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Antimanic drug sensitizes breast cancer cell line to ionizing radiation

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Abstract. Breast cancer is one of the most prevalent types of cancer among women. Lithium chloride (LiCl) is an FDA-approved drug for bipolar disorder. Breast cancer is reported to occur with higher rate in women with bipolar disorder. The effect of LiCl on the response of breast cancer cells to ionizing radiation has not been studied. We studied the effect of LiCl on the radiosensitivity of radioresistant T47D breast cancer cell line. Treatment of T47D cells with 20 mM LiCl for 24 hours decreased the radioresistance of these cells indicated by clonogenic survival assay. Comet assay demonstrated decreased DNA repair in LiCl-treated cells. LiCl treatment also decreased the meiotic recombination 11 (Mre11) mRNA level. Mre11 is an essential protein for DNA repair whose transcription is regulated by β-catenin protein. Western blot analysis indicated that the β-catenin protein level was decreased in LiCl-treated cells. LiCl increased glycogen synthase kinase-3β (GSK-3β) protein that is involved in β-catenin degradation. The results demonstrated that LiCl could radiosensitize T47D cells by decreasing DNA repair, partially through Mre11 repression. GSK-3β/β-catenin/Mre11 pathway might be the connection between LiCl treatment and the decreased DNA repair in T47D cells.

Key words: LiCl — T47D — Radiosensitivity — DNA repair — Mre11

Abbreviations: DSB, double strand break; GSK-3β, glycogen synthase kinase-3β; HRR, homologous recombination repair; IR, ionizing radiation; LQ, linear-quadratic; Mre11, meiotic recombination 11; MRN, Mre11/Rad50/Nbs1; %T, percent DNA in tail; TLK, two-lesion kinetic.

Introduction

Breast cancer is one of the most common and life-threatening types of cancers in women worldwide (Dumitrescu and Cotarla 2005). Eradicating tumor cells by ionizing radiation (IR) is an efficient approach in breast cancer therapy. Yet, sensitizing radioresistant breast cancer cells to IR is a challenge in cancer radiotherapy. T47D is an epithelial human breast cancer cell line obtained from metastatic sites of a patient with ductal carcinoma. This cell line is a radioresistant human breast cancer cell line (Siles et al. 1996; Coco Martin et al. 1999).

Lithium is an antimanic drug used for treatment of bipolar disorder (Quiroz et al. 2004). Breast cancer occurrence is higher in women with bipolar disorder (BarChana et al. 2008; McGinty et al. 2012). Lithium chloride (LiCl) has been shown to have antiproliferative and apoptotic effects in some breast cancer cell lines (Gustin et al. 2009; Higgins et al. 2011; Kaufmann et al. 2011). There are a few reports about the effect of LiCl on radioresistance and double strand break (DSB) repair in cancer cells (Yazlovitskaya et al. 2006; Yang et al. 2009). However, these effects have not been studied in breast cancer cells.

IR induces lethality mainly by induction of DSBs in DNA molecules. Tumor cells are more proficient in DNA DSB repair and therefore are more resistant to IR (Ding et al. 2006). Hence, targeting DSB repair pathways would be a beneficial strategy for overcoming radioresistance of cancer cells (Ding et al. 2006). The meiotic recombination 11 (Mre11) is a key protein of the mammalian three-component complex Mre11/Rad50/Nbs1 (MRN). This complex is pivotal for DSB recognition and repair (Stracker and Petrini 2011). *Mre11* mutation results in ATLD (ataxia-telangiectasia-like dis-

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order) accompanied with high sensitivity to IR (Stewart et al. 1999). It is demonstrated that Mre11 is a key protein for targeting DSB repair and increasing tumor radiosensitivity (Deng et al. 2011; Yuan et al. 2012). We have studied the effect of LiCl on the radiosensitivity, DNA repair and *Mre11* expression in T47D breast cancer cell line. This is the first study about the relationship between LiCl treatment and *Mre11* expression.

Materials and Methods

All chemicals were purchased from Merck unless otherwise mentioned.

Cell culture and treatments

The T47D breast cancer cell line was obtained from National Cell Bank of Iran (NCBI)-Pasteur Institute of Iran. Cells were maintained in RPMI-1640 medium (Gibco) in 10% FBS (Gibco) containing streptomycin and penicillin (Sigma) antibiotics and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated at 2×10^4 cells/cm^2 density. After three days, cells were treated with LiCl (0 or 20 mM) for 24 hours. Radiation was performed by a linear accelerator X-ray machine (Siemens Primus) at 2 Gy/min dose rate and 6 MV energy. The 20 mM LiCl concentration was a non-toxic concentration (Fig. S1) that did not induce DNA break (Fig. S2).

Clonogenic survival assay

LiCl-treated and control cells were exposed to 2, 3, and 6 Gy of IR or mock-irradiated. Cells were plated in triplicates in 60 mm plates in suitable densities. After 13 days of incubation, colonies were fixed with formaldehyde and stained with crystal violet. Colonies containing more than 50 cells were counted. Surviving fractions were calculated by dividing the number of counted colonies to the expected number of colonies. Changes in surviving fractions were indicator of variation in radiosensitivity. These survival curves were fitted to the linear-quadratic (LQ) model by the OriginPro8 software (Barendsen 1992).

We also analyzed the clonogenic survival data using the two-lesion kinetic (TLK) model by virtual cell radiobiology software (VC 2.00A) provided by Dr. Robert D. Stewart (Department of Radiation Oncology, University of Washington). The virtual cell input parameters were set to find the most reliable results based on the least function of merit (FOM) score. In TLK model a simple DSB refers to a 10–20 bp section of DNA that contains one break in each strand. A complex DSB contains additional defects including base damage, strand break and base deletion. The repair rate of simple and complex DSBs is λ_1 and λ_2 respectively. The probability of a misrepair to be lethal is denoted by φ. Besides, two DSBs can interact with the rate η (Stewart 2001; Guerrero et al. 2002).

Alkaline comet assay

LiCl-treated and control cells were exposed to 3 Gy of IR or mock-irradiated. Cells were incubated for 15 or 45 min at 37°C before performing comet assay. About 2×10^4 cells were mounted within 0.5% low melting point agarose (Sigma) on slides previously coated with 1% agarose. The alkaline comet assay was carried out as described previously (Singh et al. 1988). Briefly, slides were incubated in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and 1% Triton X-100; pH 10) for 1 hour. Denaturation buffer (300 mM NaOH, 1 mM EDTA; pH 13) was added on slides for 30 min. Slides were electrophoresed at 1 V/cm within fresh denaturation buffer for 30 min. Neutralization buffer (400 mM Tris-HCl; pH 7.5) was added on slides for about 5 min. All these stages were performed at 4°C. Cells were stained with Ethidium Bromide and observed under a fluorescence microscope (Zeiss, Axioskop 2 plus).

At least 200 cells were analyzed by CometScore freeware (version 1.5; TriTek Corp.). Percent DNA in tail (%T) was utilized as an indicator of the amount of DNA damage (Kumaravel and Jha 2006). To omit the effect of time and treatment, we calculated the "IR-induced %T" as: %T of irradiated cells – %T of unirradiated similarly treated cells at the same time point.

Western blot analysis

Total cell extracts were obtained by scraping cells in lysis buffer (200 mM NaCl, 2% w/v SDS, 50 mM Tris-HCl, 2 mM EDTA, 5 mM DTT, and 1 mM PMSF; pH 8). SDS-PAGE was performed and proteins were blotted on PVDF membranes (BioRad). Antibodies against GSK-3β and Lamin B2 were obtained from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The antibody against β-catenin was kindly gifted by Dr. S. Mahmoud A. Najafi (Department of Cell & Molecular Biology, Faculty of Biology, University of Tehran) (Salmanian et al. 2010). Protein bands were detected using enhanced chemiluminescence kit (Amersham Bioscience). Bands were analyzed by TotalLab software.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted by High Pure RNA Isolation Kit (Roche) based on manufacturer's protocol. RT-PCR was performed by Thermo Hybrid PCR machine using 2-steps RT-PCR Kit (vivantis). We designed *Mre11* primers (Forward: 5'-ggcaat-

catgacgatcccaca-3', Reverse: 5'-tgttcatggccccagataacaa-3') by Primer3 software (Ye et al. 2012) to amplify a 362 bp fragment located within exons 5 to 8. β-actin mRNA was amplified as the internal control using previously designed primer set (Forward: 5'-GGCGGCACCACCATGTACCCT-3', Reverse: 5'-AGGGGCCGGACTCGTCATACT-3') that gives a 202 bp PCR product (Lango et al. 2002). Bands were analyzed by TotalLab software.

Statistical analysis

All results were reported as mean ± SEM (standard error of mean) of three independent experiments. The statistical significance of clonogenic and comet assay data was checked with Two-Way repeated mode ANOVA followed by *post hoc* Tukey analysis. The One-Way repeated mode ANOVA followed by *post hoc* Tukey analysis was used to check the significance of the Western blot and RT-PCR data. The statistical analyses were performed by the OriginPro8 software. A p value less than 0.05 was considered significant.

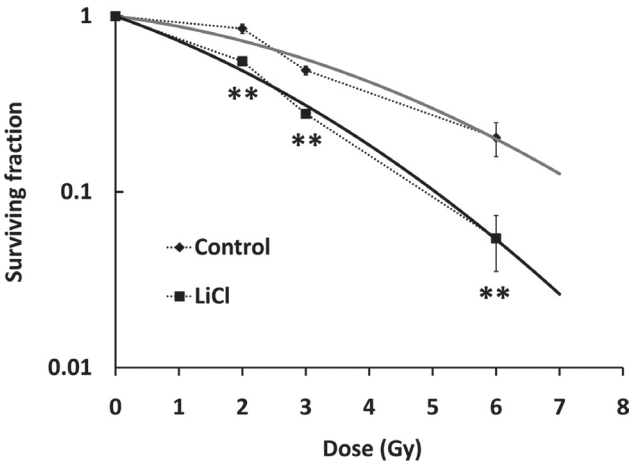

Figure 1. LiCl decreased the radioresistance of T47D cells. Clonogenic survival assay was performed for control cells and cells treated with LiCl (20 mM) for 24 hours before exposure to different doses of X-ray. Data represent mean ± SEM of three independent experiments. Solid lines are the LQ model fits. ** $p < 0.01$ compared with control.

Results

The effect of LiCl on the radiosensitivity of T47D cell line

Based on *post hoc* Tukey test, at 0.05 significance level, the samples treated with lithium had significantly lower surviving fractions after exposure to radiation when compared to samples in regular medium (Fig. 1). Pairwise comparison Tukey analysis revealed significant differences between surviving fractions of control and LiCl-treated cells at all doses of X-ray ($p < 0.01$). Clonogenic survival curve was fitted to the LQ model. α, β and SF2 (surviving fraction at 2 Gy) parameters of the LQ model are summarized in Table 1. LiCl treatment induced a 2.7-fold increase in the α parameter and sensitized the cells even at low doses of ionizing radiation.

Effect of LiCl pretreatment on the repair of IR-induced DNA damages

Alkaline comet assay was performed to determine the effect of LiCl on DNA repair in irradiated cells. Percent DNA in tail (%T) was measured in control and LiCl-treated cells mock-irradiated or irradiated with 3 Gy of X-ray (Fig. 2A). The "IR-induced %T" was calculated as described in Materials and Methods (Fig. 2B). LiCl did not induce any DNA damage in unirradiated cells (Fig. 2A, S2). However, based on Two-Way repeated mode ANOVA followed by *post hoc* Tukey analysis LiCl treatment significantly increased the "IR-induced %T" at 15 ($p < 0.01$) and 45 ($p < 0.001$) min post-IR (Fig. 2B).

During the chased period (30 min), 81% of "IR-induced %T" faded in control cells ($p < 0.001$). However, this feature was 33% for LiCl-treated cells ($p < 0.01$) (Fig. 2B). Therefore, LiCl decreased the repair rate of IR-induced DNA damages.

To estimate the kinetics parameters of DNA repair, we analyzed clonogenic survival data with the TLK DSB repair model (Stewart 2001; Guerrero et al. 2002). Parameters that were different between control and LiCl-treated cells are summarized in Table 2. LiCl treatment induced a 4.5-fold

Table 1. Parameters obtained from fitting clonogenic survival data to the LQ model

	α (1/Gy)	β (1/Gy^2)	SF2
Control	0.11	0.027	0.72
LiCl	0.29	0.033	0.49

SF2, surviving fraction at 2 Gy.

Table 2. Parameters obtained from fitting clonogenic survival data to the TLK model

	λ_1 (1/h)	λ_2 (1/h)	φ	η (1/h)
Control	69.3	0.10	0.011	0.0014
LiCl	69.3	0.03	0.049	0.0006

λ_1, simple DSB repair rate; λ_2, complex DSB repair rate; φ, the probability of a misrepair to be lethal; η, DSB-DSB pairwise interaction rate.

increase in the φ parameter (the probability that misrepaired DSB is lethal). According to the TLK model outputs, LiCl decreased the repair rate of complex DSBs (λ_2) and the rate of DSB-DSB interaction (η) by 3.6- and 2.3-folds, respectively.

Alterations in GSK-3β and β-catenin protein levels and Mre11 mRNA level in response to LiCl treatment

We evaluated total GSK-3β protein level in control and LiCl-treated T47D cells by Western blot analysis (Fig. 3). Treatment of T47D cells with 10 and 20 mM of LiCl increased GSK-3β protein level by 12 and 56% ($p < 0.01$), respectively.

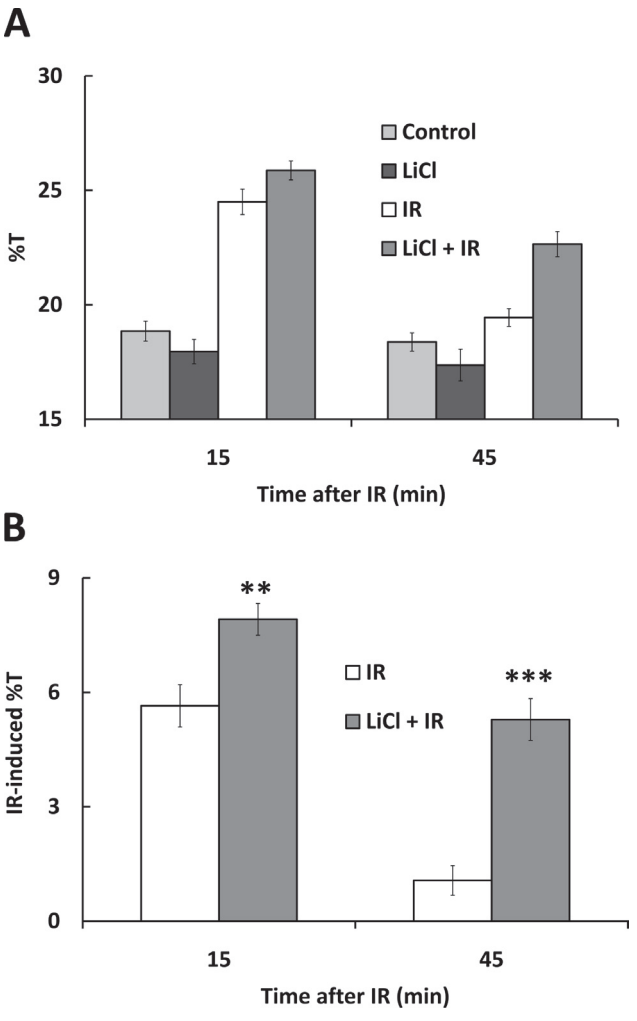

Figure 2. LiCl hindered the repair of IR-induced DNA damages in T47D cell line. Control and LiCl (20 mM)-treated cells were subjected to alkaline comet assay at 15 and 45 min after exposure to 0 or 3 Gy of X-ray. Percent DNA in tail (%T) (**A**) and "IR-induced %T" (**B**) are depicted at both time points. Data represent mean ± SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared with control irradiated cells.

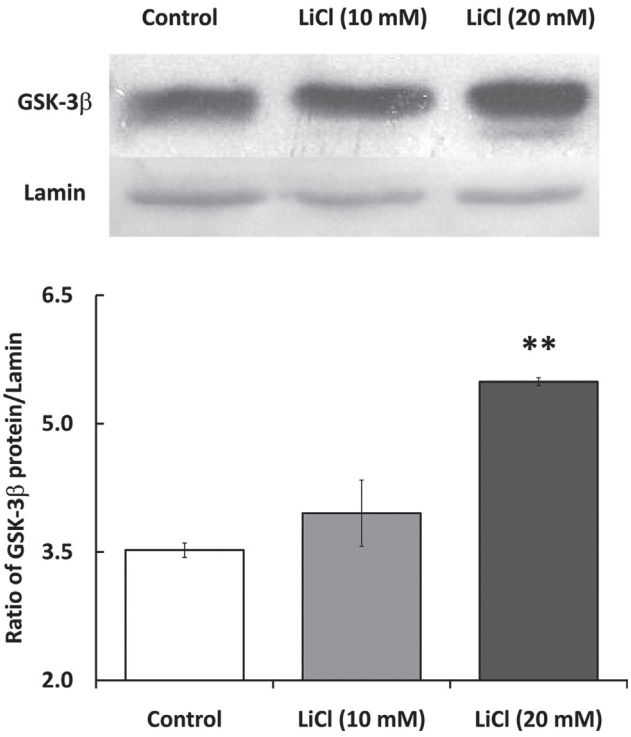

Figure 3. LiCl treatment increased the total level of GSK-3β protein. GSK-3β level was determined by immunoblotting in lysates of control cells and cells treated with 10 mM and 20 mM LiCl for 24 hours. Lamin B2 was used as the loading control. Data represent mean ± SEM of three independent experiments. ** $p < 0.01$ compared with control.

We assessed β-catenin protein and Mre11 mRNA levels in control and LiCl-treated T47D cells (Fig. 4). Western blot analysis indicated a 17% decrease in the amount of β-catenin protein in cells treated with LiCl (20 mM) ($p < 0.05$) (Fig. 4A). On the other hand, RT-PCR analysis revealed a 20% decrease in Mre11 mRNA level in cells treated with LiCl (20 mM) ($p < 0.05$) (Fig. 4B).

Discussion

We have demonstrated the radiosensitization effect of LiCl, as a common antimanic drug, on the radioresistant T47D breast cancer cell line. Treatment of these cells with LiCl suppressed DNA repair after exposure to IR. LiCl decreased the *Mre11* expression involved in DSB repair processes. Alterations in GSK-3β/β-catenin/Mre11 pathway components were in accordance with the decreased DNA repair and increased radiosensitivity in LiCl-treated T47D cells.

Although LiCl did not induce DNA damage in unirradiated cells, it predisposed T47D cells to higher DNA damage at 15 and 45 min post-IR (Fig. 2A). The repair of DNA damage was slower in T47D cells treated with LiCl compared with control untreated cells (Fig. 2B). The lower repair rate in LiCl-treated cells might underlie the higher radiosensitivity of these cells observed in clonogenic survival assay.

We observed higher GSK-3β and lower β-catenin protein levels in LiCl-treated T47D cells. Although LiCl is known as an inhibitor of GSK-3β protein (Quiroz et al. 2004), it increased total level and decreased the inactive form of GSK-3β in some breast cancer cells (Gustin et al. 2009). It was mentioned that the LiCl-induced increase in GSK-3β protein level occurred through an unknown mechanism. GSK-3β phosphorylates and thereby targets β-catenin molecule for proteasomal degradation (Liu et al. 2002). Therefore, the decreased β-catenin protein level (Fig. 4A) verified the functionality of upregulated GSK-3β. GSK-3β/β-catenin pathway might play an important role in LiCl-induced alterations in radioresistance of breast cancer cell lines. It is also possible that LiCl inhibits DSB repair by affecting other LiCl targets (Quiroz et al. 2004).

LiCl treatment decreased the Mre11 mRNA level in T47D cells (Fig. 4B). It is mentioned that a direct correlation exists between Mre11 mRNA and its protein level (Garner and Eastman 2011). The Mre11 repression was accompanied by decrease in β-catenin protein level. β-catenin protein level has been shown to be in direct correlation with *Mre11* transcription (Deng et al. 2011).

Based on the estimated results of TLK model, LiCl decreased the complex DSB repair rate (λ_2) and the DSB-DSB pairwise interaction rate (η) but it did not change the simple DSB repair rate (λ_1). The λ_2 and η lower repair rates in LiCl-treated cells could increase the remaining DSBs as observed in comet assay experiments (Fig. 2B). Among the IR-induced DNA lesions, DSBs are the most lethal. Two major DSB repair pathways are non-homologous end joining (NHEJ) and homologous recombination repair (HRR). NHEJ repair is subdivided into canonical NHEJ (C-NHEJ) and alternative end joining (A-EJ). Mre11 is one of the three components of the MRN complex involved in the C-NHEJ repair of complex DSBs (Huang and Dynan 2002; Riballo et al. 2004). Simple DSBs that undergo fast repair (Stewart 2001; Reynolds et al. 2012) need Ku70/80 and ligase IV/XRCC4 complexes and

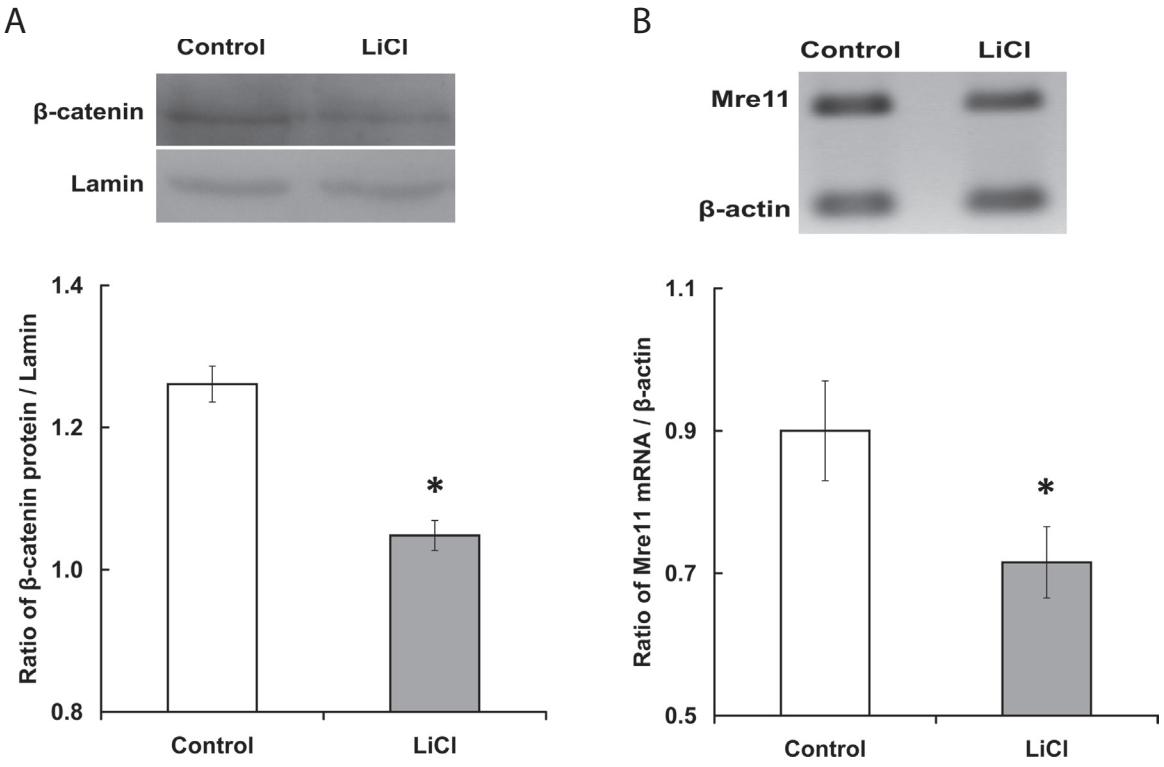

Figure 4. LiCl treatment decreased β-catenin protein and Mre11 mRNA levels. Lysates of control cells and cells treated with 20 mM LiCl for 24 hours were used for assessing β-catenin protein level by immunoblotting (**A**) and Mre11 mRNA level by RT-PCR analysis (**B**). Lamin B2 and β-actin were used as loading controls. Data represent mean ± SEM of three independent experiments. * $p < 0.05$ compared with control.

probably are not dependent on MRN for their repair process (Yamaguchi-Iwai et al. 1999; Reynolds et al. 2012). MRN complex is also involved in HRR (Limbo et al. 2007; Stracker and Petrini 2011) and A-EJ (Rass et al. 2009; Xie et al. 2009) *via* the end resection process. Consequently, MRN is essential for processing complex DSBs. Mre11 down-regulation leads to a decreased number of MRN complexes (Takemura et al. 2006). Therefore, decrease in Mre11 level slows down the repair of complex DSBs either when ends to be joined belong to one DSB or to two interacting DSBs.

The TLK model also predicted a 4.5-fold increase in the φ parameter (the probability that misrepaired DSB is lethal) in LiCl-treated T47D cells. This increase in lethality was consistent with the decreased survival in LiCl-treated cells (Fig. 1). The MRN complex localizes at DSBs soon after radiation (Haince et al. 2008). This complex is involved in tethering DSB ends together or to the template chromatid during HRR (Williams et al. 2008). Therefore, Mre11 down-regulation might decrease the available linker complexes at the time of radiation. Hence, there would be some free DSB ends that may move about and juxtapose other free ends or template sequences. These ends might be recognized by MRN complex later and linked and repaired erroneously. Misrepaired DSBs can cause chromosomal aberrations and increase cell lethality.

MRN complex also participates in initiating signaling pathways that lead to checkpoint activation (D'Amours and Jackson 2002; Carson et al. 2003; Difilippantonio et al. 2007). Therefore Mre11 repression hinders cell cycle arrest and decreases the available time for repairing DSBs. The decreased repair rate and repair opportunity due to Mre11 deficiency leads to the accumulation of DSBs as observed previously (Costanzo et al. 2001). DSB accumulation along with higher amount of chromosomal aberrations increases cell lethality and IR sensitivity in Mre11 deficient cells. Therefore, the observed Mre11 repression in LiCl-treated T47D cells was in agreement with TLK model-derived parameters. Because Mre11 is significant for DSB repair, the observed Mre11 repression might be an important reason for DNA break repair defect and lower clonogenic survival in LiCl-treated T47D cells.

We suggested LiCl as a radiosensitizer capable of decreasing *Mre11* expression in radioresistant T47D breast cancer cell line. We also proposed a molecular pathway that links LiCl treatment to the decrease in DNA repair and the relationship between LiCl and Mre11 repression. LiCl down-regulates *Mre11* expression possibly through GSK-3β/β-catenin pathway. LiCl increased the radiosensitivity of T47D breast cancer cell line by decreasing *Mre11* expression involved in DSB repair processes.

The findings of this study must be viewed in light of its limitations. Firstly, in this study the alkaline comet assay was used to measure the amount of both single and double strand breaks. Changes in the amount of double strand breaks can be determined by more specific assays. Secondly, the possible involvement of other molecular pathways in LiCl-induced radiosensitization can not be ruled out. Specific siRNA-mediated downregulation of GSK-3β together with LiCl treatment can clarify to what extent the GSK-3β protein is involved in the LiCl-induced radiosensitization. On the other hand, overexpression of β-catenin or Mre11 together with LiCl treatment can certify the involvement of these proteins in the sensitization induced by LiCl.

In the current study, LiCl was indicated to decrease the radioresistance of a breast cancer cell line. This radiosensitization was apparent even at therapeutic doses of ionizing radiation. Further work is necessary to investigate the effect of LiCl on other breast cancer cells. *In vivo* studies would further clarify the effect of LiCl on the response of breast tumors to ionizing radiation. Eventually LiCl might be used in combination with radiotherapy for eradicating breast tumors.

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Supplementary Figures

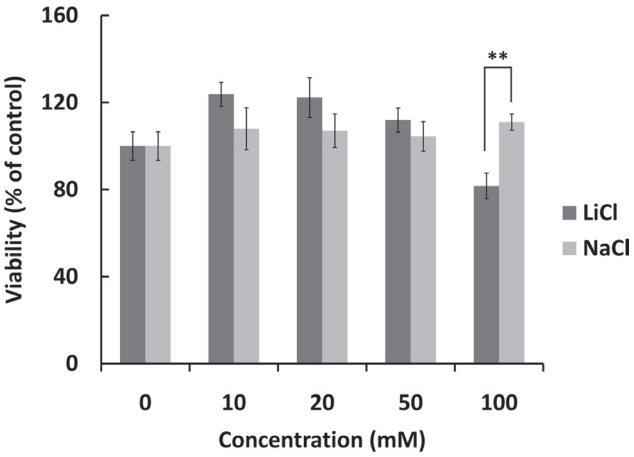

Figure S1. LiCl did not decrease cell viability up to 50 mM concentration. Cells were treated with 0, 10, 20, 50 and 100 mM concentrations of NaCl (as control salt) or LiCl for 24 hours and viability was determined by MTT assay (Mosmann 1983). Briefly, 50 µl MTT solution (2 mg/ml) was added to each well. Cells were incubated at 37°C for 3 hours. 100 µl dimethyl sulfoxide was added to each well. Absorbance was determined at 540 and 630 nm wavelengths using BioTek plate reader. The differences between these two absorbances were used for determining viability. The viability was set to 100% for cells without any treatment. Data represent mean ± SEM of three independent experiments. ** $p < 0.01$ compared with NaCl-treated cells based on Two-Way repeated mode ANOVA followed by *post hoc* Tukey analysis.

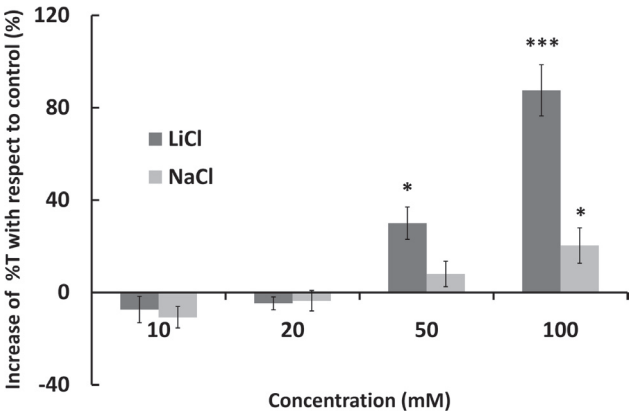

Figure S2. LiCl did not induce DNA break up to 20 mM concentration. Alkaline comet assay was performed on control cells and cells treated with different concentrations of LiCl and NaCl for 24 hours as described in Materials and Methods. Data represent mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control cells based on Two-Way repeated mode ANOVA followed by *post hoc* Tukey analysis.

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