/ml RNase, 50 mg/ml propidium iodide and incubated for 30 min in the dark at room temperature. PI fluorescence intensity was measured using the BD C6 Accuri cytometer and data were analyzed using the BD C6 Accuri Software (Becton Dickinson).

Statistical analysis. All data were obtained from at least three independent experiments and are reported as the mean \pm SEM. Statistical differences between groups was determined by the t test and/or Kruskal-Wallis test followed by a Dunn's post hoc test at a significance level of P<0.05.

Results

LiCl decreases the viability of irradiated cancer cells. Using the MTT assay, we first evaluated the effect of high-energy photon irradiation on the viability of T88 primary colon cancer

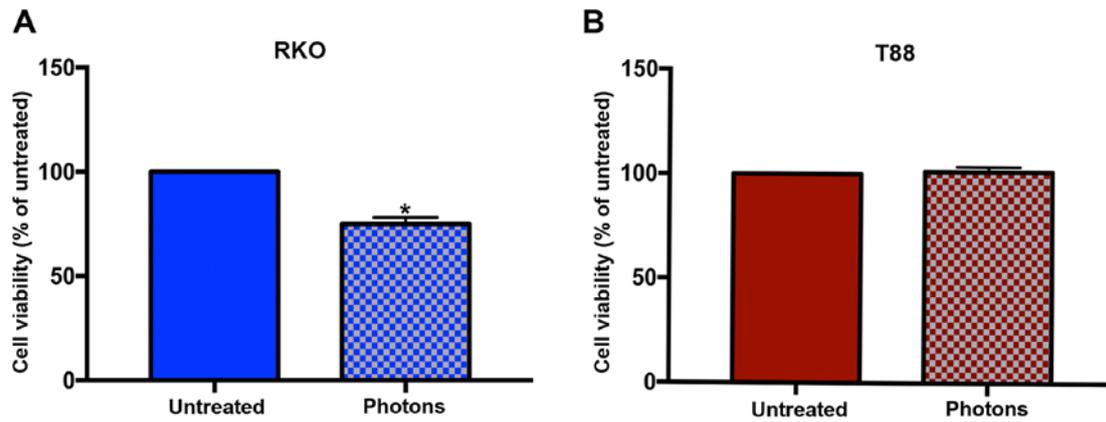


Figure 1. Effect of high energy photon irradiation on RKO and T88 cells. An MTT assay was performed in untreated and high energy photon- irradiated (A) RKO and (B) T88 cells. Bars represent the mean absorbance \pm SEM of experiments performed in triplicate. * $P < 0.05$.

cells and of commercially available RKO colon cancer cells. We initially irradiated cells with 2 Gy, which is the dose generally used in clinical practice. However, neither the T88 primary colon cancer cells or the commercially available colon cancer RKO cells responded to this treatment (data not shown). Thus, we irradiated cells with 7 Gy of high-energy photon beams, and obtained a response, in terms of cell viability, only in RKO cells. As shown in Fig. 1, the photon irradiation affected the viability of RKO cells, but not that of T88 cells, which appear completely unresponsive. To determine whether LiCl would sensitize T88 cells to high-energy photon irradiation, we evaluated the viability of T88 cells after i) incubation with LiCl, ii) high energy photon irradiation and iii) combined treatment immediately after completion of treatment (T0) and 48 h after completion of treatment (T48). As shown in Fig. 2 and Table I, LiCl decreased the viability of T88 cells versus untreated cells, at both T0 (Fig. 2A) and T48 h (Fig. 2B). As expected, high-energy photon irradiation affected cell viability only at T48. Notably, the maximum effect on cell viability was observed when cells were irradiated after LiCl pretreatment, which suggests that LiCl and high-energy photon irradiation may have an additive effect.

LiCl sensitizes colon cancer cells to high energy photon irradiation altering the balance between pro-apoptotic and survival signaling. Since both LiCl and high-energy photon are known to activate pro-apoptotic signals in colon cancer cells, we investigated the effects of each of these treatments alone and in combination on the expression of proteins involved in apoptosis and death escape, such as p53, Bax, and Survivin. To this aim, we performed Western blot assay on total protein extracts from untreated cells, cells treated with LiCl or high energy photons, and cells irradiated with high-energy photons after pretreatment with LiCl. Samples were collected 48 h after completion of treatments. As shown in Fig. 3A-C, p53 and Bax protein expression was upregulated in both LiCl and LiCl plus high-energy photon treated cells, and the highest increase was observed after combined treatment. On the contrary, Survivin expression was greatly reduced after each treatment (Fig. 3A and D). As LiCl induces GSK-3 β inhibition, we investigated β -catenin expression level in treated and untreated cells. As shown in Fig. 3A and in Fig. 3E, β -catenin

expression was stabilized in both LiCl-treated cells and in cells treated with LiCl plus high-energy irradiation. Interestingly, in LiCl-treated cells, western blot immunostaining showed upregulation of an isoform of β -catenin a little higher in molecular weight than that observed in cells irradiated without LiCl pre-treatment. Additional experiments are necessary to shed light on this result. To verify whether these molecular changes result in differences in apoptosis levels, we also performed a FACS analysis to evaluate the percentage of subdiploid cells. As expected, the percentage of subdiploid cells increased significantly after the combined LiCl and high-energy photon treatment versus control cells (Fig. 4). Taken together, these data indicate that LiCl sensitizes T88 primary CRC cells to the effects of high-energy photon treatment by altering the expression pattern of pro-apoptotic and pro-survival proteins.

Discussion

The epithelial-to-mesenchymal transition, a biological process by which epithelial cancer cells lose their epithelial phenotype and acquire a mesenchymal phenotype, is a physiological mechanism developed by cancer cells during cancer progression and metastases. Indeed, cells that have undergone the EMT-transcription factors (EMT-TFs) acquire all the features needed to complete the metastatic process, such as, motility, stem cell features, cell plasticity, resistance to apoptosis and to therapy. This process is orchestrated by the EMT-TFs TWIST1 and Snail. It has been suggested that high levels of these EMT-TFs can also cause resistance to apoptosis by altering the TGF- β - and p53-mediated programmed cell death pathways (21-24). Apoptosis is an energy-dependent mechanism of programmed cell death by which organisms maintain tissue homeostasis (25). Several pathways regulate apoptosis and several mechanisms are used by tumor cells to survive, suppressing apoptotic program. The most frequent apoptotic pathways are the extrinsic and intrinsic pathways. Both are usually characterized by early activation of the caspases proteolytic cascade that causes cleavage of cellular proteins and of other components essential for cell survival, thereby triggering programmed cell death. In the extrinsic pathway, death domain-containing proteins, such as the tumor necrosis factor family of receptors, which are direct

Table I. Statistical analysis of MTT assay.

Statistic	T0	T48h
Un. (mean ± SEM)	100.00	100.00
LiCl (mean ± SEM)	66.00±3.46	61.00±19.08
High energy photons (mean ± SEM)	91.33±8.41	83.00±10.60
LiCl + high energy photons (mean ± SEM)	63.00±3.00	31.00±14.18
P-value: Un. vs. LiCl	0.1161	0.3312
P-value: Un. vs. high energy photons	>0.9999	>0.9999
P-value: Un. vs. LiCl+high energy photons	0.0476 ^a	0.0262 ^a

Results are representative of three independent experiments. ^aP<0.05 (Kruskal-Wallis test). Un, untreated; LiCl, lithium chloride.

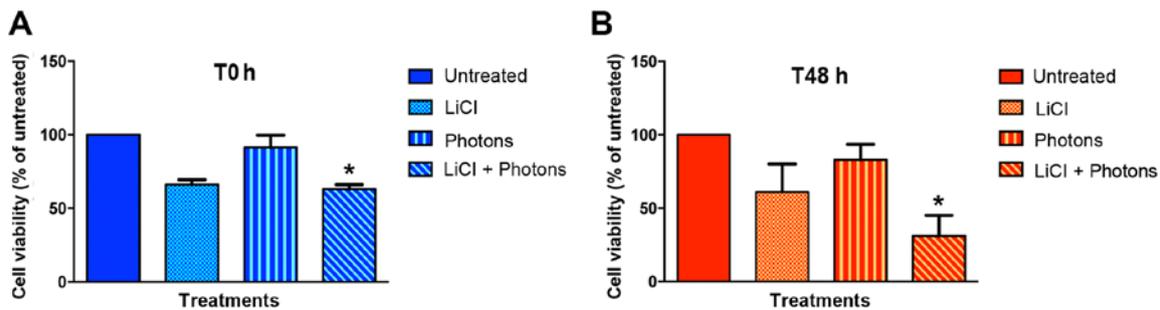


Figure 2. Effect of LiCl on high energy photon irradiation sensitivity of T88 cells. MTT assay performed in T88 cells (A) immediately after (T0) or (B) 48 h (T48 h) after treatment with LiCl and/or high energy photons. Bars represent the mean absorbance ± SEM of a representative experiment performed in triplicate. *P<0.05 vs. untreated cells, using Kruskal-Wallis test. LiCl, lithium chloride.

targets of caspase cleavage, are activated (26). In the intrinsic pathway, mitochondria play an essential role in triggering apoptotic signals by releasing cytochrome-c into the cytosol, which induces 'apoptosome' assembly. Subsequently, the 'apoptosome' complex is able to activate the caspases proteolytic cascade. This pathway is known as the 'BCL-2-regulated apoptotic pathway', because the cell death program is triggered by the upregulation of the BCL-2 protein family, that, in turn, activates the cell death effectors Bax and Bak (27). Cytochrome c can also induce the release of other proteins, i.e., endonuclease G and apoptosis-inducing factor, that may promote caspase-independent cell death (28,29).

The intrinsic pathway is altered in most cancer cells and is closely regulated by cellular metabolism. Indeed metabolic changes occurring in cancer cells, consequent to oncogenic activation or stress-induced therapy, promote resistance to apoptosis and therapy via alterations of BCL-2 family expression (25). P53, a protein often altered in tumor progression, has been implicated in the activation of the intrinsic and extrinsic pathways, and its ability to induce apoptosis depends on NF- κ B activation (30). Furthermore, alteration in the extrinsic and intrinsic pathways could cause resistance to anoikis, which is programmed cell death induced by detachment of cells from the extracellular matrix, that is often altered in cancer cells with metastatic potential (31-34). Upon activation of proapoptotic cellular pathways, the survivin protein, a member of a family of apoptosis inhibitors, is released from mitochondria and inhibits caspase-9 thereby enhancing the effects of inhibitor of apoptosis proteins (35).

In this scenario, we previously isolated and characterized epithelial colon cancer cells endowed with mesenchymal features, and together with high EMT-TFs expression, in the attempt of identifying a therapeutic target able to sensitize colon cancer cells to specific therapies. These cells were able to grow for long periods in suspension as tumorspheres, which suggests they are anoikis-resistant (16). We have also demonstrated that LiCl induces differentiation, MET, and downregulation of the EMT-TFs TWIST-1 and Snail, in primary colon cancer cells (15). In the present study, we demonstrate that T88 primary colon cancer cells are completely unresponsive to high-energy photon irradiation in terms of cell viability, and that LiCl sensitizes them to such treatment.

Development of radiation resistance has been correlated with two main mechanisms, one consisting in disequilibrium between pro-apoptotic signaling transduction pathways (mediated by p53 and Bax) and pathways mediating cell survival, in which the protein Survivin plays an essential role (36-38). On the other hand, DNA double strand break repair pathways, which maintain genomic integrity and prevent mis-repair and chromosomal rearrangements, could be responsible for radio-resistance and failure of radiation treatment. Rouhani *et al* (39) reported that LiCl increases radio-sensitivity in breast cancer cells in vitro by abrogating DNA repair. Indeed, in contrast to the expected effect of LiCl, i.e., GSK-3 β inactivation and β -catenin stabilization, the authors observed GSK-3 β upregulation and β -catenin down-regulation together with mRNA downregulation of its transcriptional target MR11, which is a crucial protein of DSB repair system (39). As expected, we observed

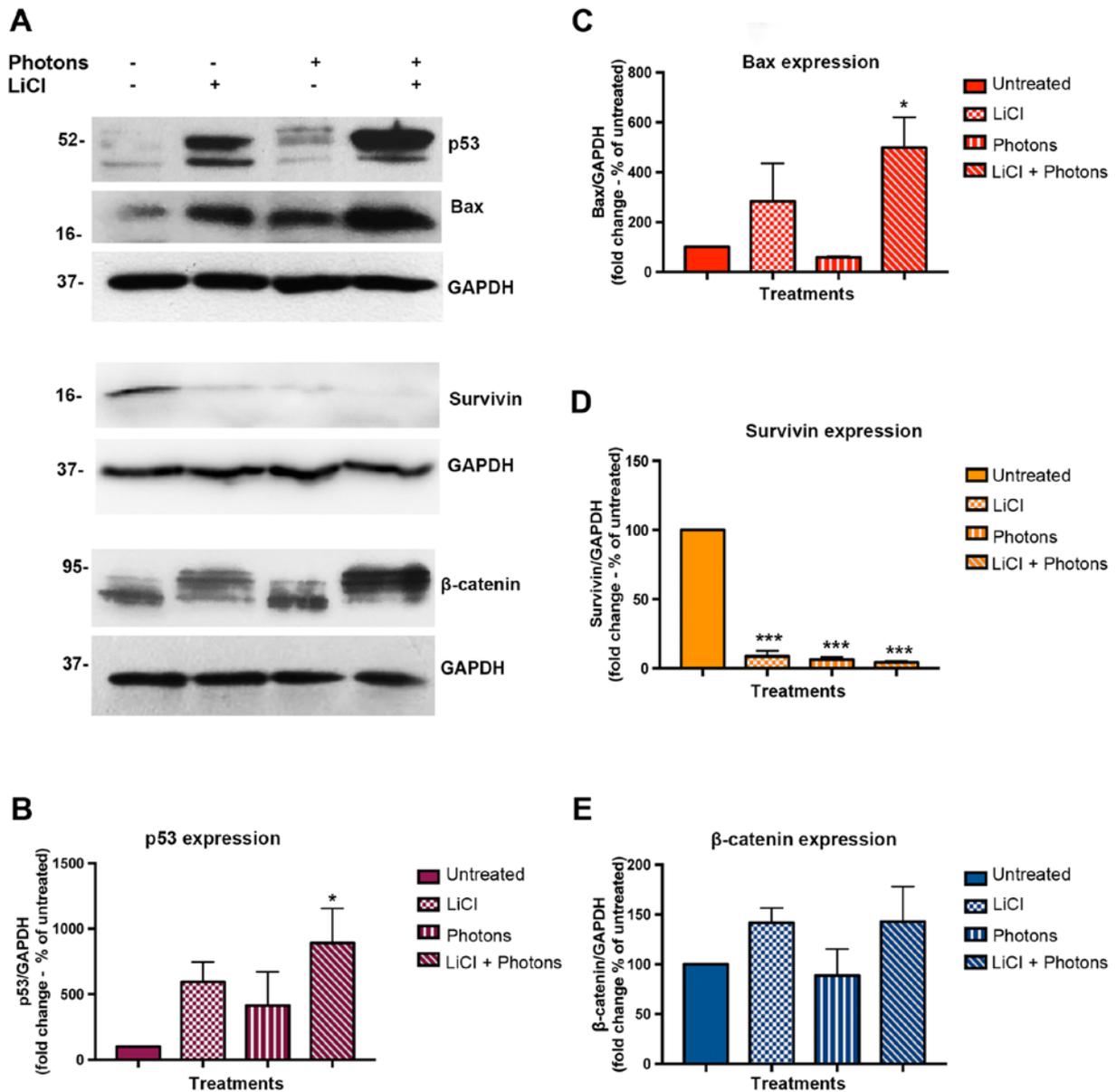


Figure 3. Effect of LiCl and high energy photon irradiation on p53, Bax, Survivin and β-catenin protein expression in T88 cells. (A) Representative western blot images revealing the protein expression levels of p53, Bax and Survivin in treated and untreated T88 cells. GAPDH was used as the loading control. The graph shows the densitometric analysis of (B) p53, (C) Bax, (D) Survivin and (E) β-catenin compared with GAPDH. Bar graphs represent mean ± SEM (3 independent experiments). *P<0.05 and ***P<0.0001 vs. untreated cells, using Kruskal-Wallis test. LiCl, lithium chloride.

that LiCl induces apoptosis by activating cell death signaling and down-regulating survival signaling in T88 cells. Indeed, LiCl induced upregulation of the p53 and Bax proteins and strong downregulation of the survivin protein. In addition, combined cell treatment with LiCl and high energy photons was more effective than high energy photons or LiCl used alone in reducing the viability of colon cancer cells. In fact, under combined treatment, cells show the highest percentage of apoptotic subdiploid cells between all treatments analyzed, associated with the highest expression of p53 and Bax protein, and absence of survivin protein expression. A diagrammatic summary representing a model for the action of LiCl by which it sensitizes resistant colon cancer cells to radiotherapy is shown in Fig. 5. In contrast to breast cancer (39), but in accordance with the LiCl mechanism of action, we observed stabilization of high molecular weight β-catenin isoforms in

cells treated with LiCl alone and in cells treated with LiCl plus high energy photons. We speculated that these isoforms could be those phosphorylated by pyruvate dehydrogenase kinase 1 in Thr112 and Thr120, which are selectively directed to the plasma membrane, where they interact with the E-cadherin protein (40). We previously observed that the effect exerted by LiCl on the level of β-catenin expression was time-dependent and LiCl promoted β-catenin membrane localization (15,16). Furthermore, β-catenin is heterogeneously distributed in CRC cells; indeed, well-differentiated parenchymal cells, located in the tumor center, retain β-catenin membranous expression comparable to that of normal colon epithelium, while nuclear β-catenin expression predominates in tumor cells localized at the invasion front (41). It would be interesting to investigate further the role of β-catenin in LiCl-induced sensitization to photon irradiation therapy and, more generally, in the antineoplastic

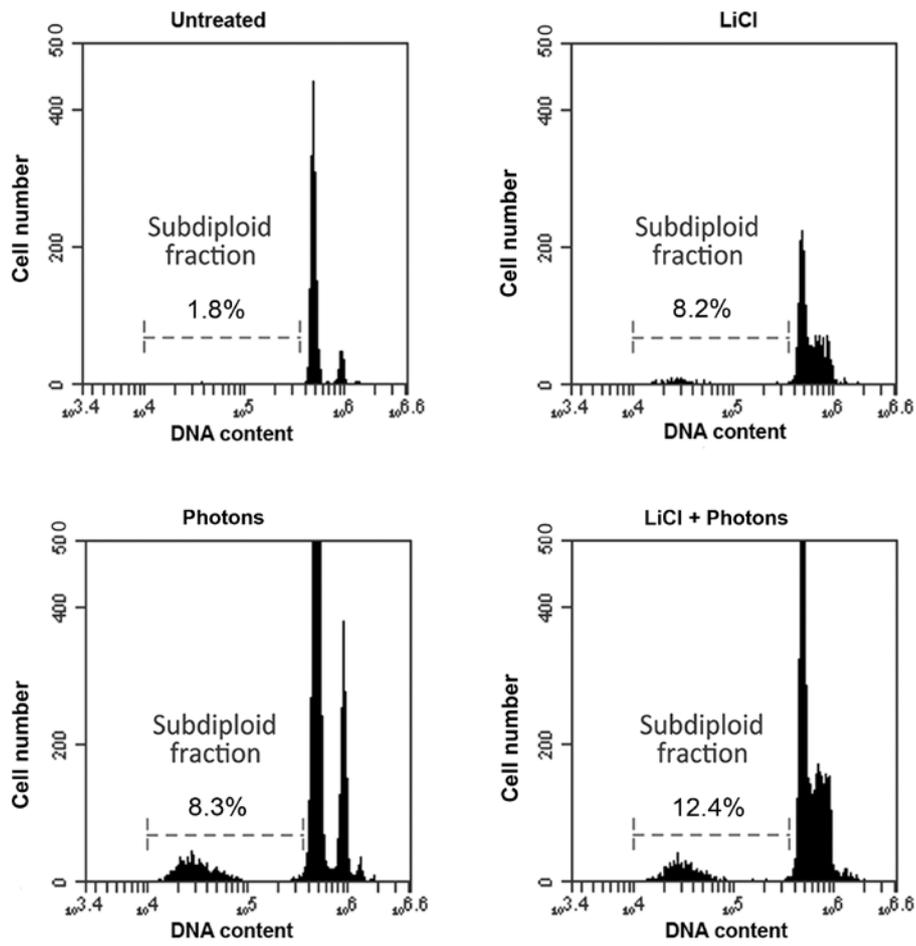


Figure 4. Effects of LiCl and high-energy photon irradiation on the subdiploid-apoptotic fraction of T88 cells. Propidium iodide was used to stain cellular DNA and flow cytometry was performed to analyze cell cycle distribution. LiCl, lithium chloride.

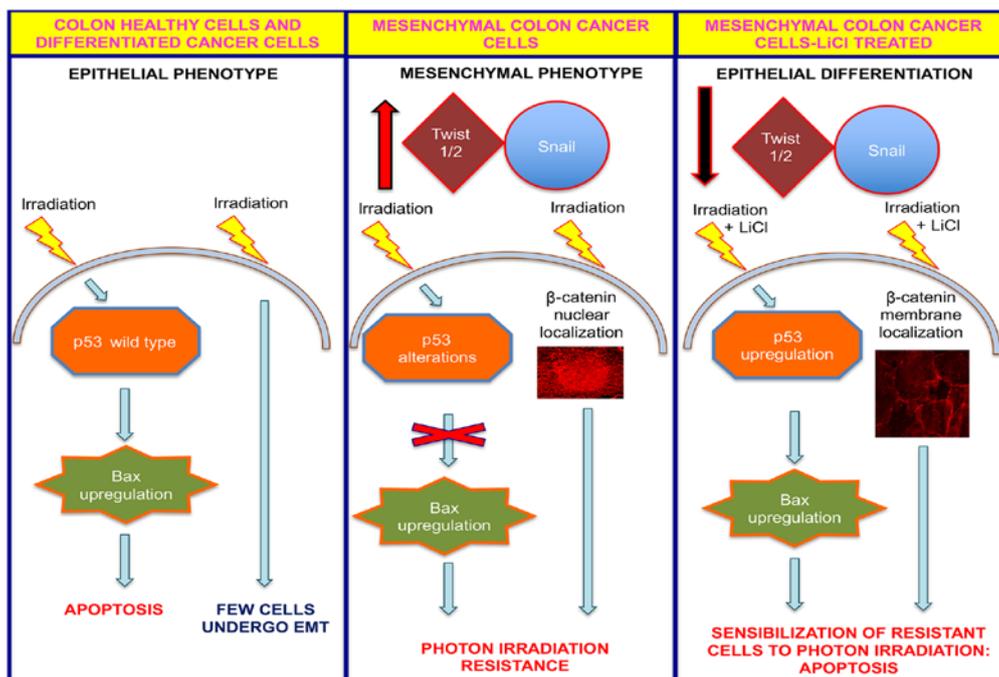


Figure 5. Model of LiCl action in sensitizing resistant colon cancer cells to radiotherapy. Photon irradiation induces apoptosis, in the majority of differentiated cancer cells and healthy cells, via p53 upregulation. Only few cells survive; the cells which underwent EMT. Mesenchymal colon cancer cells show nuclear localization of β-catenin and p53 alterations and, consequently, are resistant to apoptosis after irradiation. LiCl induces mesenchymal to epithelial transition and differentiation of colon cancer cells. The EMT-TFs are downregulated and β-catenin is selectively directed to the plasma membrane. Consequently, photon irradiation causes p53 upregulation and cancer cell death by apoptosis. LiCl, lithium chloride; EMT, epithelial-mesenchymal transition.

effect of LiCl in colon cancer. Could the LiCl-sensitizing effect to photon irradiation on colon cancer cells be also mediated by a decrease in the activity of the DSB repair system, as observed in breast cancer cells? Additional experiments are required to shed light on these intriguing and controversial points.

As we discussed in a previous paper (15), cell culture models obviously do not mimic the complex interactions that occur in the intestinal mucosa, and a functional change in cell cultures may not equate with the effect observed *in vitro*. On the other hand, complex functions can be reproduced in cell cultures and thus become amenable to investigation. However, the data reported herein could have important clinical significance and clinical applications and need, in a next future, to be improved and reinforced with animal models experiments.

In conclusion, we demonstrate that T88 mesenchymal colon cancer cells are resistant to radiotherapy, and that LiCl sensitizes these cells to apoptosis in response to high-energy photons, probably by acting on the balance between pro-apoptotic and survival signaling transduction pathways. In light of our finding, we suggest that LiCl could be used to increase sensitivity of resistant colon cancer cells to radiotherapy.

Acknowledgements

The authors would like to thank Dr Jaen Ann Gilder for the text editing.

Funding

The present study was supported by a grant from Fondo Straordinario di Ateneo-2018, Università di Napoli Federico II; POR Campania FESR 2014-2020 'SATIN'.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GMP, MDR and PR designed the study. FC, AC, AA, VV and MT performed the cellular and molecular experiments. PM, GA and PR performed the high energy photon irradiation. MDR and PR performed the statistical analysis of the data. PD provided the surgical sample for primary cell isolation. GMP and MDR coordinated the work. GMP, MDR, PI and PD contributed to data interpretation. PI provided funding. MDR, PI and PD wrote the manuscript. GMP and MT critically revised the manuscript. All authors edited and approved the final version of the manuscript.

Ethics approval and consent to participate

The patient study was approved by the Ethics Committee of the University of Naples Federico II, 'Comitato etico per le attività Biomediche-Carlo Romano' (no. 35/17). The patient provided written informed consent for the study. All methods were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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